PhD értekezés

Az aktin konformációs és dinamikai tulajdonságainak tanulmányozása fluoreszcencia spektroszkópiás módszerekkel

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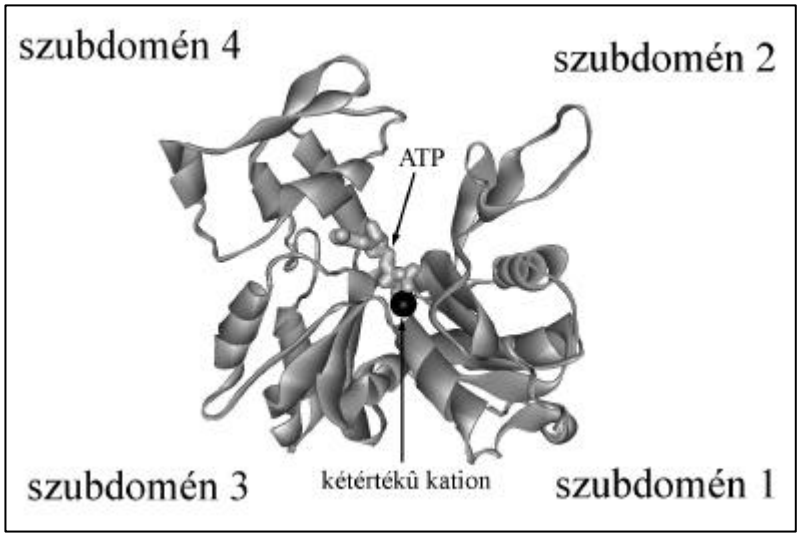
TARTALOMJEGYZÉK

1. BEVEZETÉS

1.1. Az aktin szerkezeti felépítése

Az élet alapvetô jellemzôi között szereplô irányított mozgás alapjainak megértése már az ókortól kezdve foglalkoztatta az embert. A probléma jelentôségét és bonyolultságát jól szemlélteti, hogy a világon máig a legtöbbet vizsgált fehérjék az izomfehérjék, és róluk évrôl évre óriási számban jelennek meg publikácók. A vizsgálatukba fektetett hatalmas energiák ellenére azonban az izommûködés molekuláris alapjainak megértése a mai napig hiányos, és számos fontos kérdés vár még megválaszolásra.

Az aktin felfedezése Straub F. Brúnó nevéhez fûzôdik, aki 1942-ben elsôként izolálta és nevezte el ezt az izomfehérjét (Straub, 1942). A késôbbi vizsgálódások során derült ki, hogy ez a fehérje nem csak az izomban található meg (Hatano és Oosawa, 1966), hanem például a sejtekben található citoszkeletonnak is alapvetô alkotóeleme (Brown és mtsai., 1976). Elektronmikroszkópos felvételekbôl már korán nyilvánvalóvá vált, hogy a monomerekbôl felépülô aktin filamentumot füzérszerûen elhelyezkedô gyöngyök sorozatának tekinthetjük (Hanson és Lowy, 1963). A fehérje elsôdleges szerkezetének tisztázása 1973-ban történt meg (Elzinga és mtsai., 1973). Az aktin szálakat felépítô monomerek nagyfelbontású, röntgendiffrakciós kísérletekkel történô feltérképezése azonban nehézségekbe ütközött. Az alapvetô problémát az jelentette, hogy a röntgendiffrakciós kísérletekhez nem sikerült a szükséges kristályosított monomereket elôállítani. A probléma megoldására olyan fehérjéket kerestek, melyek az aktin monomerekkel komplexet képeznek és a fehérjét monomer formában tartják a kristályosítás során. Kitartó munkájuk eredményeképpen Kabsch és munkatársai 1990-ben bemutatták az aktin monomer DN-áz I-el alkotott komplexének háromdimenziós atomi szerkezetét (Kabsch és mtsai., 1990). Még ugyanebben az évben Holmes és munkatársai orientált aktin filamentumokból álló géleken végzett röntgendiffrakciós kísérleteik eredményét összevetve a monomerekre kapott háromdimenziós képpel, megalkották az aktin filamentum atomi modelljét



1. ábra: Az aktin monomer sematikus ábrázolása

(Holmes és mtsai., 1990). A 375 aminosavból felépülô, 43 kDa relatív molekulatömegû fehérje két formában, monomer vagy más néven globuláris (G-aktin), illetve a monomerekbôl nem kovalens kötéssel létrejövô polimer vagy más néven filamentális (F-aktin) formában fordulhat elô. A természetben valószínûleg az “aktív” (mûködô) formának tekinthetô polimer forma kialakulása az ionerôsség növelésével könnyen elérhetô. A monomer szerkezete két doménre osztható, melyek közül a kisebb domént a szubdomén 1 és 2, míg a nagyobb domént a szubdomén 3 és 4 alkotja (1. ábra). A két domén közötti hasadékban helyezkedik el egy nagy affinitású kationkötô hely. Míg az aktin a nagy affinitású kationkötô helyen *in vivo* valószínûleg magnézium iont tartalmaz (Estes és mtsai., 1992), a szokásos preparálási eljárással nyert fehérjéhez kalcium ion kötôdik. A nagy affinitású kationkötô hely a következô erôsségi sorrendben köti a különbözô kationokat: Ca>Mn>Cd>Mg>Zn>Ni>Sr. A fehérjén további kationkötô helyek is találhatók, melyekre jellemzô, hogy kisebb, és közel azonos affinitással kötik a kationokat mint a domének közötti hasadékban elhelyezkedô nagy affinitású kötôhely. A szubdomének közötti hasadékban a fehérjéhez, illetve az ott elhelyezkedô kétértékû kationhoz kötve található egy ATP molekula is. A négy szubdomént hidrogén és ionos kötések kötik össze az ATP-vel és a kétértékû kationnal, stabilizálva ezzel a molekula szerkezetét. Straub F. Brúnó és munkacsoportja már 1949-ben felfedezte, hogy a G-aktin ATP-t tartalmaz, mely az aktin polimerizációja során ADP-vé és anorganikus foszfáttá (P_i) alakul. Az aktin ATP hidrolízissel kísért polimerizációjának szerepe lehet bizonyos aktinon alapuló sejtmozgások kivitelezésében (Loisel és mtsai., 1999). Az aktin filamentumok felépülését követôen az aktin szálak két végén dinamikus egyensúly alakul ki a filamentumokba beépülô és onnan leváló monomerek között. Mivel a filamentumok két végén a monomerek beépülésének és kiválásának sebessége eltérô, az egyik végén a molekula folyamatos növekedése (“szakállas vég”), míg a másik végén folyamatos leépülése figyelhetô meg (“hegyes vég”). Ezt a dinamikus folyamatot nevezzük “taposómalom effektusnak” (“treadmilling”) (Wegner, 1982). A “szakállas” és “hegyes vég” elnevezések abból adódnak, hogy amikor az aktin filamentumokhoz miozint adunk, a filamentumokon a miozin molekulák egy irányba mutatva rendezôdnek el, és ily módon nyílhegyhez hasonló alakot vesznek fel. Az aktin filamentum szerkezetét közelebbrôl szemügyre véve megfigyelhetô, hogy a filamentum geometriailag kétféle módon is jellemezhetô. Leírható mint egy balmenetes, egyszálú hélix (“genetikus hélix”), melyen belül 13 monomer alkot egy periódust, azaz egy 36 nm-es menetemelkedésû csavarmenetet. Leírható továbbá két, egymásra tekeredô szálból álló jobbmenetes hélixként is, ahol a teljes menetemelkedés felénél, azaz 36 nm-es távolságban találunk azonos pozícióban monomereket. A helikális formába rendezôdô filamentumok átmérôje 9-10 nm.

1.2. Az aktin dinamikus tulajdonságainak jelentôsége

Az aktin szerepét, konformációs állapotait és a hozzájuk rendelhetô dinamikai tulajdonságait sokan és sokféleképpen vizsgálták. A technika fejlôdésének köszönhetôen ma már betekintést nyerhetünk az izommozgás alapjainak molekuláris részleteibe is. A harántcsíkolt izomban az izomkontrakció legkisebb önállóan mûködô egysége a szarkomer. A szarkomert felépítô fehérjék között a vékony filamentumok felépítésében résztvevô aktin szerepét az izomösszehúzódás folyamatában legtöbben passzívnak tekintették. Ezen elképzelés szerint a vékony filametumok képezik az alapját a vastag filamentumokat alkotó miozin molekulák rajtuk történô, ciklikus kapcsolódások révén létrejövô elmozdulásának. Az aktív résztvevô a “motor” fehérjének tekintett miozin, mely az ATP molekulák hidrolízisével nyert kémiai energiát mozgási energiává átalakítva mozdul el az aktin filamentumok biztosította vázon. Ezzel szemben Straub. F. Brúnó már az általa elôször használt “aktin” elnevezéssel is a fehérjének az izomkontrakcióban betöltött alapvetô és aktív szerepére utalt.

Az aktin filamentum eltérô konformációs állapotai és flexibilitása közötti kapcsolat az izom összehúzódásában fontos szerepet játszhat (Prochniewicz és Yanagida, 1990; Rayment és mtsai., 1993; Kim és mtsai., 1998a). Az aktin dinamikai tulajdonságait vizsgálva, a különbözô keresztkötô molekulák valamint az aktin környezetének fiziko-kémiai tulajdonságaiban beállt változások hatásait részletesen vizsgálták. Prochniewicz és Yanagida megfigyelték, hogy egyes keresztkötô molekulák alkalmazásakor az aktin filamentum mozgása a nehéz meromiozinnal fedett üvegfelületen jelentôsen lelassul (Prochniewicz és Yanagida, 1990). Véleményük szerint a keresztkötô molekulának közvetlenül az aktin filamentumra kifejtett hatása érvényesül ezekben az esetekben. Hegyi György és munkatársai az aktin filamentumot felépítô monomerek összekötésére alkalmas keresztkötô molekulák alkalmazása esetén a filamentumokat merevebbnek találták (Kim és mtsai., 1998a; Kim és mtsai., 1998b; Hegyi és mtsai., 1998). A megváltozott flexibilitással párhuzamosan az in vitro motilitási próbával vizsgált polimerek mobilitása csökkent. Véleményük szerint megfigyeléseik az aktin dinamikájának az izom kontrakciójában betöltött alapvetô szerepét támasztják alá. Az aktin filamentum dinamikai tulajdonságait és aktív részvételét az izommûködésben más kutatók is alapvetô fontosságúnak gondolják (Janmey és mtsai., 1990; Schutt és Lindberg, 1993).

A fehérje dinamikai tulajdonságai, a környezeti paraméterek változásainak hatására is módosulhatnak. Egyes kutatók szerint jelentôs flexibilitásbeli különbségek mutathatók ki az aktin monomerek és filamentumok eltérô kationtartalmú formái között (Isambert és mtsai., 1995; Orlova és Egelman, 1993; Scharf és Newman, 1995; Steinmetz és mtsai., 1997; Yasuda és mtsai., 1996; Orlova és Egelman, 1995). Az aktin monomerekhez kötôdô kation minôsége

alapvetô hatással van a fehérje szerkezeti és funkcionális tulajdonságaira (Estes és mtsai., 1992; Carlier, 1990). Mihashi és munkatársai méréseik során arra a következtetésre jutottak, hogy az aktin filamentum torziós flexibilitása jelentôsebb, mint az aktin filamentumok hajlíthatóságát jellemzô flexibilitás (Mihashi és mtsai., 1983). Yasuda és munkatársai vizsgálataik során arra az eredményre jutottak, hogy az aktin filamentum torziós merevsége nagyobb, ha a nagy affinitású kötôhely magnézium helyett kalcium ionnal van telítve (Yasuda és mtsai., 1996). A filamentumok hajlíthatósága méréseik során a kation minôségétôl szinte teljesen függetlennek adódott. Orlova és Egelman az aktin filamentumok hajlíthatóságát jellemzô flexibilitást nagyobbnak találták abban az esetben, amikor az aktint magnézium tartalmú monomerekbôl polimerizálták (Orlova és Egelman, 1993). A megváltozott flexibilitás hátterében véleményük szerint a kettes számú szubdomén (1. ábra) elfordulása áll. Késôbbi vizsgálataik során egy általuk “bridge of density”-nek nevezett képletet találtak az aktin két szála között kalcium ion jelenlétében preparált filamentumok esetében. Magnézium ion jelenlétében preparált filamentumok esetén az elôbb említett képlet nem volt kimutatható. Mivel a kalcium ion hatására, az aktin szálak röntgendiffrakciós képében bekövetkezô változás rendkívül hasonló a miozin molekulák vékony filamentumokkal történô összekapcsolódásakor létrejövô változáshoz, ezért a kalcium-F-aktin a vékony filamentum “aktivált”, míg a magnézium-F-aktin a vékony filamentum “relaxált” formáját modellezheti (Orlova és Egelman, 1995). Más, eltérô technikákat alkalmazó kutatócsoport az eltérô kationok jelenlétében polimerizált filamentumok esetében nem talált különbséget (Scharf és Newman, 1995). A vizsgálatok nagy száma ellenére nem alakult ki egységes álláspont a kötött kétértékû kationoknak az aktin filamentum flexibilitására kifejtett hatásáról. Az eddigi eltérô eredmények a mérési módszerek és preparálási technikák különbözôségébôl is származhatnak.

Az aktin filamentumokat körülvevô környezet fiziko-kémiai jellemzôinek egyike a pH, mely mind fizilógiás, mind patológiás körülmények között jelentôsen lecsökkenhet az izomsejten belül. Mindkét esetben elmondható, hogy az érintett izomrész funkciójának károsodása, a kifejtett erô csökkenése a pH csökkenésének egyik alapvetô következménye (Edman és Mattiazzi, 1981). A vázizom sejtjein belül a pH fiziológiásan, például intenzív munkavégzés hatására csökkenhet le (Edman és Mattiazzi, 1981; Thompson és mtsai., 1992b; Thompson és mtsai., 1992a; Metzger és Fitts, 1987b; Metzger és Fitts, 1987a; Stevens, 1980; Renaud és mtsai., 1986). A csökkent intracelluláris pH az izomkifáradás jeleként értékelhetô. A pH csökkenés hátterében a felgyorsult ATP hidrolízis, valamint a sejten belüli energiatermelés aerob útról anaerob útra történô átállása áll (Wolfe és mtsai., 1988; Garlick és mtsai., 1979; Bailey és mtsai., 1982). A csökkent hatékonyságú energiatermelés nyilvánvaló

szerepe mellett az intracellulárisan csökkent pH szerepe az izom megváltozott mûködésében még nem tisztázott kérdés. Az intracelluláris pH csökkenés másik fontos, patológiásnak tekintett példája a szívizom iszkémiát kísérô intracelluláris pH csökkenése, mely a szívizom különbözô alkotórészeire lehet hatással (Mohabir és mtsai., 1991). Az akut miokardiális iszkémia során bekövetkezô pH csökkenés jelentôsen lelassítja a szomszédos sejtek közötti ingerületterjedést (Hermans és mtsai., 1995), így a kóros szívizomrészek elektromosan elszigetelôdnek az ép szívizomsejtektôl. A pH változás a szívizomsejteken belüli kontraktilis és szabályozó fehérjék mûködését is jelentôsen befolyásolja. Orchard és Kentish (Orchard és Kentish, 1990) munkájuk során arra a következtetésre jutottak, hogy az izommûködést szabályozó regulációs rendszer részét képezô troponin-C molekula mûködése a citoszólon belüli pH csökkenésével párhuzamosan jelentôsen megváltozhat. Véleményük szerint a csökkent pH befolyással van a kalcium ion sejten belül betöltött szerepére azáltal, hogy a nagyobb számban jelenlévô hidrogén ionok versengeni fognak a kalcium ionokkal a fehérje kalcium-kötô helyeiért, melynek következményeként a troponin-C molekula mûködésének hatékonysága csökken. Véleményük szerint a megváltozott pH direkt hatással is lehet az aktin és miozin közötti kötésekre. A csökkent intracelluláris pH érték következménye lehet a miokardium merevségének fokozódása is, melynek alapját az aktin és miozin molekulák megváltozott kapcsolata képezheti (Krayenbuehl és mtsai., 1989). Az akto-miozin komplex tulajdonságainak a változó pH értékek hatására bekövetkezô változását mutatta ki Kron és munkatársa (Kron és Spudich, 1986). Aktin filamentumok miozinnal fedett üvegfelületen történô in vitro motilitását vizsgálták a környezet egyes paramértereinek függvényében. Megfigyelték, hogy az aktin filamentumok mozgása leállt, amint a puffer oldat pH-ját 6.5–re csökkentették. A pH értékének 8.5-re történô emelésével a filamentumok motilitása fokozódott, míg a pH további emelésével a filamentumok mozgása ismét csökkent. Mindezen változások függetlennek bizonyultak az alkalmazott puffer fajtájától. Ezen kísérletes megfigyelések felvetik a kérdést, hogy noha a változó pH miozin molekulákra kifejtett hatását kizárni nem tudjuk, vajon az oldat pH értékének változása hatással van e az izom mechanikai funkciójára az aktin molekulák dinamikai tulajdonságainak befolyásolásán keresztül. A környezet változó pH értékének az aktin molekulákra kifejtett közvetlen hatása számos kutató által vizsgálatra került. A csökkenô pH érték meggyorsítja a monomerek polimerizációját (Straub, 1942), megnô a monomerek dimerizációs készsége (Zimmerle és Frieden, 1988a), valamint lecsökken az aktin kritikus koncentrációja (kritikus koncentráció: az a fehérje koncentráció, mely felett jelenlévô aktin monomerek polimerré történô átalakulása spontán bekövetkezik) (Wang és mtsai., 1989). A változó pH érték hatással lehet továbbá az aktin monomerek konformációs állapotára és kationkötô képességére is. Zimmerle és Frieden

vizsgálatai azt mutatják, hogy a kalcium ionnak a nagy affinitású kationkötô helyhez történô kötôdésének ereje tízszeresére nô, amint az oldat pH-ját lecsökkentették (Zimmerle és Frieden, 1988b). Mindezen megfigyelések tovább erôsítik azt a feltevést, mely szerint az aktin molekulák változó pH hatására bekövetkezô szerkezeti és/vagy dinamikai változásai fontos szerepet játszhatnak az izommûködés során.

Az eddigi vizsgálatok eredményei alapján alapvetô fontosságúnak tekinthetô az aktin filamentumok “passzív” jelenléte mellett, a fehérje dinamikai (“aktív”) tulajdonságaiban beállt változások szerepe az izommûködés molekuláris alapjainak pontos megértésében mind fiziológiás, mind patológiás körülmények között.

2. CÉLKITÛZÉSEK

Kísérleteink során a környezeti paramétereknek az aktin filamentumok dinamikai tulajdonságaira kifejtett hatását vizsgáltuk.

- Kísérleteink elsô felében arra az eddig nem tisztázott kérdésre kerestük a választ, hogy a kalcium ion jelenlétében polimerizált aktin filamentumok flexibilitása milyen mértékben különbözik a magnézium ion jelenlétében polimerizált filamentumok dinamikus tulajdonságaitól.
- Válaszolni kívántunk továbbá arra a kérdésre is, hogy a fellelhetô dinamikus különbségek vajon az aktin filamentumot felépítô monomereken belüli, illetve a monomerek közötti dinamikus kapcsolatot milyen mértékben érintik.
- Kísérleteket végeztünk annak eldöntésére, hogyan befolyásolja a fiziológiás és patológiás körülmények között egyaránt változó intracelluláris pH az F-aktint felépítô protomerek (az aktin filamentumba beépült monomerek megnevezése) közötti dinamikus kapcsolatot.
- A változó pH értékeknek az aktin filamentumokon belüli inter-monomer flexibilitásra kifejtett hatását mind a filamentumok kalcium ionnal telített (“aktivált filamentum” modell), mind magnéziummal telített (“relaxált filamentum” modell) állapotában jellemezni kívántuk.

3. ALKALMAZOTT MÓDSZEREK

3.1. Az aktin preparálása

Méréseinkhez az aktint nyúl harántcsíkolt izomból preparáltuk (Feuer és mtsai., 1948; Spudich és Watt, 1971). A fehérjekoncentráció meghatározásához a G-aktin esetében abszorpciós koefficiensnek a 0.63 ml mg^{-1} cm^{-1} értéket használtuk 290 nm-es hullámhosszon (Houk és Ue, 1974), a relatív molekulatömeget pedig 42.300 Da-nak tekintettük (Elzinga és mtsai., 1973).

3.2. Az aktin fluorofórral történô jelölése

Az aktin monomeren a 374-es pozícióban lévô cisztein, IAEDANS-al (N-(((iodoacetyl)amino(ethyl)-5-naphthylamine-1-szulfonat) történô jelölését a Masao Miki és munkatársai által leírt módon végeztük el (Miki és mtsai., 1987).

Az aktin IAF-el (5-(iodoacetamido)fluoreszcein) történô jelölése során a monomer formában jelenlévô aktinhoz a 0.1 normálos NaOH-ban feloldott fluorofórt tízszeres moláris feleslegben, szobahômérsékleten, a pH állandó értéken tartásával adtuk hozzá. A jelölés elsô négy órája szobahômérsékleten történt, majd a következô 8-12 órában a jelölés 4 ^{o}C-on folytatódott. A jelölés leállítása a minta polimerizálásával, valamint 1mM MEA hozzáadásával történt. Ezután a minta 80.000 g -vel, 2 órán keresztül való ultracentrifugálása, a pellet puffer oldatban történô duzzasztása, homogenizálás, majd a minta dializálása következett.

Az aktin monomeren a 41-es pozícióban lévô glutamin FC-vel (fluoreszcein-kadaverin) történô jelölése a Takashi által leírt módon történt (Takashi, 1988).

3.3. Az aktin spinjelölôvel történô módosítása

Az F-aktin molekulák MSL-el (N-(1-oxyl-2,2,6,6-tetrametil-4-piperidinil)-maleimid) történô módosítását a Mossakowska és munkatársai által leírt módon végeztük el (Mossakowska és mtsai., 1988).

3.4. Ioncsere az aktinon; az aktin polimerizációja

A kalcium tartalmú G-aktinból történô, magnézium iont tartalmazó G-aktin preparálása a Strzelecka-Golaszewska és munkatársai által leírt módon történt (Strzelecka-Golaszewska és mtsai., 1993). A G-aktin polimerizációja 100 mM KCl és a megfelelô kétértékû kation 2 mM-os végkoncentrációjának beállításával történt.

A szaturáció transzfer ESR mérésekhez a minták polimerizálása polimerizáló puffer (a megfelelô puffer 100 mM KCl-al, 0.2 mM EGTA-val és 2 mM $MgCl_2$-al vagy 100 mM KCl-al, és 2 mM $CaCl_2$–al kegészítve) elleni dialízissel történt 4^{o}C-on egy éjszakán keresztül.

3.5. Spektrofotometria

A vizsgált minták abszorpcióját termosztálható mintatartójú Shimadzu UV–2100 típusú spektrofotométerrel határoztuk meg.

3.6. Steady-state fluoreszcencia mérések

A steady-state fluoreszcencia méréseket termosztálható mintatartóval ellátott Perkin-Elmer LS50B típusú spektrofluoriméterrel végeztük. A steady-state fluoreszcencia anizotrópia számolása a polarizált fénnyel történô gerjesztés után mért emittált és egyben polarizált fény komponenseibôl a következô módon történt:

$$r = (F_{VV} - GF_{VH}) / (F_{VV} + 2GF_{VH}) \quad (1)$$

ahol az alsó indexek betûi közül az elsô a gerjesztési polarizátor, míg a második az emissziós oldali polarizátor állását jelenti (V: függôleges, H: vízszintes), G pedig a mûszerállandó ($G = F_{HV} / F_{HH}$).

3.7. Fluoreszcencia-élettartam és anizotrópia lecsengés mérések

Idôfüggô fluoreszcencia méréseinket termosztálható mintatartóval ellátott ISS K2 típusú (ISS Fluorescence Instrumentation, Champaign, IL, USA) multi-frekvenciás fázisfluoriméterrel végeztük.

Méréseink során a fluoreszcencia átlagélettartamot, diszkrét élettartam eloszlás feltételezése esetén a következô módon határoztuk meg:

$$\tau_{átlag} = (\sum \alpha_i \tau_i^{\,2}) / (\sum \alpha_i \tau_i) \quad (2)$$

ahol az α_i és a τ_i az egyes élettartam komponensek amplitúdóját és értékét jelentik. Az átlagélettartamok hômérséklettôl való függésének jellemzésére az Arrhenius analízist alkalmaztuk:

$$(\tau_{átlag})^{-1} = k_0 + A \exp (- E^* / RT) \quad (3)$$

ahol k_0 egy hômérséklettôl független sebességi állandó, A a frekvenciafaktor, E* az aktivációs energia, T az abszolút hômérséklet, R pedig az egyetemes gázállandó. Az anizotrópia lecsengés mérések kiértékelése során kettôs exponenciális fügvény szerinti lecsengést feltételeztünk:

$$r(t) = r_1 \exp(- t / \varphi_1) + r_2 \exp(- t / \varphi_2) \quad (4)$$

ahol a φ_i a rotációs korrelációs idô r_i amplitúdóval.

3.8. Fluoreszcencia rezonancia energiatranszfer (FRET) mérések

Fluoreszcens donor-akceptor párt alkotó fluorofórok esetén az energiatranszfer hatásfokát a következô módon határozhatjuk meg:

$$E = 1 - (F_{DA} / F_D) \quad (5)$$

ahol F_{DA} a donor intenzitása akceptor jelenlétében, F_D pedig akceptor nélkül. Az energiatranszfer hatásfokának ismeretében kiszámolható a donor és az akceptor molekulák közötti távolság (R) a következô egyenlet alkalmazásával:

$$E = R_0^6 / (R_0^6 + R^6) \quad (6)$$

ahol R_0 az úgynevezett Förster-féle kritikus távolság, ahol a donor és akceptor molekulák közötti energiatranszfer hatásfoka 50%-os. Kiszámítása a következô összefüggés alapján lehetséges:

$$R_0^6 = (8.79 \times 10^{-11}) n^{-4} \kappa^2 \phi_D J \quad (7)$$

ahol n a közeg törésmutatója, κ^2 a donor-akceptor molekula relatív pozícióját jellemzô orientációs faktor, ϕ_D a donor molekula fluoreszcencia kvantumhatásfoka, és J a donor fluoreszcencia spektruma és az akceptor abszorpciós spektrumának átfedési integrálja M^{-1} cm^{-1} nm^4 egységben kifejezve.

Az átfedési integrál kiszámítására a következô összefüggés nyújt segítséget:

$$J = \int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda / \int F_D(\lambda)d\lambda \quad (8)$$

ahol a $F_D(\lambda)$ a donor molekula fluoreszcencia emissziós spektruma, $\varepsilon_A(\lambda)$ az akceptor molekula abszorpciója a hullámhossz (λ) függvényében.

Méréseink során a fluoreszcencia spektrumokat mindig korrigáltuk az adott minta abszorpciós spektruma segítségével, az “inner filter” effektus figyelembe vételével:

$$F_{corr} = F_{obs} \text{ antilog } (OD_{ex} + OD_{em}) \quad (9)$$

mely összefüggésben az F_{corr} és F_{obs} értékek a korrigált és mért fluoreszcencia intenzitások, az OD_{ex} és OD_{em} pedig a minta abszorpciós értékei a gerjesztési és emissziós hullámhosszakon mérve.

3.9. Elméleti megfontolások a normalizált transzferhatásfok alkalmazásával kapcsolatban

A FRET hatékonyságának definíciója a kövekezô:

$$E = k_t / (k_t + k_f + k_0) \quad (10)$$

ahol a k_t az energiatranszfer sebességi állandója, a k_f a fluoreszcencia donor emisszióra jellemzô sebességi állandó, míg a k_0 a donor molekula gerjesztett állapotának megszûnésében

résztvevô, egyéb folyamatok sebességi állandóinak összege. Az energiatranszfer sebességi állandója (k_t) a következô módon számolható:

$$k_t = d\ J\ n^{-4}\ k_f\ R^{-6}\ \kappa^2 \qquad (11)$$

A fenti egyenletben használt d az alkalmazott mértékegységektôl függô állandó. Számolásaink során a törésmutatóra (n) 1.4-et, az orientációs faktorra (κ^2) pedig 2/3–os értéket használtunk.

Fluoreszcens festékkel jelölt fehérjék esetén meghatározható egy paraméter (f vagy f': a fluoreszcencia rezonancia energiatranszfer hatásfokának és a donor molekula akceptor jelenlétében mért kvantumhatásfokának vagy intenzitásának hányadosa), mely arányos az energiatranszfer sebességi állandójának átlagértékével ($<k_t>$) (Somogyi és mtsai., 1984):

$$f = <E>/<\phi_{DA}> \text{ vagy } f' = <E>/F_{DA} \qquad (12)$$

A fenti kifejezésben ϕ_{DA} a donor kvantumhatásfoka az akceptor molekula jelenlétében. Az f, illetve f' értéke egyaránt függ a vizsgált rendszer szerkezeti és dinamikai tulajdonságaitól, és az alkalmazott donor és akceptor molekulák spektrális paramétereitôl (Somogyi és mtsai., 1984):

$$f = <E>/<\phi_{DA}> = d\ n^{-4}\ J\ <R_i^{-6}\ \kappa_i^{\ 2}> \qquad (13)$$

A fenti megfontolások alapján információt nyerhetünk a fehérjék dinamikai és konformációs tulajdonságairól az f paraméter hômérséklettôl való függését nyomon követve. Vizsgálataink során az f paraméter relatív értékének változását vizsgáltuk a hômérséklet függvényében. Az f relatív értékét az f aktuális értékének a legkisebb hômérsékleten meghatározott f értékkel történô normálásával kaptuk meg. Az f paraméternek a hômérséklet függvényében történô változása tájékoztathat bennünket a vizsgált molekulák dinamikai tulajdonságairól. Alapvetôen két molekuláris mechanizmus felelôs az f paraméter értékének hômérséklettôl való függéséért. Az elsô a fluoreszcens donor és akceptor molekula közötti átlagos távolság (R) megváltozása (13. egyenlet), mely a fehérje szerkezetében beállt konformációs változást tükröz. Ebben az esetben az f paraméter hômérséklettôl való függésének meredekségében kis hômérséklet tartományon belül jelentôs változás ("törés") áll be, ezáltal elvesztheti szigorúan monoton növekvô jellegét. A második mechanizmus, mely felelôssé tehetô az f paraméter hômérséklettôl való függéséért, a fluoreszcens akceptor molekula relatív, "oszcilláló" mozgásának megváltozása a hômérséklet hatására. A hômérséklet növelésével a donor és akceptor molekula relatív fluktuációjának növekedése az f paraméter szigorúan monoton növekedését eredményezi, az ábrázolt hômérsékletprofilban beállt "törés" nélkül.

Mindezek alapján megállapítható, hogy az f paraméter hômérsékletprofilja flexibilisebb fehérjerész esetén meredekebb a merevebb formával összehasonlítva, abban az esetben ha a

fehérje szerkezetében bekövetkezô jelentôs konformációváltozás kizárható. Az említett elméleti megfontolások alapján az f paraméter hômérséklettôl való függésének nyomonkövetésével lehetôség nyílik a fehérje eltérô flexibilitású és/vagy konformációs állapotú formái közötti különbség kimutatására.

3.10. ESR spektroszkópia

A konvencionális és szaturáció traszfer ESR (ST-EPR) spektrumokat ESP 300E (Bruker, Germany) spektrométerrel mértük. A konvencionális ESR méréseknél az abszorpciós spektrum rögzítése 20 mW mikrohullámú teljesítménnyel, 100 kHz-es térmodulációval és 0.1-0.2 mT amplitúdóval történt. A szaturáció transzfer ESR abszorpciós spektrum rögzítése 63 mW teljesítménnyel 50 kHz es modulációval, 0.5 mT amplitúdóval valamint 100 kHz –es “out-of-phase” detektálással történt. A spektrumok analizálására a WIN-EPR 3.0 -t, valamint önállóan kifejlesztett szoftvert használtunk.

4. EREDMÉNYEK ÉS KÖVETKEZTETÉSEK

4.1. Kétértékû kationok hatása az aktin filamentumok dinamikai tulajdonságaira

Attól függôen, hogy az aktin monomerek nagy affinitású kationkötô helye milyen kétértékû kationnal telített, a belôlük polimerizált filamentumok flexibilitásában jelentôs különbségek mérhetôk. Vizsgálataink során célunk volt annak tanulmányozása, hogy nanoszekundumos (fluoreszcenciás mérések) és milliszekundumos (EPR mérések) idôskálán milyen konformációs, illetve flexibilitásbeli változások figyelhetôk meg az F-aktinban, ha a fiziológiás körülmények között jelenlévô magnézium ion helyett kalcium ionnal telítjük a nagy affinitású kationkötô helyet. A különbözô idôskálákon elvégzett vizsgálatok a fehérje eltérô méretû szegmenseinek tulajdonságairól adhatnak felvilágosítást.

Az aktin monomer C-terminális végén lévô Cys-374 aminosavhoz kapcsolt IAEDANS molekula fluoreszcencia-élettartamát és anizotrópia lecsengését mértük a hômérséklet függvényében. A hômérsékletfüggô fluoreszcencia-élettartam mérések Arrhenius analízise (3. egyenlet) során mind az aktivációs energia (E*), mind a frekvencia faktor (A) értéke kisebb volt a magnézium ionnal telített F-aktin esetén. A fluoreszcencia anizotrópia lecsengés mérések során mért rotációs korrelációs idôk hosszú komponensének értékét 6 és 28^{o}C között nagyobbnak találtuk a nagy affinitású kationkötô helyen magnézium ionnal telített fehérje esetén. Ez a különbség azonban 28^{o}C fölötti hômérséklettartományon elhanyagolhatóvá vált. Ezen eredmények egyértelmûen arra utalnak, hogy a Cys-374 aminosav környezetében lévô fehérjerész szerkezeti, illetve flexibilitásbeli tulajdonságai érzékenyek az aktin által kötött kation minôségére. A mérések alapján a magnézium ion tartalmú F-aktin molekulák merevebbnek mutatkoztak a kalcium iont tartalmazó formával összehasonlítva.

Az aktin monomer Cys-374 aminosavjához kapcsolt MSL molekula segítségével az aktinon szaturáció transzfer elektron paramágneses rezonancia méréseket végeztünk. A paramágneses szondával jelölt filamentumokon végzett mérések azt mutatják, hogy a kalcium ionnak magnézium ionra történô cseréje a spinjelölô mozgékonyságának csökkenését eredményezte a milliszekundumos idôskálán.

Eredményeink alapján arra a következtetésre jutottunk, hogy a kalcium ion jelenlétében polimerizált aktin filamentumok flexibilitása nagyobb, mint a magnézium ionok jelenlétében preparált filamentumoké. Az említett különbség mind a nanoszekundumos, mind a milliszekundumos idôskálán kimutatható volt.

4.2. Az aktin filamentumok flexibilitásának vizsgálata fluoreszcencia rezonancia energiatranszfer segítségével

További méréseink során az f’ paraméter felhasználásával arra a kérdésre kerestük a választ, hogy a kalcium ion hatására bekövetkezô flexibilitás növekedésért milyen mértékben tehetô felelôssé a filamentumon belüli intra-monomer és/vagy inter-monomer flexibilitás megváltozása. Méréseink során az aktin monomerek 374-es számú cisztein aminosavjához fluoreszcens donor (IAEDANS) vagy fluoreszcens akceptor (IAF) molekulát kapcsoltunk, majd az eltérô módon jelölt monomereket a monomerek polimerizációja elôtt összekevertük. Ezen kísérleteinkben a monomerek polimerizációjával nyert filamentumokban az IAEDANS-IAF fluoreszcens donor-akceptor pár között számolt f’ paraméter hômérséklettôl való függésének vizsgálatával a monomerek közötti flexibilitás tanulmányozására nyílt módunk. Egy másik jelölési folyamat során az IAEDANS-szel (fluoreszcens donor) már megjelölt monomerek 41-es számú glutamin aminosavjához FC (fluoreszcens akceptor) molekulát kapcsoltunk. Az IAEDANS-FC donor-akceptor pár esetében számolt f’ paraméter hômérséklettôl való függésének vizsgálatával az intra-monomer flexibilitást tanulmányoztuk. A relatív f’ mind az intra-monomer, mind az inter-monomer flexibilitás vizsgálata során nagyobb flexibilitást mutatott a kalcium ionnal telített filamentumok esetén. Az inter-monomer flexibilitás mind a kalcium ionnal, mind a magnézium ionnal telített aktin filamentum esetén nagyobb volt az intra-monomer flexibilitásnál. A 41-es glutamin és a 374-es cisztein aminosavak között mért távolság mind a kation minôségétôl, mind a polimerizációtól függetlenül állandónak bizonyult, igazolva ezzel a két pont közötti fehérjeszerkezetben beállt konformációs változás hiányát.

Méréseink segítségével megállapítható volt, hogy a nagy affinitású kationkötô helyen kalciummal telített aktin filamentumok nagyobb flexibilitásának kialakulásában mind az intra-monomer, mind az inter-monomer kapcsolatok flexibilitásának növekedése szerepet játszik.

4.3. A kalcium vagy magnézium iont tartalmazó aktin filamentumok inter-monomer flexibilitásának vizsgálata fiziológiás és patológiás pH értékeken

Annak tisztázására, hogy az aktin filamentum dinamikája az intracelluláris pH változásának hatására megváltozik-e, hômérsékletfüggô fluoreszcencia rezonancia energiatranszfer méréseket végeztünk aktin filamentumokon különbözô pH értékeken. Kísérleteink során a korábbiakban már ismertetett módon az aktin egyik populációját a 374-es számú cisztein aminosavon jelöltük meg IAEDANS–szel (fluoreszcens donor), míg egy másik populációt ugyanezen az aminosavon IAF-fel (fluoreszcens akceptor). Az inter-monomer flexibilitás vizsgálata során az f paraméter hômérséklettôl való függésének meghatározását mind kalcium-F-aktinon, mind magnézium-F-aktinon, pH 6.5, valamint pH 7.4 értékeknél végeztük el.

Magnézium-F-aktin esetén a relatív f paraméter változása szerint az alacsonyabb pH értéken a fluoreszcens donor-akceptor pár közötti proteinmátrix flexibilitása kisebb volt, azaz a vizsgált fehérjerész merevebbé vált, a magasabb pH értéken jellemzett flexibilitáshoz képest. Ezen megfigyeléssel összhangban, az alacsonyabb pH értéken a kalcium ionnal telített F-aktin esetében is merevebb fehérjestruktúrát tudtunk kimutatni, a fehérje magasabb pH értéken jellemzett flexibilitásával összevetve.

Méréseink során az inter-monomer flexibilitás az alkalmazott mindkét pH érték esetén nagyobbnak bizonyult a kalcium ionnal telített aktin filamentumok esetén, ami alátámasztja korábbi, pH 8.0 érték mellet mért eredményeinket (ld. 1. és 2. sz. közlemények).

Eredményeinkbôl egyértelmûen kimutatható, hogy az intracelluláris pH megváltozásának alapvetô hatása van az aktin filamentum dinamikai állapotára, mely változás feltehetôen az aktin és a miozin molekulák közötti kapcsolatot is jelentôsen befolyásolhatja.

5. AZ EREDMÉNYEK ÖSSZEFOGLALÁSA

Vizsgálataink során részleteiben tártuk fel a kalcium ionnak az aktin filamentum flexibilitására kifejtett hatását, melynek során az izomkontrakcióban résztvevô egyik kulcsszereplô fehérje alapvetô dinamikai tulajdonságainak alaposabb megértésére nyílt módunk. Megállapítottuk, hogy a nagy affinitású kationkötô helyén kalcium iont tartalmazó aktin filamentum flexibilisebb a magnézium iont tartalmazó F-aktinnal összehasonlítva mind a nanoszekundumos, mind a milliszekundumos idôskálán. Ezen következtetésünk igaznak bizonyult pH 8.0, pH 7.4 és pH 6.5 értékek mellett is.

Megállapítottuk, hogy a kalcium-F-aktin nagyobb flexibilitásának létrejöttében a filamentumon belüli inter-monomer, valamint az intra-monomer flexibilitás növekedése egyaránt szerepet játszik. Az inter-monomer flexibilitás nökedése erôteljesebbnek bizonyult a monomereken belüli flexibiltás növekedésével szemben.

Vizsgálatokat végeztünk a fiziológiás és patológiás körülmények között egyaránt változó intracelluláris pH-nak, az aktin filamentumok flexibilitására kifejtett hatásának tisztázása érdekében. Méréseinkkel kimutatható volt, hogy a csökkent intracelluláris pH hatására az aktin filamentumokon belüli inter-monomer kapcsolatok merevebbé válnak. A filamentumok flexibilitásának alcsony pH hatására bekövetkezô csökkenése kimutatható volt a modellezett “aktivált” (kalcium-F-aktin) és “relaxált” (magnézium-F-aktin) állapotban egyaránt.

Eredményeink alapján kijelenthetjük, hogy mivel a kalcium-F-aktin a miozinnal történô összekapcsolódásban résztvevô aktin filamentumot reprezentálja (“aktivált” filamentum), valószínûleg a húzási ciklus azon fázisában, ahol az aktin és a miozin közötti közvetlen kapcsolat létrejön, az erôkifejtésben az aktin filamentum flexibilisebb szereplôként vesz részt, a “relaxált” állapotával összevetve. Az eredményes erôkifejtés alapját képezi, hogy az aktin filamentum megfelelô dinamikus állapotban legyen. Ezen megállapítást erôsíti az a megfigyelés is, hogy az intracelluláris pH értékének csökkenését az izom teljesítménycsökkenése kísérheti, mely teljesítménycsökkenésre az intracelluláris pH csökkenés hatására az aktin filamentumokban kialakuló csökkent flexibilitás is jellemzô. Az aktin filamentum megváltozott dinamikai állapota az aktin és a miozin molekula közötti kapcsolatot jelentôsen befolyásolhatja, melyet az akto-miozin komplex megváltozott mûködése egyértelmûen tükrözhet.

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9. A PHD ÉRTEKEZÉS ALAPJÁT KÉPEZÕ KÖZLEMÉNYEK

9.1. The Influence of Divalent Cations on the Dynamic Properties of Actin Filaments: A Spectroscopic Study (1. számú közlemény)

The Influence of Divalent Cations on the Dynamic Properties of Actin Filaments: A Spectroscopic Study

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ABSTRACT The principal aim of this investigation was to study the change of the protein flexibility and/or conformational properties of actin filaments upon the replacement of Ca^{2+} by Mg^{2+}. The temperature dependence of the fluorescence lifetime and the anisotropy decay of *N*-(iodoacetyl)-*N′*-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS) attached covalently to the Cys^{374} residue of actin were measured. Saturation transfer electron paramagnetic resonance (ST-EPR) experiments were also carried out using *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide (MSL) attached to the same residue (Cys^{374}). The Arrhenius analysis of the temperature dependence of the fluorescence lifetimes shows that for Mg-F-actin, both the activation energy (E^*) and the frequency factor (A) are smaller than they are for Ca-F-actin. The longer rotational correlation times resolved in the fluorescence experiments are larger in the Mg^{2+}-loaded form of the actin filament between 6°C and 28°C, but this difference becomes negligible above 28°C. The results of saturation transfer electron paramagnetic resonance measurements on maleimide spin-labeled actin filaments indicate that the replacement of Ca^{2+} by Mg^{2+} induced a decrease of the mobility of the label on the sub-millisecond time scale. Based upon these results, we concluded that the filaments polymerized from Ca-actin are more flexible than the filaments of Mg-actin.

INTRODUCTION

The conformational state and flexibility of the actin filament play an important role in muscle contraction (Prochniewicz and Yanagida, 1990; Rayment et al., 1993). The rotational correlation times characteristic of different modes of internal motion within the actin filament are generally distributed across a wide time scale from the nanosecond to the millisecond range. Depending on the experimental method applied, various types of these intramolecular motions can be identified.

The relaxation time of the actin filament bending motion is on the order of 10 ms (Fujime and Ishiwata, 1971). The results of saturation transfer electron paramagnetic resonance (ST-EPR) experiments revealed that a spin label rigidly attached to F-actin has a correlation time in the range of 10^{-4} s (Thomas et al., 1979; Hegyi et al., 1988). The correlation time expected for the uniaxial rotation of the F-actin molecule as a whole is $\sim 20-40$ μs (Kawamura and Maruyama, 1970; Tawada et al., 1978), which range is therefore comparable with the range obtained from the data of saturation transfer EPR experiments. Mihashi and coworkers concluded from transient absorption anisotropy experiments that the actin filaments are more flexible in twisting motion than in bending motion (Mihashi et al., 1983). The correlation times resolved in these measurements was in the microsecond range, reflecting some kind of internal motion of F-actin. The torsional motion is faster than the above modes and has been studied by fluorescence techniques (Mihashi and Wahl, 1975; Wahl et al., 1975; Kawasaki et al., 1976; Tawada et al., 1978; Ikkai et al., 1979; Miki et al., 1982a,b). The longer correlation times were resolved in the range of several hundreds of nanoseconds according to fluorescence anisotropy decay measurements.

The binding of Ca^{2+} to the actin in the presence of 100 mM KCl increased the mobility of the fluorescence label attached to the Cys^{374} residue of the protein (Miki et al., 1982a,b). The authors concluded that the change to the longer correlation time must be due to a conformational transition. Recent results of nuclear magnetic resonance (NMR) experiments on actin filaments revealed cation-dependent changes in the mobility of the *N*-terminal segment (first 21 amino acids) of actin (Heintz et al., 1996). The torsional rigidity of actin filaments, however, is larger when Ca^{2+} occupies the high affinity binding site than when Mg^{2+} occupies the site (Yasuda et al., 1996). Orlova and Egelman found that the bending flexibility of filaments polymerized from Mg-actin is greater than that of filaments polymerized from Ca-actin (1993). A "bridge of density" was found between the two strands of filament when the high affinity cation-binding sites were occupied by Ca^{2+}, whereas this "bridge of density" was not detected in Mg-F-actin (Orlova and Egelman, 1995). However, other laboratories found essentially no cation dependence of the flexibility of filaments using either dynamic light scattering measurements (Scharf and Newman, 1995), or other techniques (Isambert et al., 1995; Steinmetz et al., 1997) to determine the persistence length of filaments. Direct measurement of the flexibility of single actin filaments corroborates this conclusion (Yasuda et al., 1996). The apparent inconsistency of these data was attributed in part to subtle differences in the polymerization conditions (i.e., in terms of nucleotide and cation compositions and/or concentrations) (Steinmetz et al., 1997).

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Accordingly, there is no consistent picture of the effect of bound metal ion (i.e., Ca^{2+}, Mg^{2+}) on the flexibility of actin filaments. In this paper we direct attention to the effect of replacement of Mg^{2+} with Ca^{2+} on the flexibility and/or conformational properties of the actin filament, using fluorescence and EPR spectroscopic methods. We measured the temperature dependence of the fluorescence lifetime and the anisotropy decay of *N*-(iodoacetyl)-*N′*-(5-sulfo-1-naphthyl) ethylenediamine (IAEDANS) and the saturation-transfer EPR data obtained by using MSL, both attached covalently to the Cys^{374} at the C-terminus on subdomain-1 of actin. These spectroscopic experiments provided evidence that filaments polymerized from Mg-actin are more rigid than filaments of Ca-actin.

MATERIALS AND METHODS

Materials

KCl, $MgCl_2$, $CaCl_2$, Tris, glycogen, IAEDANS, MSL, and EGTA were obtained from Sigma Chemical Co. (St. Louis, MO). ATP and α-mercaptoethanol were obtained from Merck (Darmstadt, Germany), and NaN_3 from Fluka (Buchs, Switzerland).

Protein preparation

Acetone-dried powder of rabbit skeletal muscle was obtained as described earlier (Feuer et al., 1948). Rabbit skeletal muscle actin was prepared according to Spudich and Watt (1971) and stored in a buffer containing 2 mM Tris-HCl (pH 8.0), 0.1 mM ATP, 0.1 mM $CaCl_2$, and 0.02% NaN_3 (buffer A). The concentration of G-actin was determined spectrophotometrically using the absorption coefficient of 0.63 mg ml^{-1} cm^{-1} at 290 nm (Houk and Ue, 1974), with a Shimadzu UV-2100 type spectrophotometer. Relative molecular mass of 42,300 was used for G-actin (Elzinga et al., 1973).

Fluorescence labeling of actin

Actin labeled fluorescently with IAEDANS at Cys^{374} was prepared according to the method of Miki and co-workers (1987). 2 mg/ml F-actin (in buffer A without α-mercaptoethanol, supplemented with 100 mM KCl and 2 mM MgCl2) was incubated with tenfold molar excess of IAEDANS at room temperature for 1 h. The label was first dissolved in dimethylformamide; its final concentration in the labeling solution did not exceed 0.6% (v/v). The solution was diluted $\sim 50\times$ with buffer A before being added to the protein. Labeling was terminated with 1 mM α-mercaptoethanol. After ultracentrifugation of the sample at 100,000 *g* the pellet was incubated in buffer A for 1 h and gently homogenized with a Teflon homogenizer. The homogenated sample was exhaustively dialyzed overnight against buffer A at 4°C. The concentration of fluorescent dye in the protein solution was determined by using the absorption coefficient of 6100 M^{-1} cm^{-1} at 336 nm for actin-bound IAEDANS (Hudson and Weber, 1973). The extent of labeling was determined to be 0.85 $\pm$ 0.05 mol/mol of actin monomer.

Spin labeling of actin

G-actin was polymerized by adding KCl and $MgCl_2$ to final concentrations of 100 and 2 mM, respectively. Actin was labeled in F-form with MSL as described earlier (Mossakowska et al., 1988). One mole of spin label per mole of actin monomer was reacted for 90 min over ice. Unreacted labels were removed as described above. The degree of labeling ranged from 0.2–0.55 mol/mol of actin.

Cation exchange on actin

Mg-G-actin was prepared from Ca-G-actin following the method of Strzelecka-Golaszewska and colleagues (1993). The protein solution was dialyzed overnight against buffer A (in which the concentration of $CaCl_2$ was 50 μM). EGTA and $MgCl_2$ were added from stock solutions to reach final concentrations of 0.2 mM and 0.1 mM, respectively, in the protein solution. The sample was incubated for 10 min at room temperature. Mg-F-actin and Ca-F-actin were prepared from Mg-G-actin and Ca-G-actin, respectively, by the addition of 100 mM KCl and 2 mM of the appropriate cation ($MgCl_2$ or $CaCl_2$) to the sample. Incubation time was 2 h for Mg-F-actin and at least 4 h for Ca-F-actin at room temperature.

For saturation-transfer EPR measurements the concentration of F-actin was at least 100 μM. In some cases the precipitation of the protein was observed when a highly concentrated solution of salts (2–3 M solution of KCl, $CaCl_2$, or $MgCl_2$) was added to the Mg-G-actin or Ca-G-actin to initialize polymerization. To avoid this condensation, we obtained the filaments with different cations by dialysing the samples in the appropriate polymerization buffer (i.e., buffer A supplemented with 100 mM KCl, 0.2 mM EGTA, and 2 mM $MgCl_2$ for Mg-F-actin, or buffer A with 100 mM KCl and 2 mM $CaCl_2$ for Ca-F-actin). Polymerization was carried out overnight at 4°C.

Light-scattering experiments

The polymerization capability of Ca-actin and Mg-actin was examined by measuring the change in the intensity of Rayleigh-scattered light on actin during the polymerization process. The experiments were performed on a Perkin-Elmer LS50B luminescence spectrometer (Norwalk, CT) equipped with thermally controlled cell holder at 22°C. The intensity of the scattered light was measured perpendicular to the incident light. Both the excitation and emission wavelengths were 600 nm with 3-nm slits.

Fluorescence lifetime and emission anisotropy decay measurements

The fluorescence measurements were made with an ISS K2 multifrequency phase fluorometer (ISS Fluorescence Instrumentation, Champaign, IL) using the frequency cross-correlation method. The excitation light was provided by a 300-W Xe arc lamp and was modulated with a double-crystal Pockels cell. Excitation wavelength was set to 350 nm and the emission was monitored through a KV 370 high-pass filter. The modulation frequency was changed in 10 steps (linearly distributed on a logarithmic scale) from 2 to 80 MHz in fluorescence lifetime measurements and from 2 to 150 MHz in anisotropy decay measurements. The phase delay and demodulation of the sinusoidally modulated fluorescence signal were measured with respect to the phase delay and demodulation of a standard reference substance. Freshly prepared glycogen solution was used as a reference (lifetime = 0 ns). The fluorescence lifetimes of the fluorophore were determined by the use of nonlinear least-square analysis. In anisotropy decay measurements the sample was excited with sinusoidally modulated and polarized light. To resolve the anisotropy decay parameters, the difference between the phase angle and modulation ratio of the parallel and perpendicular components of the emission was used. The data were analyzed by the ISS187 decay analysis software assuming a constant, frequency-independent error in both phase angle ($\pm$ 0.200°) and modulation ratio ($\pm$ 0.004). The goodness of fit was determined from the value of the reduced χ^2 defined as in Lakowicz (1983):

$$\chi^2 = \Sigma \frac{[(P_c - P_m)/\sigma^P]^2 + [(M_c - M_m)/\sigma^M]^2}{2n - f - 1} \qquad (1)$$

where the sum is carried over the measured values at n modulation frequencies and f is the number of free parameters. The symbols P and M correspond to phase shift and relative demodulation values, respectively. The indices c and m indicate the calculated and measured values respectively. σ^P and σ^M are the standard deviations of each phase and modulation measurements.

Average fluorescence lifetimes were calculated from the results of the analysis assuming discrete lifetime distribution as described by Lakowicz (1983):

$$\tau_{aver} = (\Sigma\alpha_i\tau_i^2)/(\Sigma\alpha_i\tau_i) \quad (2)$$

where τ_{aver} is the average fluorescence lifetime and α_i and τ_i are the individual amplitudes and lifetimes, respectively. Assuming that the temperature-dependence of the fluorescence of IAEDANS is represented by a single Arrhenius factor, the average fluorescence lifetime was analyzed as a function of temperature according to the equation:

$$(\tau_{aver})^{-1} = k_o + A\exp(-E^*/RT) \quad (3)$$

where k_o is the temperature-independent rate constant (both radiative and nonradiative), A is the frequency factor, E^* is the activation energy, T is the absolute temperature, and R is the molar gas constant.

The anisotropy is expected to decay as a sum of exponentials (Belford et al., 1972). The experimentally obtained data were fitted to a double exponential function:

$$r(t) = r_1\exp(-t/\varphi_1) + r_2\exp(-t/\varphi_2) \quad (4)$$

where φ_i are the rotational correlation times with amplitudes r_i. The limiting anisotropy recovered at zero time is given by:

$$r_o = r_1 + r_2 \quad (5)$$

The concentration of actin was 23 μM (1 mg/ml) during the fluorescence measurements. Temperature-dependent experiments were carried out using a thermostated sample holder, which was flushed with dry air to avoid condensation. The temperature was maintained with a HAAKE F3 heating bath and circulator (HAAKE Mess-Technik GmbHu. Co., Karlsruhe, Germany) and the temperature of the solution was continuously monitored in the sample holder.

EPR measurements

Conventional and ST-EPR spectra were taken with an ESP 300E (Bruker, Karlsruhe, Germany) spectrometer. First harmonic, in-phase absorption spectra were obtained using 20 mW microwave power and 100 kHz field modulation with amplitude of 0.1–0.2 mT. Second harmonic, 90° out-of-phase absorption spectra were recorded with 63 mW and 50 kHz field modulation of 0.5 mT amplitude detecting the signals at 100 kHz out of phase. The microwave power of 63 mW corresponds to an average microwave field amplitude of 0.025 mT in the center region of the standard tissue cell of Zeiss (Carl Zeiss, Jena, Germany), and the values were obtained using the standard protocols (Fajer and Marsh, 1982; Squire and Thomas, 1986). The samples ($\sim$ 50 μl) were thoroughly mixed on the surface of the flat cell to avoid the effect of orientation of associated filaments. The spectra were recorded at 22.0°C. The conventional EPR spectra were characterized with the splitting of the outermost extreme ($2A'_{ZZ}$), the accuracy of the estimation of splitting was $<$0.025 mT. In the saturation transfer EPR time domain, the diagnostic peak heights L and L″ were used to calculate the rate of molecular motions. The spectra were scaled to the same peak-to-peak amplitude or normalized to an identical double integral. Evaluation of the ST-EPR spectra was performed by a computer program written in our laboratory that calculates the extremum of the spectrum in a given interval. Varying the endpoints of the selected interval in the neighborhood of the extremum and running the program three to five times, it is possible to get a reliable mean value for L″ and L even in the case of noisy spectra.

RESULTS

The completion of polymerization was tested by light-scattering measurements for both Ca-actin and Mg-actin (Fig. 1). These data indicate that the polymerization process was complete after 4 h and 2 h in the case of Ca-actin and Mg-actin, respectively. Fluorescence lifetimes were measured in Ca^{2+}- or Mg^{2+}-loaded filaments as a function of temperature in five steps ranging from 9 to 33°C. Fluorescence data were analyzed by assuming either discrete or continuous (Gaussian) lifetime distributions. The adequacy of the assumptions was tested by monitoring the value of the reduced χ^2 (Eq. 1). In the case of Gaussian distribution the values of χ^2 were larger than that for the two components discrete analysis in both Mg-F-actin and Ca-F-actin. The analysis assuming discrete components was carried out for one-, two-, or three-exponentials decay model. Better fits were obtained by accepting the two-exponential decay model (with a typical χ^2 of 1.5–2.5) than by assuming a single exponential decay (with χ^2 of 11–19) in both Mg-F-actin and Ca-F-actin. Involving a third lifetime component in the analysis improved χ^2 negligibly. Therefore, in agreement with previous observations (e.g., Ikkai et al., 1979; Miki et al., 1982a,b), the two-exponential fluorescence decay with discrete distribution seems to be the best model for characterizing the fluorescent lifetime of IAEDANS attached to either Ca-F-actin or Mg-F-actin (Table 1).

The average fluorescence lifetime values were calculated for further analysis (Eq. 2, Table 1). This parameter is longer at temperatures below 28.5°C in Ca-F-actin than in Mg-F-actin but the difference disappears at $\sim$ 33°C. As the temperature increased the values of lifetimes decreased in both forms of F-actin; this change was more intense in the

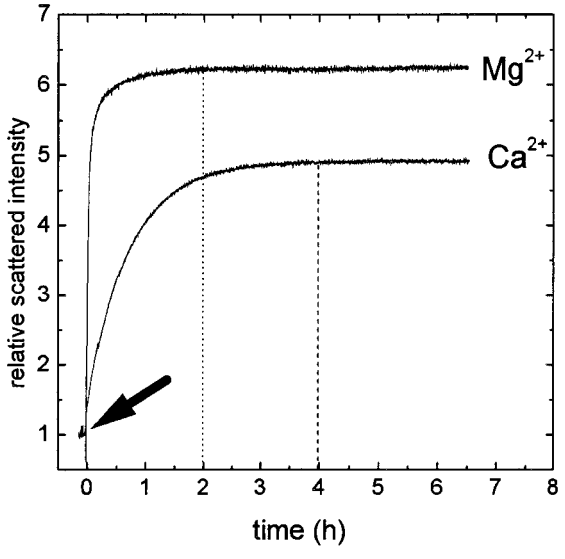

FIGURE 1 The relative intensity of the scattered light as a function of time during the polymerization process in Ca-actin and Mg-actin. Polymerization was initiated by the addition of 100 mM of KCl and 2 mM of the appropriate cation (final concentrations). The arrow indicates the time of addition of salts.

TABLE 1 Lifetime parameters for IAEDANS in Mg-F-actin and Ca-F-actin analyzed with the assumption of two-component discrete distribution of fluorescence decays

Parameter	Mg-F-actin				Ca-F-actin			
t (°C)	τ_1* (ns)	τ_2 (ns)	α_1	$\tau_{average}$ (ns)	τ_1 (ns)	τ_2 (ns)	α_1	$\tau_{average}$ (ns)
9.5	19.63 (± 0.07)	3.11 (± 0.74)	0.984 (± 0.002)	19.58 (± 0.06)	20.64 (± 0.54)	5.49 (± 3.30)	0.972 (± 0.013)	20.51 (± 0.46)
15.0	19.35 (± 0.05)	2.94 (± 0.59)	0.985 (± 0.003)	19.31 (± 0.04)	20.22 (± 0.48)	4.85 (± 2.48)	0.974 (± 0.014)	20.11 (± 0.40)
22.0	19.10 (± 0.04)	3.10 (± 0.41)	0.983 (± 0.002)	19.06 (± 0.03)	19.63 (± 0.23)	4.80 (± 1.57)	0.971 (± 0.010)	19.52 (± 0.18)
28.5	18.86 (± 0.08)	3.37 (± 0.68)	0.980 (± 0.004)	18.80 (± 0.07)	18.97 (± 0.15)	3.33 (± 1.51)	0.980 (± 0.006)	18.91 (± 0.14)
33.0	18.72 (± 0.07)	2.989 (± 0.693)	0.981 (± 0.004)	18.67 (± 0.05)	18.74 (± 0.23)	3.98 (± 1.57)	0.976 (± 0.007)	18.66 (± 0.21)

*τ_1 and α_i are the lifetimes and pre-exponential factors, respectively, where $\alpha_1 + \alpha_2 = 1$. τ_{aver} is the average fluorescence lifetime calculated according to Eq. 1. The standard deviations are presented in parentheses.

Ca^{2+}-loaded than in the Mg^{2+}-loaded form. Arrhenius plots (Eq. 3) were used for further characterization of the temperature-dependence of lifetime values for Mg-F-actin and Ca-F-actin. Plots of $\ln(\tau_{aver}^{-1} - k_o)$ vs. $(RT)^{-1}$ were constructed (e.g., Fig. 2) and the analysis of the fits were made by applying Eq. 3 to calculate the Arrhenius parameters. The k_o was assumed to be cation-independent and was fixed during the fitting procedure. The value of this parameter was changed in 0.01-ns^{-1} steps between 0 and 0.04 ns^{-1} in separate fitting processes (Table 2). It should be noted here that the value of k_o theoretically cannot be larger than ~ 0.05 (see Eq. 3) due to the relatively long fluorescence lifetime of IAEDANS in actin (~ 20 ns). The other components of Eq. 3, A and E*, were taken as variables. The results, relying on the assumption of different k_o values, are shown in Table 2. Accordingly, both the activation energy E* and the frequency factor A are larger in Ca-F-actin than in Mg-F-actin, regardless of the initial assumption of the value of k_o.

To obtain further information about the dynamic properties of actin filaments the fluorescence anisotropy decay was also measured in the temperature range of 9–33°C. The analysis relying on the assumption of two decay components was made to interpret the rotational motion of actin filaments. During the analysis the value of the limiting anisotropy (r_o) was varied as a free parameter and was found to be cation-independent (0.315 ± 0.033 for Mg-F-actin and 0.315 ± 0.026 for Ca-F-actin). The values of the long rotational correlation times are larger in Mg-F-actin than in Ca-F-actin and tend to decrease as the temperature increases (Fig. 3). In contrast to these data for Mg-F-actin the values of this parameter for Ca-F-actin are nearly unchanged. The difference between the longer rotational correlation times measured in Ca-F-actin and Mg-F-actin seems to be negligible above 28°C. The values of shorter rotational correlation times are constant within the limits of experimental errors at all temperatures (ranging between 1 and 3 ns) and are identical for Ca-F-actin and Mg-F-actin (data not shown).

Comparison of conventional EPR spectra obtained on F-actin in Ca- and Mg-state showed no remarkable difference between the hyperfine splitting constants ($2A'_{ZZ}$, Fig. 4). The value of this parameter was ~ 6.67 ± 0.02 mT in both states, which agrees with earlier observations (Thomas et al., 1979, Mossakowska et al., 1988). In contrast, the ST-EPR spectra (Fig. 5) showed a significant difference in the low-field spectral parameter L″/L after the exchange of Ca^{2+} by Mg^{2+} (Table 3). The apparent rotational correlation times corresponding to the spectral parameter L″/L are 65 and 75 μs in Ca-F-actin and Mg-F-actin, respectively (Horváth and Marsh, 1983).

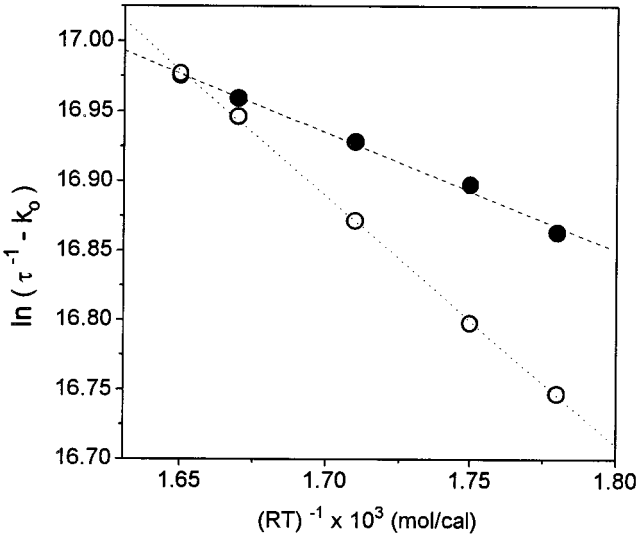

FIGURE 2 Arrhenius plots for the temperature dependence of the measured lifetimes of IAEDANS in Mg-F-actin (*filled symbols*) and Ca-F-actin (*open symbols*). The temperature-independent rate constant (k_o) was taken to be 0.03 ns^{-1} (see also Table 2).

TABLE 2 Arrhenius parameters for the temperature dependence of the measured lifetimes of IAEDANS in Mg- and Ca-F-actin at different k_o values

Parameter	Mg-F-actin		Ca-F-actin	
$k_o \times 10^{-9}$ (s^{-1})	$A \times 10^{-7}$ (s^{-1})	E* (kcal/mol)	$A \times 10^{-7}$ (s^{-1})	E* (kcal/mol)
0.04	14.99 (1.52)	1.46 (0.06)	301.10 (44.76)	3.275 (0.09)
0.03	8.85 (0.05)	0.80 (0.03)	39.56 (3.29)	1.71 (0.05)
0.02	8.62 (0.03)	0.55 (0.02)	22.70 (1.38)	1.16 (0.04)
0.01	8.75 (0.20)	0.42 (0.01)	18.49 (0.89)	0.88 (0.03)
0.00	9.42 (0.12)	0.34 (0.01)	17.13 (0.69)	0.71 (0.02)

Standard deviations are given in parentheses.

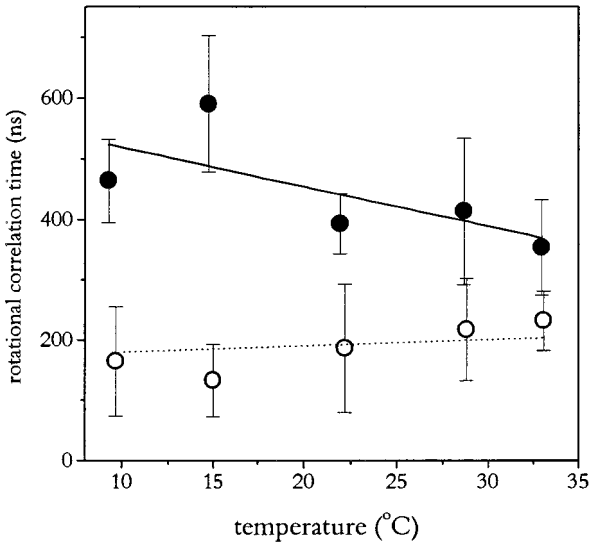

FIGURE 3 Temperature dependence of the longer rotational correlation times of the anisotropy decay of IAEDANS in Mg-F-actin (*filled circles*) and Ca-F-actin (*open circles*).

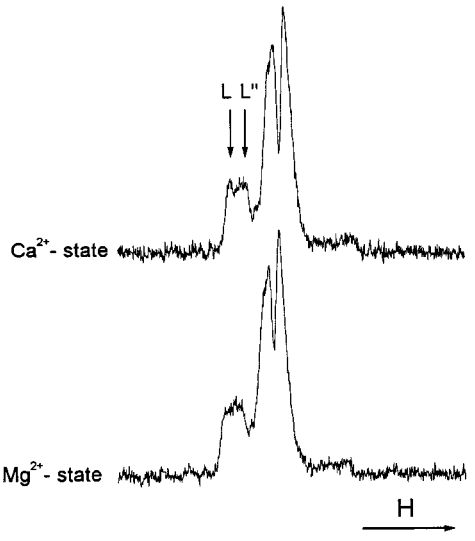

FIGURE 5 ST-EPR spectra of MSL-Ca-F-actin and MSL-Mg-F-actin. The field scan was 20 mT.

DISCUSSION

The fluorescence lifetime of the IAEDANS is longer in the Mg-F-actin than that in Ca-F-actin below 28°C (Table 1). Similar cation dependence of the fluorescence intensity (Frieden et al., 1980) and lifetime (Nyitrai et al., 1997) of IAEDANS was observed in the monomeric forms of actin as well. Frieden and colleagues concluded from the cation-induced intensity change that there is a conformational difference between the C-terminal segment of the Ca^{2+}- and Mg^{2+}-saturated forms of the monomer (Frieden et al., 1980). In the light of the data presented here, one can conclude that this conformational difference is conserved to a certain extent during polymerization.

The Arrhenius plots were applied to interpret the temperature dependence of the average fluorescent lifetimes in the Ca-F-actin and Mg-F-actin (Eq. 3). Such an analysis can provide information about the microenvironment of the reporter molecule. According to our data both the activation energy (E^*) and the frequency factor (A) are larger in the case of Ca-F-actin than in the case of Mg-F-actin (Table 3), indicating that the protein matrix around the Cys^{374} residue is more rigid in the Mg^{2+}-saturated form of the actin filament. Similar cation induced change in the flexibility was reported recently for actin monomers as well (Strzelecka-Golaszewska et al., 1993; Nyitrai et al., 1997). These data imply, in agreement with the conclusion derived from the results of fluorescence lifetime experiments, the conservation of the cation-dependent flexibility difference of the protein matrix around the Cys^{374} residue after polymerization. Three-dimensional reconstructions from electron micrographs have showed that a bridge of density exists between the two strands of the filament in Ca-actin that is absent in Mg-actin (Orlova and Egelman, 1995). According to the interpretation of these authors, this bridge of density arises from a major shift of the C terminus. Accordingly, the cation-induced difference in the flexibility of the microen-

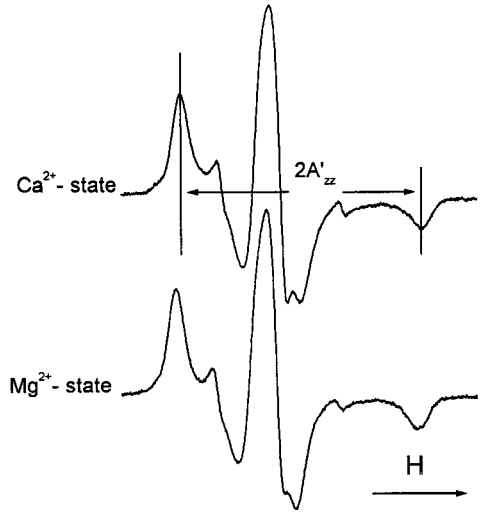

FIGURE 4 Conventional EPR spectra of Ca-F-actin and Mg-F-actin. The concentration of actin was 167 μM and field scan was 10 mT. Actin was labeled with MSL at the Cys^{374} site.

TABLE 3 Conventional and saturation transfer EPR spectral parameters of spin-labeled actin (MSL-actin) upon the replacement of Mg^{2+} by Ca^{2+}

Parameter	Ca-F-actin	Mg-F-actin
Conventional EPR	6.760	6.763
$2A'_{zz}$ (mT)	($\pm$ 0.025) (n = 10)	($\pm$ 0.022) (n = 9)
ST-EPR	0.966	1.067
L″/L	($\pm$ 0.067) (n = 8)	($\pm$ 0.055) (n = 8)

Standard deviations are presented in parentheses.

vironment of Cys^{374} in actin filaments might reflect the presence of the proposed high-density bridge between the two strands of the Ca-F-actin.

The difference in the local dynamic properties of the protein matrix around Cys^{374} may reflect a modification of a larger segment or the entire filament as a result of the cation exchange. In order to characterize global changes induced by cations, the anisotropy decay of the IAEDANS was also investigated. The longer rotational correlation times are in agreement with the values obtained by Miki and co-workers who found $\varphi_2 = 260$ ns for Ca-F-actin and $\varphi_2 = 682$ ns for Mg-F-actin at 20°C labeled with IAEDANS at Cys^{374} (1982b).

The components of the anisotropy decay can potentially characterize different regions of the actin filament. The interpretation of the long component (φ_2) resolved in these experiments is critical. This parameter obviously cannot be specific for the rotation of the whole filament because this rotation has to be much slower than the one indicated by the longer correlation time. Furthermore, the bending motion of the filament was found to occur with about 10-ms correlation times (Fujime and Ishiwata, 1971); thus, the contribution of this kind of motion should also be unnoticed in our experiments. The longer correlation times may be attributed to some restricted segmental motion in the filament (Ikkai et al., 1979; Miki et al., 1982a,b). The segment may be the protomer, but smaller components of actin cannot be ruled out as contributors to this correlation time. The value of the longer correlation time is assumed to be inversely proportional to the flexibility of the actin filaments (Tawada et al., 1978; Ikkai et al., 1979; Miki et al., 1982a,b; Hegyi et al., 1988). The decrease in this correlation time may be the result of loosening of the physicochemical links between adjacent protomers in the actin filament. Accordingly, the results of the fluorescence anisotropy decay experiments support the view that cation exchange induces conformational transition within the actin filament, resulting in a more rigid form of Mg-F-actin compared to Ca-F-actin.

Inspection of the conventional EPR spectra obtained on Ca-F-actin and Mg-F-actin revealed essentially no difference between the hyperfine splitting constants ($2A'_{ZZ}$, Fig. 4). This observation suggests the absence of a detectable change in rotational mobility on the nanosecond time scale as a result of the exchange of Ca^{2+} by Mg^{2+} (Table 3). The spin labels are sensitive reporters of the polarity of the microenvironment around the binding site as well. The slight change of the splitting constant upon replacement of Ca^{2+} with Mg^{2+} shows that the exchange of cations does not remarkably affect the polarity of the medium. This conclusion is apparently not in agreement with the results of fluorescence experiments. According to earlier publications (e.g., Stryer, 1968), the origin of this conflict might be the difference between the fluorescence and EPR methods. Furthermore, different reporter molecules were used to study the protein matrix in the two methods, and the difference between the mobility of these labels might also contribute to the difference in the observations. According to earlier data (Thomas et al., 1979), in contrast to the fluorescent probes, the maleimide spin labels are rigidly attached to the Cys^{374} residue of the actin protomer; the probe mobility relative to the protein backbone can be neglected, thus the probe molecules in F-actin report slow domain motions.

However, comparison of the spectral parameter L″/L resolved in the ST-EPR experiments showed that the local protein environment of the probe becomes more rigid after Mg^{2+} is replaced by Ca^{2+} at the high-affinity cation binding site. The inspection of the rotational dynamics of F-actin by ST-EPR experiments suggests that a complex motion is observed, which may be a superposition of twisting and torsional motions of the actin filament or a larger part of the filament (Thomas et al., 1979). Therefore, the motion of the labels in actin filaments reflects more than one mode of motion, and their motion is not isotropic. Accordingly, the apparent rotational correlation times demonstrate only the magnitude of the rotational motion of the labels in filament forms of actin in the ST time range. The apparent rotational correlation times are on the order of 10^{-4} s, much smaller than the bending motion of the entire filament and much larger than that of the overall monomer rotation; they should represent a complex intrafilament motion involving different modes. However, based on these data one cannot distinguish between the possibilities that the structure is more flexible along its entire length and that it possesses restricted regions. The change of internal flexibility induced by the exchange of Ca^{2+} for Mg^{2+} in monomer actin has been interpreted as a change in the angularly restricted motion of the label (Nyitrai et al., 1997). The increase of rotational correlation time may imply that the effect of exchange of cations in F-actin can propagate to distant parts of the protomers inducing a change of rigidity in the internal region of the protomers and/or between neighboring protomers. The interaction between the protomers might be modulated by the conformational state of the actin subunits, and the exchange of cations can stabilize the interactions between the two helical strands. This can lead to an increase of the rotational correlation time. Comparing the EPR and the fluorescence data, the more likely interpretation is that intermonomer interaction is stronger in the Mg^{2+}-saturated filament than in the Ca^{2+}-saturated form due to the increasing restriction of the twisting and torsional motions of the individual protomers.

These conclusions do not seem to agree with results obtained by other experimental methods (Orlova and Egelman, 1993; Isambert et al., 1995; Scharf and Newman, 1995; Yasuda et al., 1996; Steinmetz et al., 1997). These studies found the flexibility of Mg^{2+}-saturated actin filaments identical to (Isambert et al., 1995; Scharf and Newman, 1995; Yasuda et al., 1996; Steinmetz et al., 1997) or larger than (Orlova and Egelman, 1993) that of filaments polymerized from Ca-actin. Steinmetz and co-workers (1997) have suggested that different methods of polymerization applied by different laboratories might be responsible for the conflicting conclusions. Our present data indicate that the Mg^{2+}-loaded filaments are more rigid than the

filaments of Ca-actin. To explain the difference between this conclusion and the results cited above, one needs to consider that the earlier conclusions were based on investigations of slower motions (e.g., the bending of actin filaments) appearing on a 1-ms or longer time scale. The motion characterized in our fluorescence anisotropy and saturation transfer EPR experiments is much faster than the bending motions of filaments. Accordingly, it is not surprising that the cation-induced changes resolved by the different methods do not correlate.

CONCLUSIONS

Combining fluorescence lifetime experiments and the Arrhenius analysis enabled us to distinguish between Ca-F-actin and Mg-F-actin, indicating that the conformation and/or flexibility of the protein region around the Cys^{374} residue is sensitive to the nature of the bound cation. Based upon our data, obtained from both fluorescence anisotropy decay and ST-EPR experiments, the actin filaments appear to be more rigid when Mg^{2+} binds to the high-affinity cation-binding sites than in the case of Ca^{2+}-loaded actin. Changes in the molecular properties of the actin molecule due to the effect of Ca^{2+} ion binding can be important in different ways in the function of the muscle cells. In resting muscle cells, the Mg^{2+} ions are thought to occupy the high-affinity cation-binding sites in F-actin. We can speculate that the exchange of Mg^{2+} for Ca^{2+} during muscle contraction could enhance the activity, allowing larger structural fluctuations in the thin filaments.

Knowledge of the structure of actin in its various conformational states is important for understanding the diverse motile activities in biological systems. This statement is supported by recent suggestions about the role of actin in the force development of muscle. The actin powerstroke model is based on the length changes in actin filaments that require conformational transitions in each monomer (Schutt et al., 1995). The large free-energy change upon binding of the myosin head to actin is also able to generate conformational change in actin (Geeves, 1991). During the ATP hydrolysis cycle the myosin heads can adopt more than one conformation in interaction with actin, the multiple modes of binding can relate to different actin conformations. The exchange of Ca^{2+} by Mg^{2+} is accompanied by a change in the intramolecular motion of the actin filament, which can increase the elastic resistance to deformation. It is known that the *N*-terminal region of the LC1 light chain of myosin interacts directly with the C-terminal region of actin (Trayer et al., 1987). The altered conformation of actin might modulate the segmental mobility of the light chain that effects the force generation process.

It is important to note that the cation-induced change in filament flexibility can be the result of change in either intermonomer bonding or intramonomer flexibility. From the experimental results presented here it was not possible to identify which of these changes contribute to the flexibility difference found between the Ca-F-actin and Mg-F-actin. The separation of the cation-induced modification of intramonomer and intermonomer flexibility seems to be possible using the method of fluorescence resonance energy transfer (Somogyi et al., 1984). Such experiments are currently in progress in our laboratory.

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9.2. The Flexibility of Actin Filaments as Revealed by Fluorescence Resonance Energy Transfer: The influence of divalent cations (2. számú közlemény)

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The Flexibility of Actin Filaments as Revealed by Fluorescence Resonance Energy Transfer

THE INFLUENCE OF DIVALENT CATIONS*

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The temperature profile of the fluorescence resonance energy transfer efficiency normalized by the fluorescence quantum yield of the donor in the presence of acceptor, *f′*, was measured in a way allowing the independent investigation of (i) the strength of interaction between the adjacent protomers (intermonomer flexibility) and (ii) the flexibility of the protein matrix within actin protomers (intramonomer flexibility). In both cases the relative increase as a function of temperature in *f′* is larger in calcium-F-actin than in magnesium-F-actin in the range of 5–40 °C, which indicates that both the intramonomer and the intermonomer flexibility of the actin filaments are larger in calcium-F-actin than those in magnesium-F-actin. The intermonomer flexibility was proved to be larger than the intramonomer one in both the calcium-F-actin and the magnesium-F-actin. The distance between Gln^{41} and Cys^{374} residues was found to be cation-independent and did not change during polymerization at 21 °C. The steady-state fluorescence anisotropy data of fluorophores attached to the Gln^{41} or Cys^{374} residues suggest that the microenvironments around these regions are more rigid in the magnesium-loaded actin filament than in the calcium-loaded form.

The tension generation in the striated muscle is performed through a series of chemical reactions by cyclic interaction of myosin with ATP and actin, and at least six intermediates are proposed for actomyosin ATPase in solution (1–3). On a cellular level in supramolecular complexes where stabilizing forces may modulate the hydrolysis process, some contribution from actin flexibility and dynamics to the contraction process cannot be excluded. This statement is supported by earlier and recent suggestions about the role of actin during the force development in muscle (4).

Flexural rigidity experiments suggested that the actin filament was extensible (5). These findings were supported by electron microscopic measurements on the sarcomere in rigor fibers (6). The extensibility of the thin filaments was also suggested by the changes of the spacings of the x-ray diffraction pattern during contraction (4, 7). Actin filaments were shown to be elastic and extensible by measuring the stiffness of the actin-tropomyosin complex with *in vitro* nanomanipulation (8). Polarization studies using fluorescent phalloidine on skinned rabbit psoas fibers have demonstrated that the generation of force was associated with a conformational change in the actin filament (9). The sliding of actin filaments is diminished by the cross-linking of actin subunits (10). Egelman *et al*. (11) and later the workgroup in DeRosier's laboratory (12) emphasized the existence of variations in the twist along the axes of isolated filaments, which increases the fluctuations of approximately 10° in the azimuthal angle between adjacent monomers. The fluctuations, bending and twisting motions, are modulated by myosin and actin-binding proteins (13, 14). The tighter binding of myosin to actin reduces the torsional motion of a small section of F-actin, as reported by standard transfer-EPR measurements (13, 15). The change of the orientation of spin labels on F-actin during interaction with heavy meromyosin was also reported (10, 16). It is also known that the actin monomers undergo conformational changes or slight rotation during contraction (17). The large free energy change caused by binding of the myosin head to actin is also able to generate conformational change in actin (18).

Experimental evidences suggest that the exchange of the bound cation can also modify the dynamic and conformational state of the actin filament. Cation-dependent changes in the mobility of the N-terminal segment (first 21 amino acids) of actin were observed performing nuclear magnetic resonance (NMR) experiments (19). The torsional rigidity of actin filaments is sensitive to the nature of the bound cation, since this parameter is larger in calcium-F-actin than in magnesium-F-actin (20). Orlova and Egelman (21) have shown that the bending flexibility of filaments polymerized from magnesium-actin is approximately four times larger than in the case of calcium-F-actin. Contrary to these data, other laboratories found no essential change in the filament flexibility using dynamic light scattering measurements (22) or various other techniques to determine the persistence length of the filaments (23, 24). The direct measurement of the flexibility of single actin filaments corroborates this latter conclusion (20). Using fluorescence methods Miki *et al*. (25) observed that the binding of Ca^{2+} to actin increased the mobility of the fluorophore attached to Cys^{374}. The results of our spectroscopic experiments indicated that filaments polymerized in the presence of Ca^{2+} were more flexible than the filaments of magnesium-actin (26).

The recently published actin powerstroke model was based on the length changes in actin filaments, which require conformational transitions in each monomer (27). During the ATP hydrolysis cycle the myosin heads can adopt more than one conformation in interaction with actin, and the multiple modes of binding can relate to different actin conformations. Recently, the negative experimental results of the rotating cross-bridge

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model have led to suggestions of a more complex model of the muscle contraction. This model involves large scale conformational changes of myosin head in the light chain-binding domain that rotates relative to the actin-binding portion of the catalytic domain (28–30). The closure of the cleft on the actin-binding domains, which follows the release of the P_i, results in a specific interaction between the two proteins, and this interaction might be modulated by the actual dynamic and conformational states of both proteins.

Although there are strong indications that actin is an active part of the contracting system, we are still far from understanding the details of the biological function of this abundant protein. The lack of complete understanding of the function of the actin in the contracting system can emphasize the importance of further investigations dealing with this matter.

The principal aim of this study was to characterize the effect of divalent cations on the internal flexibility and the conformational states of actin filaments using the method of fluorescence resonance energy transfer. According to the fluorescence resonance energy transfer data presented in this paper, the calcium-F-actin is proved to be more flexible than the magnesium-F-actin in either the intermonomer or the intramonomer protein flexibility. The intermonomer flexibility is larger than the intramonomer one, regardless of the nature of the bound cation. In accordance with these flexibility data the steady-state anisotropy experiments indicate that the microenvironments of the Gln^{41} and Cys^{374} residues are more rigid in the Mg^{2+}-saturated filaments than in calcium-F-actin.

MATERIALS AND METHODS

Reagents—KCl, $MgCl_2$, $CaCl_2$, Tris, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS),[1] quinine (hemisulfate salt), dimethylformamide, guinea pig liver transglutaminase (TGase), and EGTA were obtained from Sigma. Iodoacetamide 5-fluorescein (IAF) and fluorescein cadaverine (FC) were purchased from Molecular Probes (Eugene, OR); adenosine 5′-triphosphate (ATP) and α-mercaptoethanol were obtained from Merck (Darmstadt, Germany); the Bradford protein assay reagent was purchased from Bio-Rad (München, Germany), and NaN_3 was from Fluka (Buchs, Switzerland).

Protein Preparation—Acetone-dried powder of rabbit skeletal muscle was obtained as described by Feuer *et al.* (31). Rabbit skeletal muscle actin was prepared according to the method of Spudich and Watt (32) and stored in 2 mM Tris/HCl buffer (pH 8.0) containing 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.1 mM α-mercaptoethanol, and 0.02% NaN_3 (buffer A).

Labeling of the Cys^{374} residue (Fig. 1*A*) with IAEDANS was performed as described earlier (33), and F-actin (2 mg/ml) was incubated with 10-fold molar excess of IAEDANS for 1 h at room temperature. Labeling of the same residue in separate samples with IAF was carried out in the following way: monomeric actin (2 mg/ml) was mixed with the 10-fold molar excess of IAF over the protein and incubated for 3–4 h at room temperature. Then the actin was polymerized for 12–16 h at 4 °C. After the labeling procedures, the samples were centrifuged at 100,000 × *g* for 2 h at 4 °C. The pellets were dissolved in buffer A and dialyzed overnight against buffer A (in the case of IAF-labeled actin the dialyzing buffer contained 1% (v/v) dimethylformamide as well). The Gln^{41} residue (Fig. 1*A*) was modified with FC by the use of the procedure of Takashi (34), and G-actin was incubated with 10-fold molar excess of the dye in the presence of 1 mg/ml TGase. The labeling was carried out for 16 h at 4 °C. Unbound FC was removed similarly as described in the case of IAEDANS and IAF labeling procedures.

The G-actin concentration was determined with a Shimadzu UV-2100 spectrophotometer by using the absorption coefficient of 0.63 mg ml^{-1} cm^{-1} at 290 nm (35). In the case of IAEDANS-labeled G-actin the measured absorbance at 290 nm was corrected for the contribution of the fluorescence label (using A (290 nm) = 0.21 × A (336 nm) for the bound IAEDANS). Relative molecular mass of 42,300 Da was used for monomeric actin (36). Occasionally, the actin concentration was also determined by using Bradford (Coomassie Blue) protein assay reagent (37). The assay was calibrated as described by the manufacturer using unlabeled monomeric actin. The concentrations determined according to the two methods were identical within the limits of experimental error. The concentration of the IAEDANS, IAF, and FC in the protein solution was determined by using the absorption coefficient of 6100 M^{-1} cm^{-1} at 336 nm (38), 77,000 M^{-1} cm^{-1} at 496 nm (39), and 75,500 M^{-1} cm^{-1} at 493 nm (40), respectively. The extent of labeling for IAEDANS, IAF, and FC was determined to be 0.83 ± 0.02, 0.55 ± 0.04, and 0.8 ± 0.03, respectively.

Sample Preparation and Polymerization—Studying the intermonomer flexibility, part of the actin sample was labeled with the donor molecule (IAEDANS), and the remaining part was modified separately with the acceptor (IAF). After the labeling procedure, the two samples were mixed to obtain the molar ratio of the donor labeled and not donor labeled (acceptor labeled and unlabeled) monomers of minimum 1:10 (Fig. 1*B*). In order to measure the intramonomer flexibility, the actin monomer was double-labeled (*i.e.* labeled with both the donor (IAEDANS) and the acceptor (FC)). Then the solution of labeled actin was mixed with a solution of unlabeled actin monomers to adjust the labeled:unlabeled actin ratio to a minimum of 1:10 (Fig. 1*C*). After the appropriate mixture of labeled and unlabeled actin monomers the polymerization process was initiated.

Magnesium-G-actin was obtained according to the method of Strzelecka-Golaszewska *et al.* (41). The solution of calcium-G-actin was dialyzed exhaustively against buffer A in which the concentration of $CaCl_2$ was decreased to 50 μM. EGTA and $MgCl_2$ were added and adjusted to final concentrations of 0.2 and 0.1 mM, respectively. The sample was stirred at room temperature for 10 min.

Polymerization of either calcium-G-actin or magnesium-G-actin was initiated by the addition of 100 mM KCl and 2 mM of the appropriate cation ($CaCl_2$ or $MgCl_2$) to the solutions of calcium-G-actin or magnesium-G-actin, respectively. The samples were incubated at room temperature for 2 h, then dialyzed overnight against the appropriate polymerization buffer (buffer A supplemented with 100 mM KCl and 2 mM divalent cation, while in the case of magnesium-actin, the buffer also contained 0.1 mM EGTA).

Fluorescence Experiments—The concentration of actin was between 30 and 40 μM, unless stated otherwise. The fluorescence emission spectra of the donor were recorded at temperatures ranging between 5 and 40 °C with a Perkin-Elmer LS50B luminescence spectrometer in the presence and the absence of the appropriate acceptor (FC in the experiments dealing with intramonomer flexibility and IAF in the study of intermonomer flexibility). The excitation wavelength for the IAEDANS was 360 nm. The slits were set to 3 nm in both the excitation and emission paths. The spectra were corrected for the inner filter effect as described earlier (42). To calculate fluorescence resonance energy transfer efficiency (see Equation 2), the under-curve areas of these emission spectra were calculated between 380 and 460 nm. In this wavelength range, the contribution of the acceptor (either the FC or the IAF) to the measured fluorescence is negligible.

The steady-state fluorescence anisotropy of the donor and acceptor molecules was calculated from the polarized emission components (F_{VV}, F_{VH}, F_{HV}, and F_{HH}, where the subscripts indicate the orientation of the excitation and emission polarizers) as follows,

$$r = (F_{VV} - GF_{VH})/(F_{VV} + 2\,GF_{VH}) \qquad \text{(Eq. 1)}$$

where $G = F_{HV}/F_{HH}$. In the case of actin-bound IAEDANS, the excitation wavelength was 360 nm, and the emission wavelength was 460 nm, while for the fluorescein derivatives the excitation monochromator was set to 493 nm, and the emission was measured at 520 nm. The slits were set to 3 nm. In these experiments the concentration of the actin was decreased to 5 μM after the polymerization by diluting the sample with the appropriate buffer. In this way the depolarizing effect of light scattering was reduced to a negligible level.

The corrected fluorescence emission spectra of IAEDANS-F-actin was recorded in the absence of the acceptor at an excitation wavelength of 360 nm to obtain the fluorescence quantum yield of the donor molecule. The quantum yield of quinine sulfate (0.53 in 0.1 N H_2SO_4) was used as a reference (43).

To test the reversibility of the temperature-induced changes in fluorescence, the samples were re-measured by cooling back the solution to the initial low temperature (7 °C) or repeating the measurements after overnight dialysis. The errors of the measured data presented in this paper are mean ± S.E. calculated from the results of three to five independent experiments.

Donor-Acceptor Distance—The transfer efficiency of the fluorescence resonance energy transfer occurring between a single donor and single

[1] The abbreviations used are: IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)-ethylenediamine; FC, fluorescein cadaverine; IAF, 5-iodoacetamidofluorescein; G-actin, monomeric actin; F-actin, actin filament.

acceptor can be calculated from the fluorescence intensities as follows,

$$E = \{1 - ((F_{DA}/c_{DA})/(F_D/c_D))\}/\beta \quad \text{(Eq. 2)}$$

where F_{DA} and F_D are the fluorescence intensities of the donor molecule in the presence and the absence of the acceptor, respectively; β symbolizes the acceptor/monomer molar ratio. c_D and c_{DA} are the concentrations of the donor molecule in the samples indicated by the subscripts. By knowing the fluorescence energy transfer efficiency (E), it is possible to determine the distance (R) between the donor and acceptor molecules from the following equation:

$$E = R_o^6/(R_o^6 + R^6) \quad \text{(Eq. 3)}$$

where R_o is the Förster's critical distance defined as the donor-acceptor distance where the fluorescence resonance energy transfer efficiency is 50%. The use of Equation 3 requires the calculation of R_o as follows,

$$R_o^6 = (8.79 \times 10^{-11}) n^{-4} \kappa^2 \Phi_D J \quad \text{(Eq. 4)}$$

where n is the refractive index of the medium, κ^2 characterizes the relative orientation of the donor and acceptor molecules, Φ_D is the fluorescence quantum yield of the IAEDANS in the absence of acceptor, and J is the overlap integral given in M^{-1} cm^{-1} nm^4. The overlap integral (J) is defined as follows,

$$J = \int F_D(\lambda)\epsilon_A(\lambda)\lambda^4 d\lambda / \int F_D(\lambda) d\lambda \quad \text{(Eq. 5)}$$

where $F_D(\lambda)$ is the corrected fluorescence emission spectra of the donor, and $\epsilon_A(\lambda)$ is the molar extinction coefficient of the acceptor.

Normalized Transfer Efficiency—The temperature profile of the normalized energy transfer parameter f (defined as the ratio of the transfer efficiency and the fluorescence quantum yield of the donor in the presence of acceptor) is proportional to the mean value of the energy transfer rate constant, $<k_{ti}>$, which has been shown to be an appropriate parameter for monitoring intramolecular fluctuations and/or conformational changes of a macromolecule (44),

$$f = \langle E \rangle / \langle \Phi_{DA} \rangle = \langle k_{ti}/k_f \rangle = C \langle R_i^{-6} \kappa_i^2 \rangle \quad \text{(Eq. 6)}$$

where Φ_{DA} is the fluorescence quantum yield of the donor in the presence of acceptor, and k_f is the rate constant of the fluorescence emission. According to earlier publications the value of k_f is fairly constant under a wide variety of experimental conditions (see *e.g.* Ref. 45), therefore here its value is taken as constant. The subscript "*i*" indicates the value of the given parameter for the i^{th} population, taking a momentary picture, and C is a constant involving the refractive index (n) and the overlap integral (J), which were assumed to be constant (44). The sensitivity of this parameter to temperature is able to provide information regarding the flexibility of the protein matrix between the two fluorophores. It should be noted that f is sensitive to changes in the donor-acceptor distance originating from any kind of intramolecular motions. Thus, the temperature profile of this parameter provides information about the average flexibility of the protein matrix located between the two labels.

The method was developed for systems where the energy transfer occurred between a single donor and a single acceptor (44). However, in the present experiments dealing with intermonomer energy transfer, one should take into account that the donor can transfer energy to acceptors located on more than one neighboring actin protomers, *i.e.* the transfer is directed to a multiple acceptor system. Considering the helical structure of the actin filament, it seems reasonable that the acceptor population can be divided into two characteristically different groups: 1) acceptors on the closest protomer in the single-started genetic helix and 2) acceptors affecting the fluorescence of the donor from the double-started long-pitch helix. It is also assumed that acceptors on more distant protomers are not efficient in the reduction of the donor fluorescence. Accordingly, the donor-acceptor system can be described with two different equilibrium donor-acceptor distance distributions. It could be easily shown by using simple mathematical transformations that the measured normalized energy transfer parameter of the system having a single donor interacting with two different groups of acceptor molecules is the sum of the normalized energy transfer efficiencies characterized by the individual donor-acceptor systems (see also Ref. 46),

$$f = f_1 + f_2 \quad \text{(Eq. 7)}$$

Considering that the value of the fluorescence quantum yield is proportional to the fluorescence intensity measured at a given wavelength, it is usually more convenient to determine the value of the f', which is defined as follows (44),

$$f' = \langle E_i \rangle / F_{DA} = C' \langle E_i \rangle / \langle \Phi_{Di} \rangle \quad \text{(Eq. 8)}$$

where F_{DA} is the fluorescence intensity of the donor in the presence of acceptor, and C' is a constant that is proportional to the C used in Equation 6.

RESULTS AND DISCUSSION

The actin monomer has one high-affinity and three or more lower-affinity (*i.e.* intermediate- and low-affinity) cation-binding sites (see Ref. 47 for review). It is very likely that *in vivo* the high-affinity site is occupied by Mg^{2+}, and the Mg^{2+} and K^+ ions compete for the lower-affinity binding sites (47). The ion composition of the buffer that was used in this study to prepare magnesium-F-actin can be considered as a reasonable model for the free ion concentrations of Mg^{2+} and K^+ in the cytosol (47). This preparation resulted in a magnesium-F-actin that contains Mg^{2+} at the high-affinity binding site and probably either Mg^{2+} or K^+ at the lower-affinity sites. According to earlier publications the type of the cation at the lower-affinity binding sites might have an important biological effect (48). The calcium-actin filaments were polymerized in the presence of millimolar concentration (2 mM) of $CaCl_2$. Following this procedure the Ca^{2+} in calcium-F-actin, similar to the Mg^{2+} in magnesium-F-actin samples, occupies the high-affinity binding site and probably competes with the K^+ for the lower-affinity binding sites.

In the present work we explored the differences between flexibilities of filaments polymerized from calcium-actin and magnesium-actin by investigating separately the intermonomer and the intramonomer flexibilities. To examine intermonomer flexibilities the donor IAEDANS and the acceptor IAF are attached to different actin protomers within the filament. The relatively low donor ratio in these samples (compared with that of actin without the donor) assures that there is no acceptor in the actin filament, which is in resonance transfer with two donor molecules (see Fig. 1*B*). Accordingly, in these experiments one is dealing with a single donor-multiple acceptor system (see "Materials and Methods"). In a different experimental setup, the double labeling of the actin monomer makes it possible to study intramonomer flexibility within the actin filament. In this case it was necessary to dilute the samples with unlabeled actin to exclude the possibility of interaction between donor and acceptor molecules located on neighboring protomers. Considering the atomic model of the actin filament (49), it is very likely that the 10-fold dilution of the double labeled actin monomers with unlabeled monomers accurately separates the labeled monomers within the double helix of actin filaments (Fig. 1*C*). The experiments designed to monitor the reversibility of the temperature-induced changes in the fluorescence parameters gave evidence that the changes were reversible.

The distance between the donor (IAEDANS at Cys^{374}) and acceptor (FC at Gln^{41}) molecules is 4.46 ± 0.07 nm and 4.49 ± 0.06 nm in the Ca^{2+}- and Mg^{2+}-loaded forms of the monomer, respectively, indicating that the exchange of the bound cation does not influence the relative position of the Gln^{41} and Cys^{374} residues in the actin monomer. The data are in good accordance with the results of Moraczewska *et al.* (50), who found that the replacement of Ca^{2+} with Mg^{2+} produced no essential change in the distance between Gln^{41} and Cys^{374}. These results are also in agreement with our recent observation that the distance between Lys^{61} and Cys^{374} of the actin monomer is cation-independent (51). The distance between Gln^{41} (Cα) and Cys^{374} (Sγ) residues is 4.1 nm according to the x-ray diffraction experiments (52). The value of this parameter resolved in our experiments is somewhat longer. The relatively small difference between the x-ray and the fluorescence data might be due to the size of the applied fluores-

cent probes.

The donor-acceptor distances (between residues Cys^{374} and Gln^{41}) in the filament at room temperature are 4.45 ± 0.08 nm and 4.59 ± 0.09 nm in calcium-F-actin and magnesium-F-actin, respectively (Table I.), which indicates that the polymerization does not affect significantly the donor-acceptor distance. This is in agreement with Miki's conclusion (53) that the small domain in the actin monomer is substantially rigid and compact and only slightly sensitive to the binding of DNase I or myosin subfragment 1 or tropomyosin-troponin or polymerization. Above we speculate on the basis of the filament model (49) that the 10-fold dilution of doubly labeled actin samples with unlabeled actin diminishes the intermonomer resonance energy transfer. The lack of the effect of polymerization on the donor-acceptor distance implies that this assumption was correct. The distance between the two labeled residues of the small domain was temperature-independent in magnesium-F-actin (Table I). This statement is apparently not true for the calcium-F-actin (Table I), since the value of the donor-acceptor distance shows a decreasing tendency with increasing temperature (for discussion, see below).

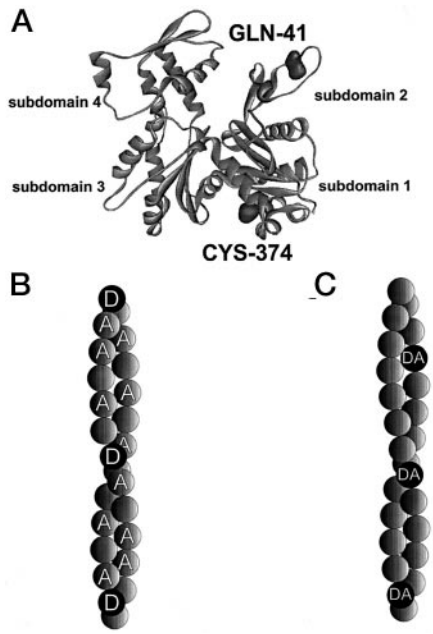

FIG. 1. *A*, the schematic representation of the atomic model of monomeric actin reconstructed according to the results of Kabsch *et al*. (60). The subdomains are labeled with numbers 1–4, and the positions of the amino acids, which were labeled to carry out the intramonomer or intermonomer fluorescence energy transfer experiments (*i.e.* Gln^{41} and Cys^{374}), are marked with *dark surfaces*. *B* and *C* show the simplified picture of the actin filament. The approximated position of the labeled monomers is shown within the actin filament in the case of intermonomer (*B*) and intramonomer energy transfer experiments (*C*). The *circles* are representing monomers within the polymer. *Capital letters within the circles* stand for the monomer with covalently attached donor (*D*) or acceptor (*A*) molecules in the intermonomer energy transfer measurements (*B*), and the doubly labeled monomers are also marked (*DA*) in the case of intramonomer measurements (*C*). The ratio of the labeled and unlabeled monomer populations in the pictures is approximately the same as it is after the preparation procedure described under "Materials and Methods."

The cation dependence of the flexibility of the actin protomer within the filament can be characterized by measuring the temperature profile of the normalized transfer efficiency (Equations 6 and 8). In experiments dealing with intraprotomer interactions the temperature dependence of the relative f' is proved to be substantially larger in calcium-F-actin than in magnesium-F-actin between 5 and 40 °C (Fig. 2*A*). The total change of 5% in the Mg^{2+}-saturated form faces the 30% increase in the Ca^{2+}-saturated form. The data set suggests that the protomer structure is more flexible in the Ca^{2+}-loaded form of the actin filament than that in the magnesium-loaded form. The change in the relative f' is very similar in calcium-F-actin to what was observed in the case of actin monomer by using a similar donor-acceptor pair (51). Accordingly, the flexibility of the small domain does not seem to be sensitive to polymerization in calcium-actin. Contrary to this, the relative change of f' is smaller in magnesium-F-actin than that in magnesium-G-actin (51), indicating that in the Mg^{2+}-loaded form this protein segment is more rigid in the filament than it is in the monomer.

According to the results of intermonomer transfer experiments, the change of the relative f' is larger in the calcium-F-actin than in the magnesium-F-actin (Fig. 2*B*), which suggests that the strength of the intermonomer interaction is stronger in the Mg^{2+}-saturated filament. By comparing the data obtained in the experiments addressing intramonomer and intermonomer fluorescence energy transfer, one can conclude that the intramonomer flexibility is smaller than the intermonomer flexibility for both the calcium-F-actin and magnesium-F-actin (Fig. 2, *A* and *B*). Considering that in intermonomer energy transfer the contributions of the two kinds of acceptor populations (see also "Materials and Methods") to the measured fluorescence energy transfer efficiency are probably similar (49), in these experiments it is not possible to separate the flexural

TABLE I

The temperature dependence of the fluorescence quantum yield of the IAEDANS (Φ_D), the Förster's critical distance of the IAEDANS-FC pair (R_o), the transfer efficiency measured in the intramonomer transfer experiments (E), and the calculated donor-acceptor distances (R) in calcium-F-actin and magnesium-F-actin

The S.E. of the mean are given in parentheses, except for the quantum yield where the error appears in the third digit.

Temperature	Φ_D		R_o		E		R	
	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}
°C			*nm*		%		*nm*	
7	0.55	0.53	5.06 (±0.05)	5.01 (±0.06)	66.8 (±1.6)	63.1 (±2.0)	4.50 (±0.08)	4.58 (±0.08)
11	0.54	0.53	5.04 (±0.04)	5.00 (±0.06)	66.3 (±2.0)	62.7 (±1.8)	4.50 (±0.09)	4.58 (±0.09)
16	0.51	0.52	4.99 (±0.06)	4.99 (±0.09)	66.1 (±2.2)	62.1 (±1.5)	4.47 (±0.08)	4.59 (±0.07)
21	0.49	0.51	4.96 (±0.08)	4.97 (±0.09)	65.7 (±2.1)	61.6 (±1.5)	4.45 (±0.08)	4.59 (±0.09)
26	0.47	0.50	4.92 (±0.08)	4.96 (±0.07)	65.6 (±1.9)	60.8 (±1.4)	4.42 (±0.08)	4.61 (±0.10)
32	0.44	0.49	4.87 (±0.06)	4.93 (±0.09)	65.2 (±1.7)	60.0 (±1.2)	4.38 (±0.10)	4.61 (±0.08)
39	0.41	0.47	4.82 (±0.9)	4.91 (±0.09)	64.5 (±1.4)	58.7 (±0.5)	4.36 (±0.09)	4.63 (±0.09)

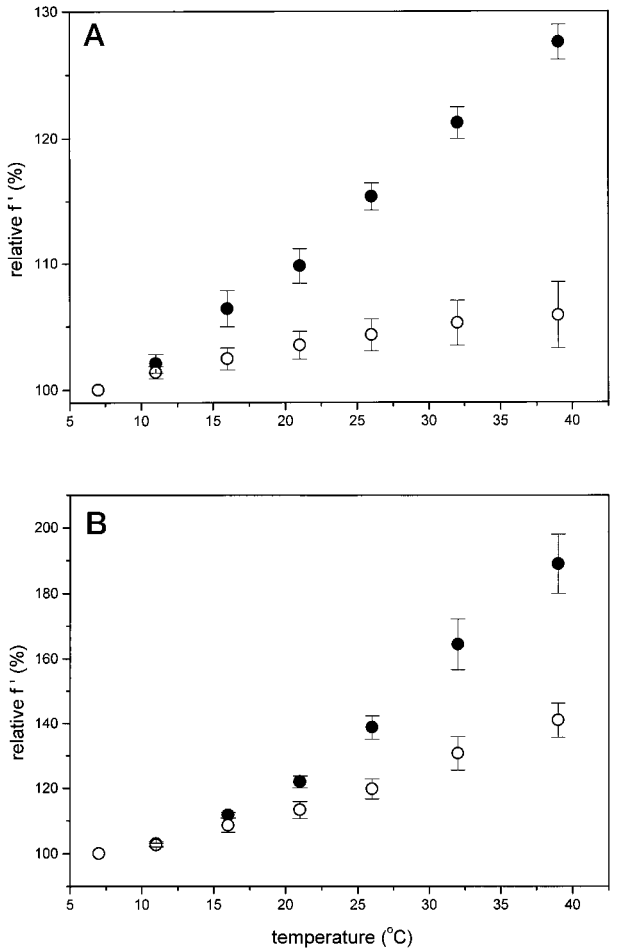

FIG. 2. *A*, the cation dependence of the temperature profile of the relative f' in F-actin resolved in the experiments dealing with intramonomer flexibility. The donor was IAEDANS, and FC served as an acceptor. The actin concentration was 30–40 μM, while the labeled actin was present at 1–3 μM. *B*, the temperature profile of the relative f' in calcium-F-actin and magnesium-F-actin resolved in the experiments dealing with intermonomer flexibility. The value of this parameter was calculated from the results of experiments with the IAEDANS-IAF donor-acceptor pair. The actin concentration was 30–40 μM.

properties of the genetic helix and the two-started long-pitch helix. The increase in the amplitude of the relative fluctuation of the donor and acceptor molecules should result in an increase of the mean value of the energy transfer rate constant, $<k_{ti}>$ and therefore the measurable donor-acceptor distance, even if the equilibrium distance between the two labels remains unchanged (44). In the light of our present data regarding the cation-dependent flexural properties of the filament, it seems possible that the slight temperature dependence of the donor-acceptor distance measured in the calcium-F-actin is partly the result of a temperature-induced increase in the amplitude of the relative fluctuation of the donor and acceptor molecules.

The interpretation of the results described above requires further spectral considerations. Both the temperature- and cation-induced changes in the shape of the emission spectra of the donor and the absorption spectra of the acceptor are negligible (data are not shown). Accordingly, the value of the overlap integral (Equation 5.) depends on neither the temperature nor the nature of the bound cation. Therefore it cannot contribute to the observed changes of f' in the filaments. However, the value of the f', and hence the relative f', might depend on the orientation factor (κ^2). Although this is the only parameter in the fluorescence energy transfer experiments which cannot be measured properly, the measurements of the steady-

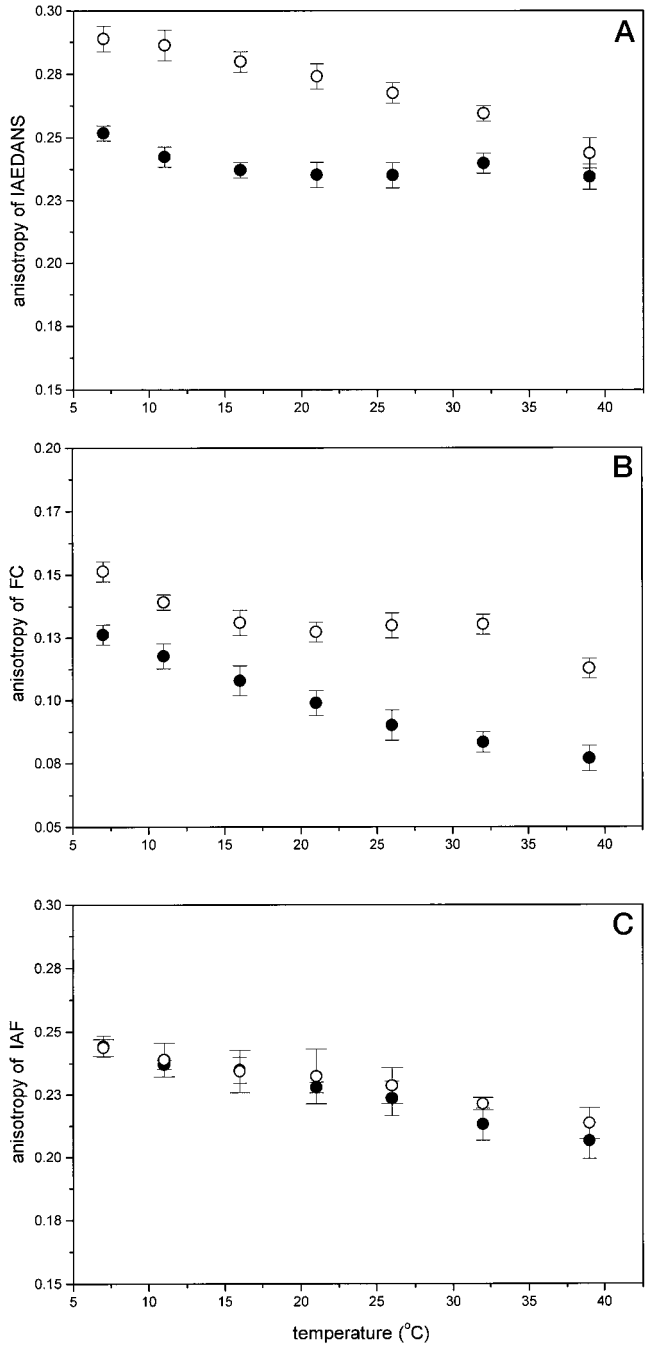

FIG. 3. **The temperature dependence of the steady-state fluorescence anisotropy of IAEDANS (*A*), FC (*B*), and IAF (*C*) in calcium-F-actin (*filled circles*) and magnesium-F-actin (*open circles*).** The concentration of actin was 5 μM in these experiments (see "Materials and Methods").

state anisotropy of both the donor and the acceptor molecules might provide information regarding the behavior of κ^2. The anisotropy of IAEDANS and FC is cation-dependent in the actin filament (Fig. 3, *A* and *B*). The measured anisotropy values are larger in the Mg^{2+}-saturated form than in the Ca^{2+}-saturated one for both IAEDANS and FC, which can be taken as an indication of conformational differences between the calcium-F-actin and magnesium-F-actin. Interestingly, similar cation-induced change was not observed in the case of IAF (Fig. 3*C*). Taking into account that both IAEDANS and IAF are connected to the same amino acid (Cys^{374}), the different cation sensitivity possibly originates from the application of different fluorophores. According to the results of Orlova and Egelman

(54), there is a high-density bridge between the two strands of filament when the high-affinity cation-binding sites are occupied by Ca^{2+}. This density bridge was not observed in magnesium-F-actin. They proposed that the presence of this bridge could be the result of the shift in the position of the C terminus. Therefore, the cation dependence of the fluorescence anisotropy in the case of IAEDANS and FC might reflect the cation-induced intramolecular rearrangement of the C-terminal segment within the actin filament. Although the exact nature of this rearrangement is not known, it seems to be possible that the formation of the density bridge in calcium-F-actin involves the modification of some of the connections between the C-terminal segment and the small domain of either the same or the neighboring protomers. The subdomain 1 (involving the Cys^{374} residue) is in close contact with the subdomain 2 (which contains the Gln^{41} residue) of the subsequent protomer within the long-pitch helix (49). Accordingly, the formation of the high-density bridge in calcium-F-actin can result in a conformation where the microenvironments of the Cys^{374} and the Gln^{41} residues are more flexible than these microenvironments in the magnesium-F-actin.

The temperature sensitivity of the fluorescence anisotropy of all fluorophores is similar (Fig. 3, *A–C*), which suggests that the temperature-induced change in the value of the orientation factor is also similar in these cases. Accordingly, the change in the κ^2 is probably not the source of the apparent cation-dependent variation in the value of the relative f' in either the intermonomer or the intramonomer energy transfer experiments. All these data allow the conclusion that both the intramonomer and intermonomer flexibilities are larger in calcium-F-actin than in magnesium-F-actin. Furthermore, the results of the steady-state anisotropy measurements support the conclusion that the microenvironments of the Gln^{41} and Cys^{374} residues are more rigid in the magnesium-F-actin than in the calcium-F-actin.

The bending and torsional flexibility of calcium-F-actin was found to be smaller (21) or similar (28–31) to that of magnesium-F-actin. However, we have shown here and in our previous work (26) that the flexibility characteristic for intramolecular motions on a nanosecond time scale is larger in calcium-F-actin than in the Mg^{2+}-saturated form of the filament. We have suggested (26) that the apparent conflict could be resolved considering that the methods applied in our experiments and those used in the cited articles (21, 28–31) provide information about intramolecular motions on a substantially different time scales.

The structure of the magnesium-F-actin can be taken as a model of the thin filament in the relaxed state. The changes in the actin-associated layer lines in x-ray diffraction pattern during muscle activation (56, 57) and the differences of the layer lines observed between magnesium-F-actin and calcium-F-actin (54) are similar. Relying on these data Egelman and Orlova (58) proposed that the structure of the calcium-F-actin was corresponding to the thin filaments in the activated state. It is very likely that due to the slow exchange of the tightly bound divalent cation in actin the replacement of Mg^{2+} by Ca^{2+} does not occur under physiological conditions (47). Accordingly, Egelman and Orlova (58) concluded that the activated state of the thin filament was probably induced by the binding of myosin. One might assume that the similarity of the structure of calcium-F-actin and the structure of the F-actin in the activated thin filaments can extend to intramolecular dynamic events occurring on a nanosecond timescale. Thus, considering that actin-myosin interaction can possibly utilize the strain energy stored in actin filaments (59), the divalent cation-dependent changes in the intramolecular flexibility described in this study might be important in the efficient energy transduction of the muscle contraction.

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9.3. Inter-monomer flexibility of Ca- and Mg-actin filaments at different pH values (3. számú közlemény)

INTER-MONOMER FLEXIBILITY OF Ca- AND Mg-ACTIN FILAMENTS AT DIFFERENT pH VALUES

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RUNNING TITLE: The effect of pH on actin filaments

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SUMMARY: The fluorescence resonance energy transfer parameter *f*, is defined as the efficiency of the energy transfer normalized by the quantum yield of the donor in the presence of acceptor. It is possible to characterize the flexibility of the protein matrix between the appropriate fluorescent probes by monitoring the temperature dependence of *f* . The inter-monomer flexibility of the Ca-actin and Mg-actin filaments was characterized by using this method at pathological (pH 6.5) and physiological (pH 7.4) pH values. The protomers were labeled on Cys^{374} with donor (IAEDANS) or acceptor (IAF) molecules. The temperature profile of *f* suggested that the inter-monomer flexibility of actin filaments was larger at pH 7.4 than pH 6.5 in both the calcium and magnesium saturated form of the actin filaments. More rigid inter-monomer connection was identified at both pH values between the protomers of Mg-F-actin compared to the Ca-F-actin. Our spectroscopic results suggest that the altered function of muscle following the change of pH within the muscle cells under physiological or pathological conditions can partly be a consequence of the modified dynamic properties of the actin filaments.

Keywords: muscle, cations, protein flexibility, protein dynamics, fluorescence resonance energy transfer.

INTRODUCTION

The dynamic properties of the actin filament have been widely investigated since it was discovered and named by Straub in 1942 (Straub, 1942). He proposed that the actin filament was an active and essential part of the muscle function. This statement was supported by a large number of experiments proving that the dynamic and/or conformational properties of actin filaments may play an important role in the force generation (Prochniewicz & Yanagida, 1990; Rayment *et al*., 1993). The better understanding of the relationship between the flexibility and the function of actin filaments seems to be important to describe the molecular details of the muscle contraction under physiological and pathological conditions.

The pH can change within the muscle cells under physiological and pathological conditions as well (Edman & Mattiazzi, 1981; Metzger & Fitts, 1987a; Metzger & Fitts, 1987b; Mohabir *et al*., 1991; Renaud *et al*., 1986; Stevens, 1980; Thompson *et al*., 1992a; Thompson *et al*., 1992b; Thompson & Fitts, 1992). In skeletal muscle cells the pH can decrease physiologically during intense exertion. This change of the pH can be a definitive mark of the fatigue accompanied by a reduced mechanical performance (Edman & Mattiazzi, 1981; Metzger & Fitts, 1987a; Metzger & Fitts, 1987b; Renaud et al., 1986; Stevens, 1980; Thompson et al., 1992a; Thompson et al., 1992b; Thompson & Fitts, 1992). The reduced pH value is considered to be caused by the continuous and increased ATP hydrolysis and the shift of the cellular energy production from the aerobic to the anaerobic way (Bailey *et al*., 1982; Bailey *et al*., 1981; Garlick *et al*., 1979; Wolfe *et al*., 1988). Besides the decreased effectivity of the energy production, the involvement of the lower pH in the development of the reduced function is not apparent yet. In heart muscle cells the pH decreases as a pathological sign of ischemic heart diseases (Mohabir et al., 1991). The decrease of the intracellular pH affects both the intercellular and sub-cellular properties of the heart muscle cells as well.

The change of the pH influences the regulatory proteins within the skeletal muscle cells (Bers & Ellis, 1982; Orchard & Kentish, 1990). On the other hand, Orchard and Kentish suggested that the decreased intracellular pH can have a direct effect on the cross bridges as well resulting in a decrease in the maximal force generation during the intracellular acidosis (Orchard & Kentish, 1990). In agreement with this conclusion the reduced intracellular pH was shown to increase the stiffness of the myocardium (Renaud et al., 1986), which could probably due to the altered interactions between the actin and myosin molecules (Krayenbuehl *et al*., 1989). Kron and his colleagues

investigated the effect of pH on the performance of the acto-myosin complex (Kron & Spudich, 1986). While investigating the movement of the actin filaments on a glass surface covered by myosin, they found that the mobility of the actin filaments diminished when the pH of the buffer decreased to 6.5. The filaments recovered from this immobile state and moved again as the pH increased up to 8.5. Beyond this pH value the velocity of the filament movement decreased again. These findings were independent of the quality of the different buffer solutions. Although the pH can have an effect on the myosin, these data can raise the question whether the pH of the environment can have an influence on the performance of the acto-myosin complex through the actin molecules. The different H^+ concentrations have a direct impact on the actin molecules. The lowered pH can enhance the polymerization of actin molecules (Straub, 1942). Zimmerle and Frieden showed that the change of pH influenced the conformation and metal affinity of G-actin (Zimmerle & Frieden, 1988b) and raised the possibility of the dynamical differences of actin at different pH values. They found that the binding of calcium at the high-affinity cation-binding site was ten fold tighter when the pH decreased from 8 to 6. The rate of dimerization, which is one of the initial steps of actin polymerization, is higher at lower pH values while the critical concentration is lower at decreased pH values (Wang *et al*., 1989; Zimmerle & Frieden, 1988a). All these results support the hypothesis that the effect of different pH values on the dynamic and conformational properties of actin can play an important role in abnormal muscle functions. The major goal of this study was to provide further information on this subject.

On the other hand the cation dependence of the flexibility of actin filaments was studied as well. Although it is thought that the F-actin is saturated *in vivo* with magnesium ions in the muscle cells (Estes *et al*., 1992) there are a lot of data accumulated about the dynamic and conformational properties of the Ca-F-actin as well (Carlier, 1991; Hild *et al*., 1998; Miki *et al*., 1982a; Miki *et al*., 1982b; Nyitrai *et al*., 1999; Orlova & Egelman, 1995). It was found that the torsional and bending flexibility of the actin filaments polymerized in the presence of calcium ions is smaller compared to Mg-actin filaments (Orlova & Egelman, 1993; Yasuda *et al*., 1996). Orlova and Egelman proposed that Ca-actin filaments are similar to the actin in the activated form of the thin filaments, while the Mg-F-actin can be taken as a relaxed form of it (Egelman & Orlova, 1995). Using spectroscopic methods we found that at pH 8.0 the intra-monomer and inter-monomer flexibility within the Ca-F-actin was greater compared to the Mg-F-actin (Hild et al., 1998; Nyitrai et al., 1999). The apparent conflict between these results could be resolved considering that the methods applied in our previous experiments (Hild et al., 1998; Nyitrai et al., 1999) and those

used in the cited articles (Orlova & Egelman, 1993; Yasuda et al., 1996) provides information about intramolecular motions on a considerably different time scales. The present FRET experiments provided further evidences that filaments of Ca-actin are more flexible than those of Mg-actin at either pH 7.4 or pH 6.5.

Our present spectroscopic results indicate that the flexibility of the actin filaments is larger at pH 7.4 than at pH 6.5 in both Ca- and Mg-F-actin as well. In previous works it was shown that the flexibility of actin filaments decreased as a result of cross-linking Gln^{41} and Cys^{374} residues on neighboring monomers with N-(4-azido-2-nitrophenyl)putrescine (Hegyi *et al*., 1998; Kim *et al*., 1998a; Kim *et al*., 1998b). These filaments lost their ability to move in the in vitro motility assay as well. Prochniewicz and Yanagida found the same effect of the cross-linking molecules on the mobility of actin filaments on a surface covered by heavy meromyosin (Prochniewicz & Yanagida, 1990). These observations and our present results may help to interpret the role of the altered dynamic properties of the actin filaments in different physiological and pathological conditions.

RESULTS AND DISCUSSION

To characterize the effect of pH on the inter-monomer flexibility of Ca- and Mg-F-actin, temperature dependent FRET experiments were performed at pathological (pH 6.5) and physiological (pH 7.4) pH values. The actin filaments were labeled on the Cys^{374} by either donor (IAEDANS) or acceptor (IAF) molecules (Fig. 1.).

Since the extent of labeling with the acceptor was less than 1 in our experiments, we had to take into account the presence of the donor molecules in the samples, which did not have a fluorescence energy transfer pair. Due to the fact that the ratio of labeling with IAF was 0.52, the probability of the presence of a monomer not labeled with an acceptor molecule was 0.48. Therefore, the possibility of not having a fluorescent acceptor of the four closest monomers around a donor was 0.05 (0.48^4). Accordingly, we considered that the effect of the actin monomers labeled by donor, which did not have an acceptor pair was negligible in the interpretation of the fluorescence measurements.

The FRET efficiency was calculated by using the fluorescence quantum yield of the donor measured in the presence and the absence of acceptor molecules (Eq. 2.). The f was calculated from the FRET data at the applied pH values on temperatures ranging from 6 to 34 ^{o}C (Eq. 6.). Prior to these calculations it was necessary to consider some spectral parameters. Because of the spectral properties of the fluorescent donor and acceptor molecules, the overlap integral of the emission spectra of the donor and the absorption spectra of the acceptor were not constant during these measurements. The change of the overlap integral can influence the temperature profile of the f (Eq. 6.). In order to eliminate this effect we normalized the f values by the overlap integral (J) determined at different temperatures and pH values for both forms of the actin filaments. Throughout this article these corrected values of f have been applied. During the analysis we monitored the relative changes of f, which is the actual value of f normalized by its value at the lowest investigated temperature. Furthermore, the f depends on the orientation factor (κ^2) as well (Eq. 6.) Unfortunately, there is no experimental way to determine the exact value of κ^2 (dos Remedios & Moens, 1995). The fact that the results of FRET experiments are in very good agreement with the distances calculated on the basis of X-ray diffraction data suggests that in these FRET experiments the effect of the orientation factor can usually be taken as negligible (dos Remedios & Moens, 1995). This conclusion is further supported by theoretical considerations indicating that the effect of the change induced by the

temperature in the donor-acceptor distances dominates that of the orientation factor in temperature dependent FRET experiments (Somogyi et al., 1984).

Two major molecular mechanisms are responsible for the temperature dependence of f. The first is that the change induced by the temperature in the equilibrium distance (R) between the donor and acceptor may have a substantial effect on the temperature profile of f and can reflect conformational transition within the protein matrix. In such a case the temperature profile of f might show transition to another curve of the plotted f data, in other words a "break" in its slope. The second is that the changes of the temperature alter the relative fluctuation of the fluorophores around an equilibrium donor-acceptor position as well. While the temperature is increasing, the amplitude of this fluctuation is expected to increase monotonously the value of f without causing any definite "break" on the slope of its temperature profile (Somogyi et al., 1984). By using the same donor-acceptor pair, the slope of the temperature profile of the f is steeper in a more flexible form of the protein if the presence of major conformational changes can be excluded (Nyitrai et al., 1999; Somogyi et al., 1984).

The change of the relative f was smooth in all cases, which suggests that there was no conformational transition induced by the temperature in the actin filaments during the measurements. At pH 7.4 the relative change of the f of Mg-F-actin showed a continuous increase up to 112 % over the whole temperature range, while at pH 6.5 it is remained in the range of the standard deviations at around 100 % (Fig. 2.). The relative change of f parameters indicates a more flexible protein matrix between the fluorescent donor-acceptor pairs at higher pH values. This conclusion is further supported by our previous results according to which at pH 8.0 the value of the relative f characteristic for the inter-monomer flexibility increased up to 130 % over the same temperature range in Mg-F-actin (Nyitrai et al., 1999).

In this study the effect of pH was characterized in the presence of calcium ions as well. In the case of Ca-F-actin the relative f showed increasing tendency at pH 6.5 up to 139 %. At pH 7.4 the relative f reached its maximum value of 167 % at 34 ^{o}C (Fig. 3.). Accordingly, the increased inter-monomer flexibility was also detected in the case of Ca-F-actin at higher pH value.

Not only the difference induced by the pH in the dynamic properties of the protein matrix was identified during these measurements but the difference between the flexibility of the actin filaments saturated with calcium or magnesium ions as well. It was already shown that the inter-monomer flexibility of the F-actin was greater if the filament was saturated with calcium ions compared to that of the Mg-F-actin at pH 8.0 (Nyitrai et al., 1999). In the

present experiments change induced by the temperature in the relative *f* was larger when the actin bound calcium than magnesium at either pH 6.5 or pH 7.4. These observations support our previous conclusion that the inter-monomer flexibility of Ca-F-actin is greater than that of Mg-F-actin (Nyitrai et al., 1999).

In the relative *f* parameters the difference induced by the pH was greater in Ca-F-actin than in Mg-F-actin. In the case of Ca-F-actin the greater effect of the pH might also indicate a smaller dynamic variability of the Mg-actin filaments.

CONCLUSIONS

Our data demonstrate that the lower intracellular pH can produce an altered and more rigid structure of actin filaments. The dynamic and conformational properties of actin are important for the proper interaction of the actin filament and myosin, and thus the alteration of this flexibility might result in inappropriate acto-myosin connections. It seems reasonable to assume that the stereo-specific interaction between the actin and the actin-binding site of the myosin head requires the proper conformational and motional freedom of the actin filaments. When these properties of actin filaments deviate from the optimum the interaction is probably modified, and therefore the force generation process is influenced as well. Considering that the pH can decrease within the muscle cells under physiological or pathological conditions it seems to be likely that the inappropriate performance of muscle contraction is attributed, at least partly, to the modified dynamic properties of the actin filaments as well. Accordingly, the present results give a possible approach to understand the molecular basis of the inadequate work of the muscle cells under physiological and pathological conditions.

MATERIALS AND METHODS

Chemicals

KCl, $MgCl_2$, $CaCl_2$, NaOH, KOH, Tris, IAEDANS, quinine (hemisulfate salt), DMF and EGTA were obtained from SIGMA Chem. Co. (St. Louis, MO, USA). IAF was purchased from Molecular Probes (Eugene, OR, USA). ATP and MEA were obtained from MERCK (Darmstadt, Germany) and NaN_3 was purchased from FLUKA (Switzerland).

Protein preparation

Acetone–dried powder of rabbit skeletal muscle was obtained as described earlier (Feuer *et al*., 1948) and actin was prepared according to the method of Spudich and Watt (Spudich & Watt, 1971). The actin was stored in a 2 mM Tris / HCl buffer containing 0.2 mM ATP, 0.1 mM $CaCl_2$ and 0.005 % NaN_3, pH 8.0 (buffer A) during the preparations and the labeling procedures. During the measurements the buffer contained 4 mM MOPS, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM MEA, 0.005 % NaN_3 and the pH was adjusted to 6.5 or 7.4. The actin concentration was determined with a Shimadzu UV-2100 spectrophotometer by using the absorption coefficient of 0.63 mg ml^{-1} cm^{-1} at 290 nm for actin monomer (Houk & Ue, 1974) with a relative molecular mass of 42.300 Da (Elzinga *et al*., 1973). In the case of the actin monomers, labeled by IAEDANS or IAF, the measured absorbency at 290 nm was corrected for the contribution of fluorescent labels. We used the $A^{290\ nm} = 0.35 \times A^{336\ nm}$ for the bound IAEDANS at both pH and $A^{290\ nm} = 0.27 \times A^{485\ nm}$ at pH 6.5 or $A^{290\ nm} = 0.23 \times A^{495\ nm}$ at pH 7.4 for the bound IAF.

Fluorescent labeling of the samples

Labeling of the Cys^{374} residue with IAEDANS was performed in buffer A as described earlier (Miki *et al*., 1987). Actin filaments (46 μM) were incubated with tenfold molar excess of IAEDANS for 1h at room temperature. The IAEDANS was dissolved in DMF and diluted with the appropriate buffer before being added to the protein solution. Adding MEA to the final concentration of 1 mM stopped the labeling procedure. After the labeling procedures the samples were centrifuged for 2 hours at 100.000 g at 4°C. The pellet was homogenized and dissolved in the appropriate MOPS buffer, and then dialyzed against this buffer for at least 12 hours.

In separate samples Cys^{374} was labeled in buffer A with IAF in the following way: monomeric actin (46 μM)

was mixed with a tenfold molar excess of IAF. IAF was dissolved in 0.1N NaOH and added to the actin solution at room temperature while the pH was kept constant. The incubation was carried out at room temperature in the first 4 hours and then the temperature of the sample was decreased to 4 ^{o}C for the next 8-12 hours. After incubation the actin was polymerized for at least one hour at room temperature and centrifuged at 100.000 x g for 2 hours at 4 ^{o}C. The pellet was treated in a way similar described in the case of labeling with IAEDANS.

The concentration of the IAEDANS in the protein solution was determined by using the absorption coefficient of 6100 $M^{-1}cm^{-1}$ at 336 nm at either pH 6.5 or 7.4 (Hudson & Weber, 1973). The appropriate values of the absorption coefficients for IAF were determined by means of the work of Takashi (Takashi, 1979). Accordingly, the absorption coefficients of 60000 $M^{-1}cm^{-1}$ at 495 nm at pH 7.4 and 49800 $M^{-1}cm^{-1}$ at pH 6.5 at 485 nm were used. In the samples the extent of labeling with IAEDANS and IAF was 0.90 ± 0.13 and 0.59 ± 0.11.

Sample preparation and polymerization

The Ca-F-actin was polymerized from the monomeric Ca-actin by adjusting the calcium concentration in the solution to 2 mM and the KCl to 100 mM. The samples were polymerized overnight at 4 ^{o}C.

During the preparation of Mg-F-actin filaments the first step was the preparation of Mg-actin monomers by the method of Strzelecka-Golaszewska and her co-workers (Strzelecka-Golaszewska *et al*., 1993). EGTA and $MgCl_2$ were added to the protein solution to set the final concentrations of 0.2 mM and 0.1 mM, respectively. During the 10 minute long cation exchange procedure the samples were stirred at room temperature. Polymerization of Mg-G-actin was started by adding KCl and $MgCl_2$ to the samples to adjust the concentrations to 100 mM and 2 mM, respectively. The samples were incubated at room temperature for at least 1 hour and then measured within one day after the preparation procedures.

While studying inter-monomer flexibility, we mixed the solutions of the actin monomers labeled by the donor (IAEDANS) or the acceptor (IAF) to obtain the molar ratio of 1 : 10 or less between the concentration of actin monomers labeled by the donor and the total actin concentration. After the appropriate mixtures of the actin monomers were obtained the polymerization process was started. The concentration of actin, IAEDANS and IAF were 23 μM, 2.3 μM and 12 μM during the measurements of f.

Fluorescence experiments

The steady-state fluorescence measurements were made with a Perkin Elmer LS50B Luminescence Spectrometer equipped with a thermostated sample holder flushed with dry air against the water condensation. The excitation wavelength for IAEDANS was 350 nm. The optical slits were set to 5 nm in the excitation light paths and 3.5, 5 or 6 nm on the emission side. In order to exclude the emission of the acceptor molecules during the FRET measurements we detected the IAEDANS fluorescence emission over the range of 400-460 nm. During the measurements of the overlap integral we recorded the fluorescent spectra of the donor molecule on the range of 350-700 nm. The fluorescence spectra were corrected with the absorption spectra of the same sample by using the following equation:

$$F_{corr} = F_{obs} \text{ antilog } (OD_{ex} + OD_{em}) \quad (1)$$

where F_{corr} and F_{obs} are the corrected and measured fluorescence intensities, and OD_{ex} and OD_{em} are the optical density of the sample on the excitation and the emission wavelengths.

Theoretical considerations

The efficiency of FRET (E) can be determined by using the following equation:

$$E = 1 - (\phi_{DA} / \phi_{D}) \quad (2)$$

where ϕ_{DA} and ϕ_{D} are the quantum yield of the donor in the presence and in the absence of the acceptor. On the other hand the FRET efficiency is defined as:

$$E = k_t / (k_t + k_f + k_0) \quad (3)$$

where k_t is the rate constant of the energy transfer, k_f is the rate constant of the donor emission and k_0 is the summarized rate constant of the other non-radiative processes de-exciting the donor molecules. The energy transfer rate constant (k_t) can be calculated as follows:

$$k_t = d\, J\, n^{-4}\, k_f\, R^{-6}\, \kappa^2 \quad (4)$$

where d is a constant depending on the units applied in the expression, J is the overlap integral between the emission spectra of the donor and the absorption spectra of the acceptor, n is the refractive index of the medium between the donor and acceptor, R is the distance between the two fluorophores, and κ is the orientation factor. During our calculations we used 1.4 for n and 2 / 3 for κ^2.

The overlap integral (J) can be calculated as:

$$J = \int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \; / \; \int F_D(\lambda) d\lambda \quad (5)$$

where $F_D(\lambda)$ is the fluorescence emission spectra of the donor molecules and $\varepsilon_A(\lambda)$ is the absorption spectra of the acceptor molecule as a function of wavelengths (λ).

The FRET parameter f, was defined as the ratio of the FRET efficiency to the quantum yield of the donor measured in the presence of the acceptor (Somogyi et al., 1984). This parameter is proportional to the mean of the rate constant that is characteristic of the FRET. The f is related to the spectral parameters of the applied donors and acceptors and also to the conformational and dynamic properties of the investigated system through the following expression (Somogyi et al., 1984):

$$f = E / \phi_{DA} = d\, n^{-4}\, J\, \{R_i^{-6}\, \kappa_i^{\,2}\} \qquad (6)$$

It is possible to obtain information regarding to the different forms of protein with respect to the protein conformation and flexibility (Somogyi et al., 1984) by monitoring the temperature dependence of f.

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ABBREVIATIONS

FRET: fluorescence resonance energy transfer;

IAEDANS: N-(((iodoacetyl)amino(ethyl)-5-naphthylamine-1-sulfonate;

IAF: 5-(iodoacetamido)fluorescein;

EGTA: ethyleneglycol-*bis*-(β-aminoethyl-ether)N,N,N',N',-tetraacetic acid;

MEA: β-mercaptoethanol;

DMF: dimethyl formamide;

NaN_3: sodium azide;

TRIS: tris-(hidroxy-methyl)amino-methane;

MOPS: 3-(N-morpholino)propanelsulfonic acid;

ATP: adenosine-5'-triphosphate;

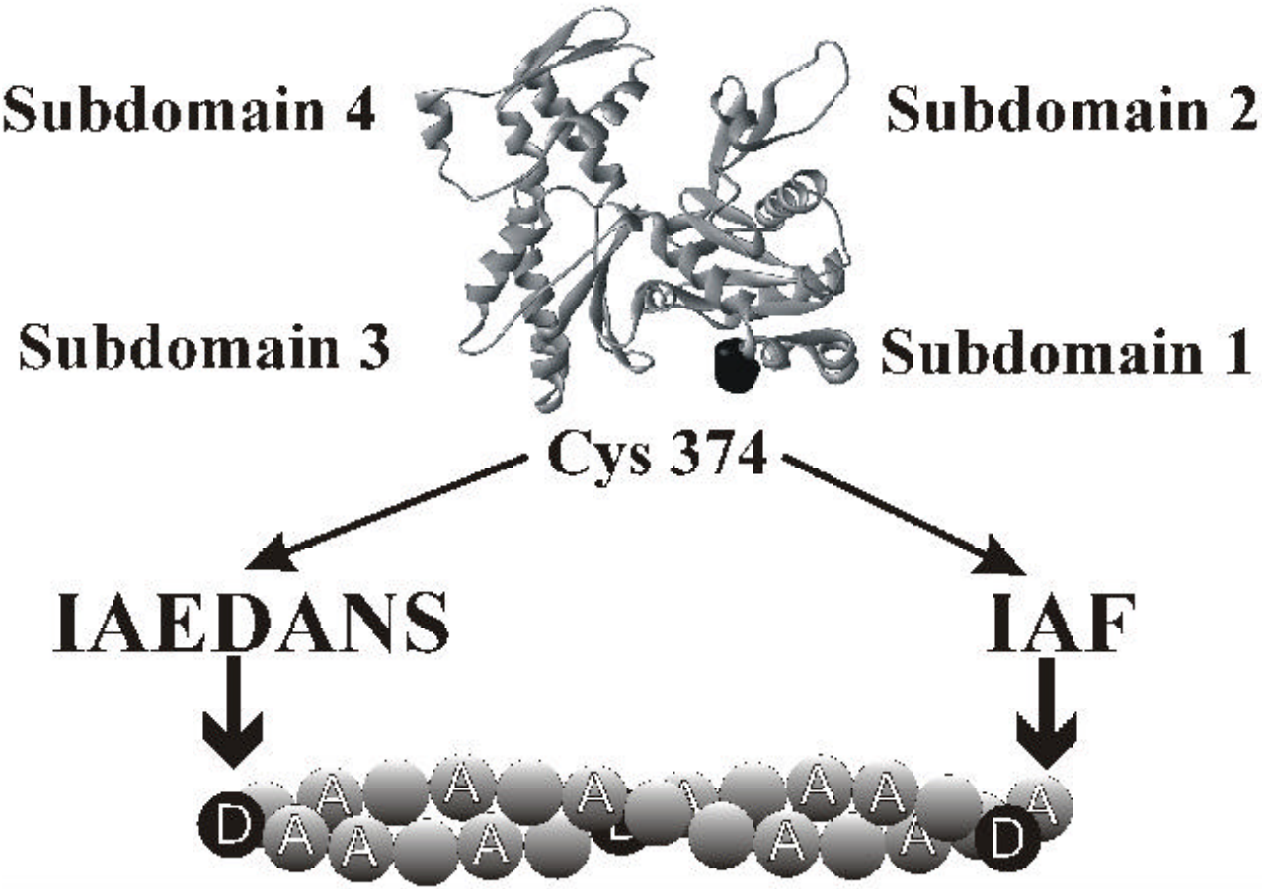

Figure 1.: Schematic representation of the actin. The actin subdomains and the approximated position of Cys^{374}, which was labeled by the fluorophores in this study, are indicated in the structure of actin monomer. The presentation of the actin filament shows a possible arrangement of protomers during the measurements of the inter-monomer flexibility of actin filaments at different pH values. The figure was constructed using the atomic coordinates of the actin monomer (Kabsch *et al.*, 1990) from the Brookhaven Protein Data Bank (file 1ATN).

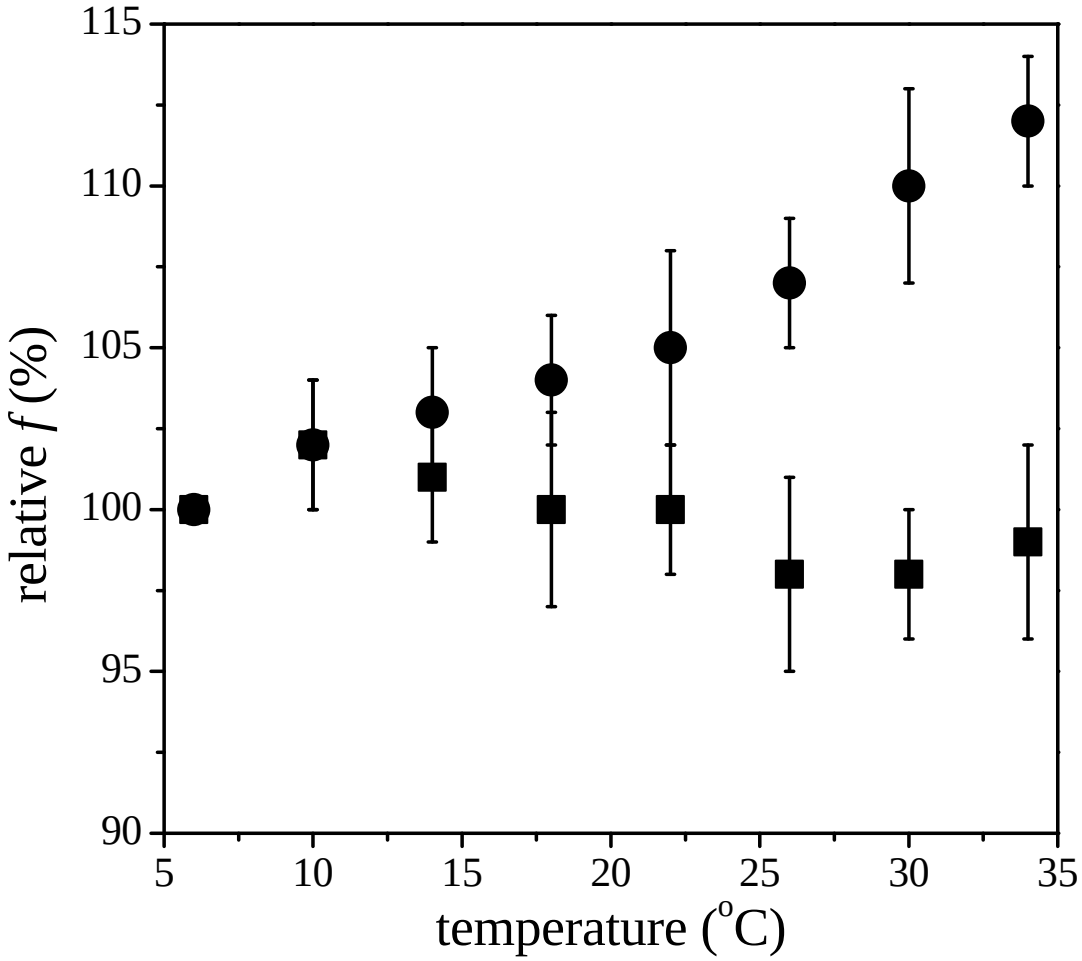

Figure 2.: The temperature profile of relative f of Mg-F-actin calculated from the FRET data calculated at pH 7.4 (filled circles) or at pH 6.5(filled squares).

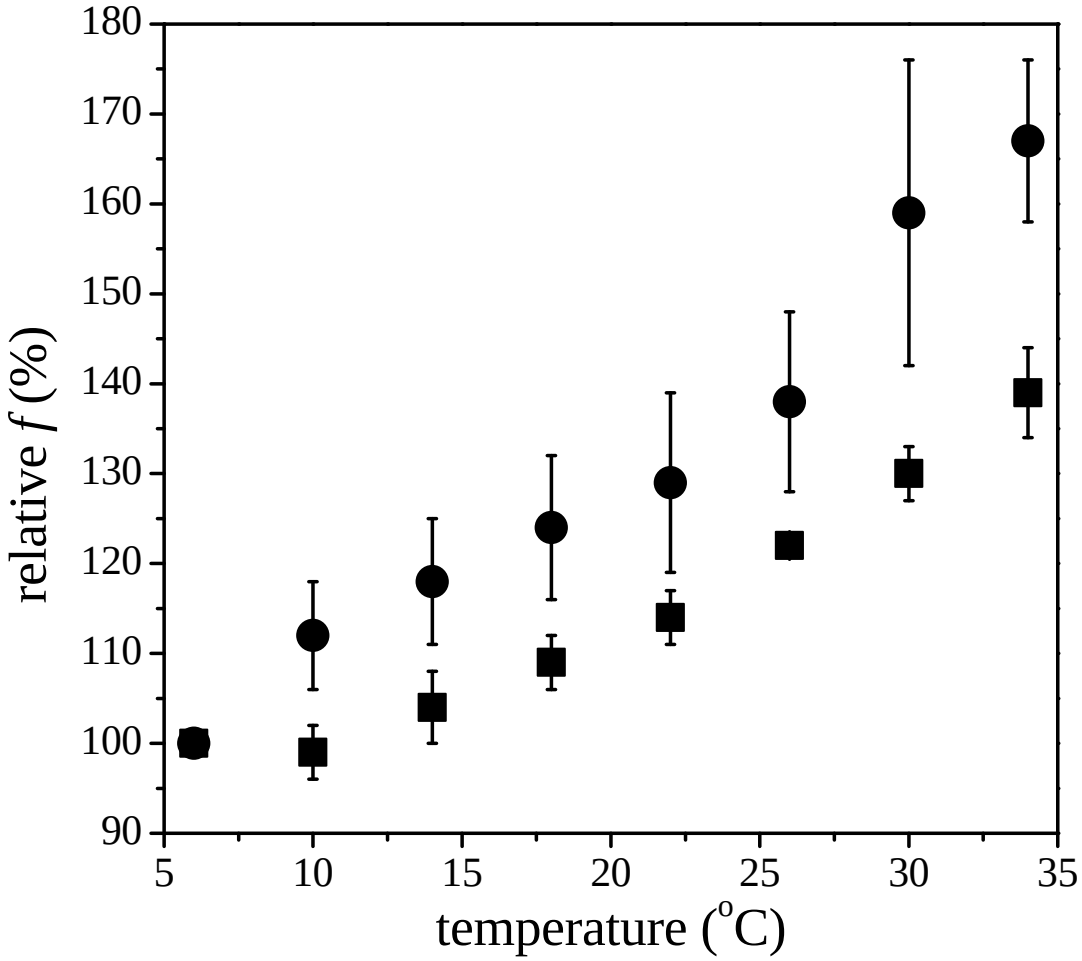

Figure 3.: The temperature profile of relative f of Ca-F-actin calculated from the FRET data calculated at pH 7.4 (filled circles) or at pH 6.5(filled squares).