Production and crystallization of lobster muscle tropomyosin expressed in Sf9 cells

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Received 19 July 1996; revised version received 14 August 1996

Abstract **A new form of muscle tropomyosin crystal has been obtained, by employing new strategies in protein preparation and crystallization. Non-polymerizable tropomyosin was prepared by removing 11 amino acids at the C-terminus. The truncated tropomyosin was expressed in Sf9 insect cells by use of the baculovirus-based expression system, to obtain highly homogeneous protein preparations. By routinely monitoring homogeneity by mass spectrometry, we found that the homogeneity played a key role in obtaining good crystals. The crystal quality was also dependent on isoforms; the crystals raised from a slow muscle-specific isoform diffracted to a higher resolution, compared with a fast muscle-specific counterpart. For crystallization, a high concentration of organic solvent was used as the precipitant; in the presence of 35% DMSO, tetragonal crystals were formed, which belong to space group $P4_{3(1)}2_12$ with cell constants of $a = b = 105.6$ Å, $c = 506.9$ Å. The crystals gave rise to reflections the intensities of which were characteristically determined by the transform of α-helical coiled-coil. Thus in the region of 10–5.5 Å resolution along the c^*-axis, the reflections were weak. For accurate measurement of these reflection intensities, beam-line ID2 in ESRF Grenoble was advantageous owing to the high brilliance and a low background. There the crystals diffracted to beyond 3.0 Å along the c^*-axis, whereas along the a^*-b^*-plane reflections were limited to 6.6 Å. Data analysis is under way on a data set from a $PtCl_4$ derivative.**

Key words: Tropomyosin; Skeletal muscle regulation; Baculoviridae genetics; Crystallization; X-ray diffraction; Mass spectrometry

1. Introduction

Tropomyosin was originally isolated as a constituent of the contractile apparatus of skeletal muscle [1]. When troponin was discovered, Ebashi and coworkers [2] rediscovered tropomyosin with its physiological role as a protein associated with troponin in the calcium-based regulatory mechanism of skeletal muscle contraction, as well as its localization in the grooves of the actin filament. It is now well known that tropomyosin isoforms are widely distributed in non-muscle cells, being always associated with actin and presumably making actin filaments more stable [3]. In skeletal muscle, tropomyosin molecules predominantly consist of two identical chains, each of 284 amino acids and about 33 kDa. Two chains, in a parallel and aligned configuration, form an α-helical coiled-coil over almost the entire length of the molecule, a rod 400 Å long and 20 Å thick.

The structure of tropomyosin at an atomic resolution is not known. Especially the structure of skeletal muscle tropomyosin is of great interest because, on one hand, this molecule is a stable α-helical coiled-coil, and yet this molecule must be flexible enough to govern the states of many actin subunits in response to Ca^{2+} binding to troponin. For the molecule to be functional in this way, segmental variation of the flexibility might be crucial, and this could be deduced from variation in the dimeric chain separation. The location of large side chains, which are mostly charged in tropomyosin, could be another clue for understanding the local stability of the molecule. It is of great interest to account for the flexibility of tropomyosin and the rigidity of the leucine zipper [4], both being α-helical coiled-coil proteins.

No tropomyosin crystal has been reported which is suitable for crystallographic studies at an atomic resolution. Bailey's crystal [1] turned out to be not suitable, because of the mesh-like packing of the molecules with a high solvent content (more than 90%). A new form of crystal grown in the presence of high concentrations of spermine was reported [5]. However, phases were not obtained from the crystals, due to difficulties in obtaining heavy atom derivatives. In the present study, new strategies were employed in preparation of the protein as well as in crystallization, and a new form of tropomyosin crystal is obtained which is suitable for structural analysis at an atomic resolution.

2. Materials and methods

2.1. Expression of lobster tropomyosin in Sf9 cells

Initially, tropomyosin was prepared either from abdominal flexor muscles (fast muscles) in the tail or from muscles in crusher claws (slow muscles) of the American lobster, *Homarus americanus*, as described previously [6]. Eleven residues were removed from the C-terminus by CPA (Sigma C9762) digestion [6].

Subsequently, cDNAs of two isoforms were obtained from lobster muscles; a slow muscle-specific isoform (*sTm1*) and a fast muscle-

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Abbreviations: CPA, carboxypeptidase A; DMSO, dimethylsulfoxide; DTT, dithiothreitol; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; EMBL, European Molecular Biology Laboratory; ESRF, European Synchrotron Radiation Facility; PMSF, phenylmethylsulfonyl fluoride; *sTm1* and *fTm1*, a slow muscle- and a fast muscle-specific isoform of lobster muscle tropomyosin; Sf9, *Spodoptera frugiperda* insect cells; Tris, tris[hydroxymethyl]aminomethane

PII S0014-5793(96)00949-0

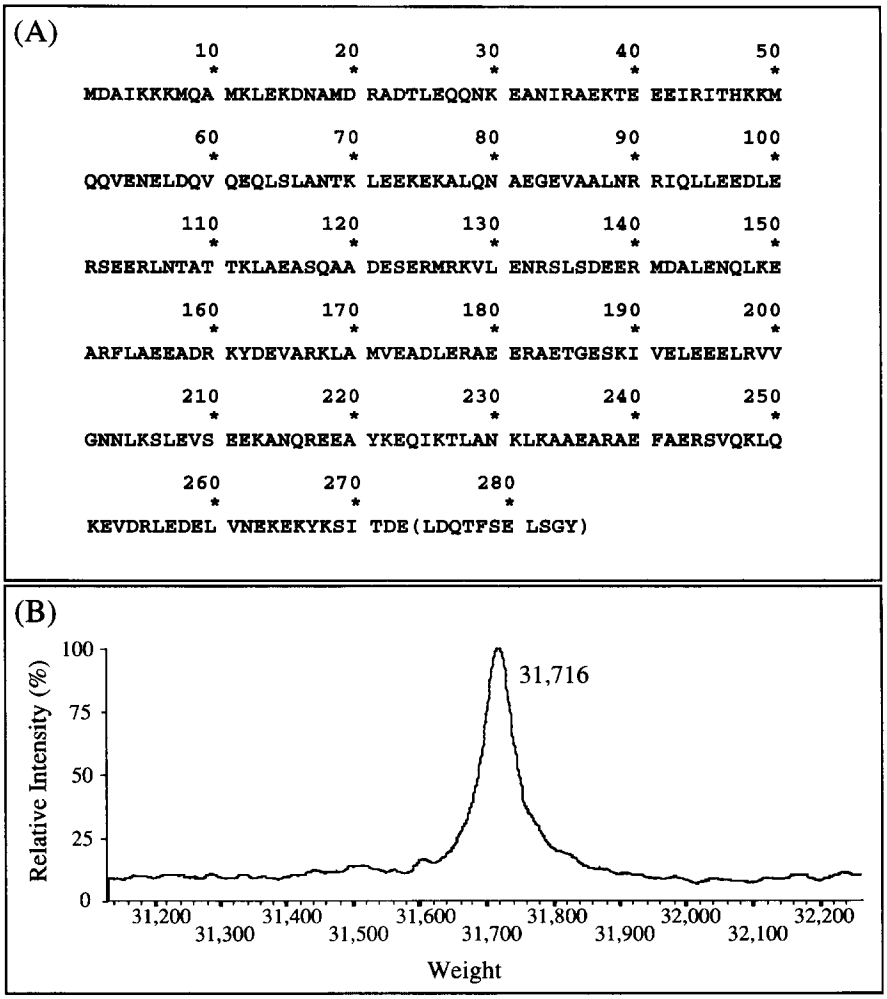

Fig. 1. A tropomyosin isoform of lobster slow muscle, *sTm1*. A: The amino acid sequence deduced from the cDNA nucleotide sequence; B: a mass spectrum of the protein expressed in the baculovirus-based expression system. Eleven residues at the C-terminus indicated in parentheses in A were not included in the expressed protein measured as in B.

specific isoform (*fTm1*), which will be described elsewhere (Mykles et al., in preparation).

By use of the baculovirus-based expression system [7], two tropomyosin isoforms were expressed, each being deprived of 11 residues at the C-terminus by replacing the codon for Leu-274 with a termination codon TAA. Extraction and purification of tropomyosin from Sf9 cells were performed as previously described [7] with some modifications. (1) The cells were cultured in suspension in medium containing 90% Sf900II (Gibco BRL), 9% Grace's insect medium (Gibco BRL) supplemented with 1% fetal calf serum (Sigma) and 0.2% Pluronic F68 (Gibco BRL). (2) The cells were disrupted not by ultrasonication but by suspension in the hypotonic lysis buffer containing 20 mM Tris-HCl (pH8.0), 0.5 mM EDTA, 5 mM DTT, 1 mM PMSF, leupeptin, pepstatin and chymostatin, each 20 μg/ml. (3) The crude tropomyosin preparation was heated, not in the presence of high concentrations of salts, but after removing salts by dialysis of the preparation against 20 mM Tris-HCl pH 8.0, 1 mM EDTA and 5 mM DTT. With these modifications the amount of contaminated materials, both proteins and non-protein factors, in the end product was substantially reduced. The expression level was high: after purification 50–100 mg per 3 liter culture contained about 10^{10} cells. Homogeneity of the preparation, including full acetylation of the N-terminus, was routinely checked using liquid chromatography/electrospray mass spectrometry (Fig. 1B) as described previously [8].

2.2. Crystallization procedures

For crystallization, the hanging-drop method was employed at 16°C by mixing the protein solution containing 30–36 mg/ml tropomyosin, 10 mM Tris-HCl, pH 7.6, 1 mM NaN_3 and the reservoir solution containing 34–38% DMSO, 10 mM Tris-HCl, pH 7.6, 1 mM NaN_3 and 0.3% 1,2,3-heptanetriol. The mixture was allowed to equilibrate against 0.5 ml reservoir solution. Resulting crystals were placed in a solution containing 40% DMSO, mounted on a rayon loop of a Crystal-Cap system of Hampton Research, and quickly frozen by plunging into liquid propane. Heavy atom labeling with $PtCl_4$ was performed by soaking the crystals in the 40% DMSO solution containing 0.2–0.5 mM K_2PtCl_4 at 20°C for 12–18 h.

The crystal density was measured on a density gradient from 50% to 20% of CCl_4 in xylene [9]. The gradient was calibrated with drops of various concentrations of CsCl in water. 0.732 was used for the specific volume of tropomyosin [10].

2.3. Data collection

Rotation photographs were recorded at ID2 (BL4) of the ESRF in Grenoble, using an image plate-based detector of MAR-research (30 cm diameter) which was placed either at 50 cm on axis or at 60 cm off axis ($2\theta=6°$) behind the crystal. The undulator and X-ray optics were tuned for $\lambda=1.00$ Å and the beam was limited by a collimator of 0.1 mm diameter. Exposures were made for 10 and 30 s, while the crystals were rotated over 3° around the spindle axis, which was vertical to the storage ring. The crystals were mounted with the *c*-axis tilted by about 10° relative to the spindle axis, and were kept at 100 K by use of a Cryostream (Oxford Cryosystems). Even at this temperature the beam damage was substantial so that the crystals were translated after every 7–10 min of cumulative exposure time.

The diffraction data were processed with the program DENZO [11] followed by scaling and merging using SCALPACK [11].

3. Results and discussion

The poor diffraction pattern of the Bailey crystal form arises from the high solvent content and/or intrinsic flexibility of the molecule. The solvent content is high as the elongated molecules form a kite-shaped mesh. The mesh is formed because of two reasons. Firstly the building block of the crystal is not a single molecule but a chain of molecules which are linked through a head-to-tail interaction, imposing extra restrictions on molecular packing. Secondly, hydrophobic interactions are emphasized by adding ammonium sulfate as the precipitant. Our crystallization strategies were therefore to find a way to make the molecules pack side-to-side by using (1) non-polymerizable tropomyosin preparations and (2) an organic solvent as the precipitant to emphasize ionic interactions and suppress hydrophobic interactions.

Non-polymerizable tropomyosin was initially prepared by CPA digestion of the protein extracted from muscles. The CPA digestion resulted in heterogeneous proteolytic products when applied to rabbit skeletal α-tropomyosin. When applied to lobster tropomyosin, however, the products were much more homogeneous, from which crystals grew in the presence of 35% DMSO [12]. Nevertheless the diffraction patterns were poor, at least partly due to insufficient homogeneity of the preparation. Mass spectrometry revealed that the tail muscle tropomyosin preparation contains more than one isoforms and that the CPA digestion gave rise to extra heterogeneity. Moreover the CPA digestion was not always reproducible.

In order to solve the heterogeneity problem finally, we expressed tropomyosin in the baculovirus-based expression system. The baculovirus system is required because only this system, not any *E. coli*-based expression system, gives rise to acetylated N-terminus, which is essential for the intact conformation of the N-terminus [7,13,14].

Expressed and compared were two isoforms, *sTm1* and *fTm1*, a slow and a fast muscle-specific isoform, respectively, which were deprived of C-terminal eleven residues. The two isoforms differ from each other at 15 residues near the N-terminus (Fig. 1A and Mykles et al., in preparation). The specific function of each isoform is not known. Both isoforms formed identical crystals, except for different morphologies (Fig. 2). *sTm1* crystals compared favorably in two respects; (1) they diffract X-rays to 3.0 Å, compared with 3.5 Å by *fTm1* crystals, and (2) the latter are too elongated to be mounted without deformations.

Table 1
Crsytallographic parameters of lobster muscle tropomyosin crystals

Parameter	Value/description	
	slow muscle type (*sTm1*)	fast muscle type (*fTm1*)
Shape	elongated octagons	prismatic needles
Density	1.104 g/cm^3	
Space group	$P4_{3(1)}2_12$	
Cell dimensions	$a=b=105.6$ Å, $c=506.9$ Å	
Cell volume	5.65×10^6 Å^3	
Volume occupied by protein	15.6%	
Chains/asymmetric unit	2 or 4 (1 or 2 dimeric molecules)	

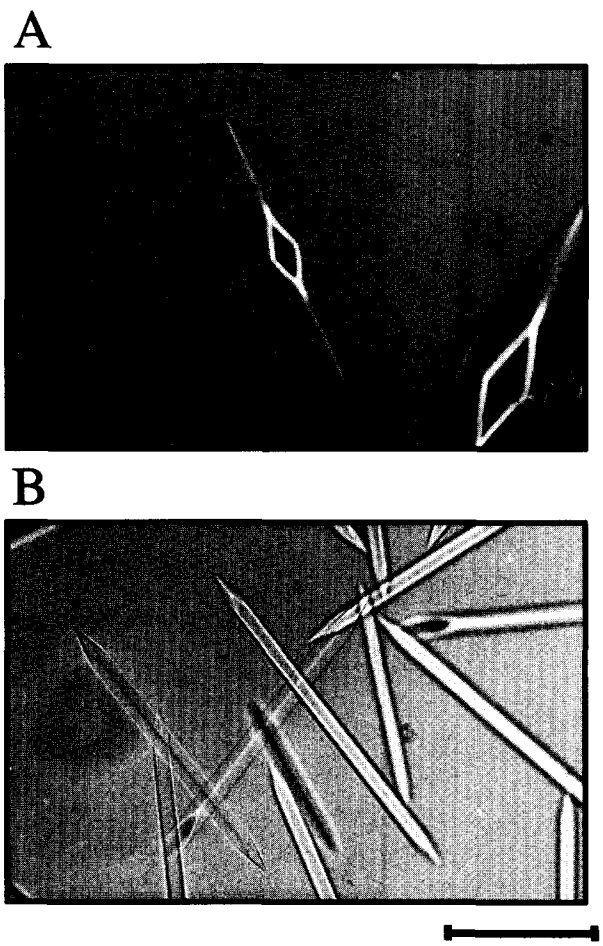

Fig. 2. Crystals of lobster muscle tropomyosin. A: *sTm1*; B: *fTm1*. The bar indicates 0.2 mm.

The use of 35% DMSO as the precipitant is uncommon for protein crystallization with a well-known exception of thermolysin. It is worth noting that 35–40% DMSO is advantageous both as a cryoprotectant and as a soaking solution for heavy atom labeling. The crystal parameters are listed in Table 1. Only crystals of poor quality were obtained when similar crystallization conditions were applied to rabbit skeletal muscle α-tropomyosin which were expressed in Sf9 cells exactly in the same way as for the lobster counterparts.

Diffraction patterns from the crystals of lobster slow muscle tropomyosin were extended beyond 3 Å along the c^*-axis, whereas in the direction of the a^*- and b^*-axes reflections were limited to 6.6 Å (Fig. 3). A prominent feature of the patterns is that the diffraction intensities are governed by the transform of a single structural motif, α-helical coiled-coil [15]. This is because the protein almost purely consists of this motif, and because within the crystal the molecules are oriented almost parallel to each other (see below). This gives rise to weak reflections in the region from 10 to 5.5 Å resolution along the c^*-axis, where accurate measurements of the intensities are crucially important. Another marked characteristic of the diffraction patterns was the intense streaks that were associated with some intense reflections. The streaks were tilted relative to the a^*-b^*-plane by on average 10–15°, suggesting that the molecules are tilted by this extent relative to the c^*-axis.

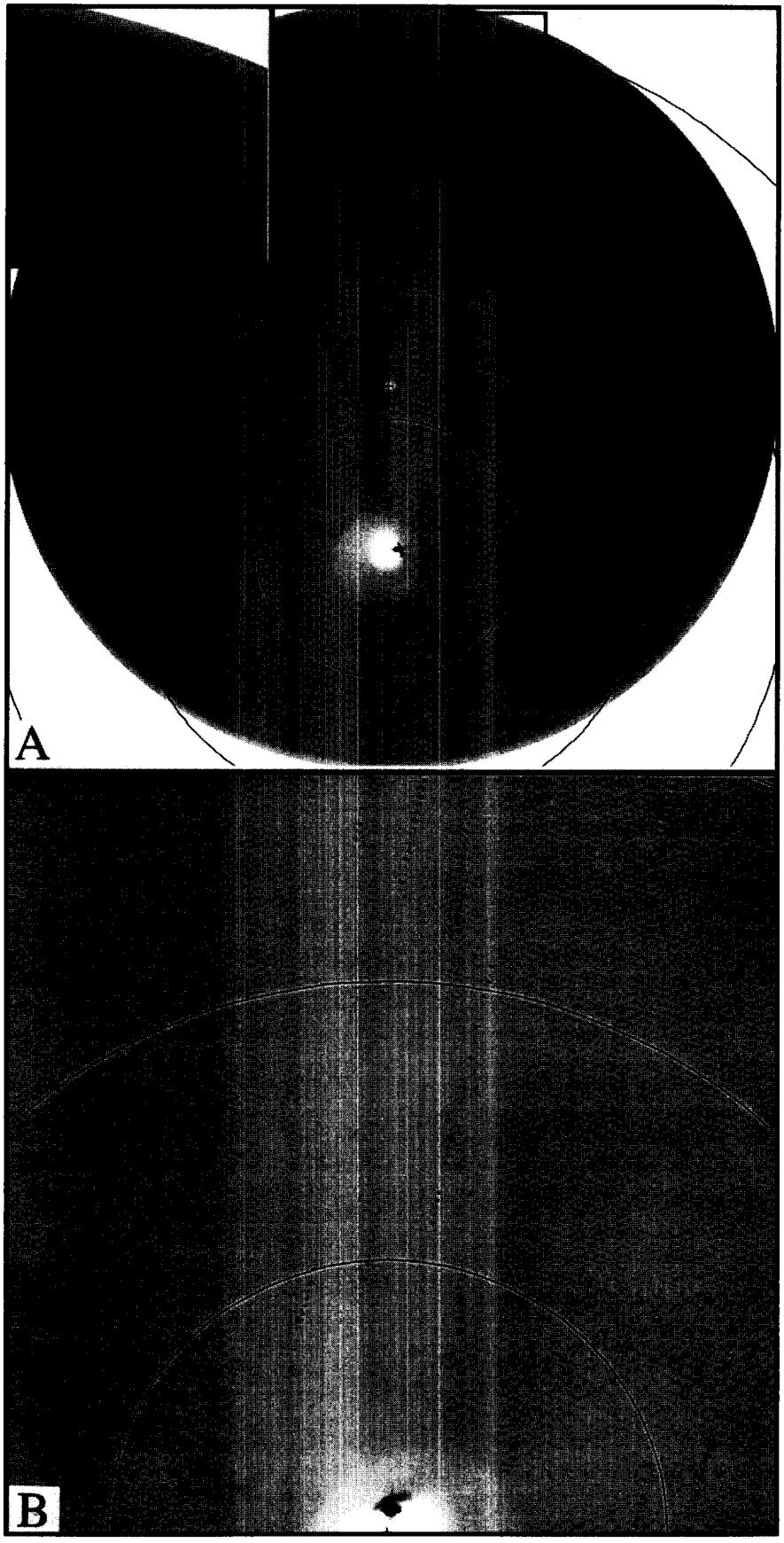
A
B

Fig. 3. Rotation photographs from an *sTm1* crystal. Exposure time was 2 min (only for these photographs) at $\lambda = 1.00$ Å at ID2 of ESRF. A: An overall pattern taken with a detector placed at 60 cm behind the crystal, with an off-set of $2\theta = 6°$. The crystal was rotated for 2° around the spindle axis, which was horizontal. This photograph has been reproduced here by rotating the original picture by 90° so that the c^*-axis is roughly vertical in the plane of this page. The circles correspond to resolutions of 12, 6, 4 and 3 Å. Note that intense reflections are observed around the equator (the plane includes the a^*- and b^*-axes) and around the plane $c^* = 1/5.4$ Å^{-1}, leaving the intermediate area much less intense. The area indicated by the square is magnified and reproduced in the inset with softer contrast to show reflections extending to 3.0 Å resolution. B: The intermediate area is enlarged to show weak reflections in the middle, intense reflections both at the bottom (around the equator) and the top (around $c^* = 1/5.4$ Å^{-1}) of the view. From the same crystal as A but with another geometry; crystal-to-detector distance 50 cm, $2\theta = 0°$, the spindle axis was vertical. The rotation angle was 3°. The circles are for 14, 7, and 4.7 Å resolutions.
←

For accurate measurements, Beamline ID2 in ESRF was advantageous, especially because of the low background and the high brilliance. In a data set obtained from a single native crystal, more than 16800 independent reflections were integrated in the area from 200 to 3.5 Å resolution, and merged with $R_{merge} = 8.6\%$. Scaling and merging of the data from a native crystal showed the crystal belongs to the tetragonal space group $P4_{3(1)}2_12$. After post-refinement, the unit cell dimensions were $a = b = 105.6$ Å, $c = 506.9$ Å. Analysis of another data set from a $PtCl_4$ derivative is in progress. Searches are under way for more heavy atom derivatives, and also for methods to prepare improved crystals which extend the diffraction limit in the direction of a^*- and b^*-axes.

Acknowledgements: We thank Z. Dauter, L. Kluwe, K. Wilson and other staff of EMBL Hamburg Outstation, as well as N. Sakabe, A. Nakagawa, N. Watanabe and other staff of Photon Factory in Tsukuba Japan, for their help during the initial stage of this work; B. Rasmussen for help with X-ray data collection and T. Tomizaki for data processing, both at ESRF in Grenoble; K. Namba and K. Imada for setting up an X-ray data collection system in the laboratory; D.L. Mykles for permitting us to publish a part of the results from a collaborative project. Financial support to M.Y. by MacScience is greatly acknowledged.

References

[1] Bailey, K. (1948) Biochem. J. 43, 271–279.
[2] Ebashi, S. and Endo, M. (1968) Prog. Biophys. Mol. Biol. 18, 123–183.
[3] Pittenger, M.F., Kazzaz, J.A. and Helfman, D.M. (1994) Curr. Opin. Cell. Biol. 6, 96–104.
[4] O'Shea, E.K., Klemm, J.D., Kim, P.S. and Alber, T. (1991) Science 254, 539–544.
[5] Whitby, F.G., Kent, H., Stewart, F., Stewart, M., Xie, X., Hatch, V., Cohen, C. and Phillips, G.J. (1992) J. Mol. Biol. 227, 441–452.
[6] Miegel, A., Kobayashi, T. and Maéda, Y. (1992) J. Muscle Res. Cell Motil. 13, 608–618.
[7] Kluwe, L., Maeda, K., Miegel, A., Fujita-Becker, S., Maéda, Y., Talbo, G., Houthaeve, T. and Kellner, R. (1995) J. Muscle Res Cell. Motil. 16, 103–110.
[8] Taniguchi, H., Suzuki, M., Manenti, S., and Titani, K. (1994) J. Biol. Chem. 269, 22481–22484.
[9] Matthews, B.W. (1985) in: Diffraction Methods for Biological Macromolecules, Part A (Wyckoff, H.W., Hirs, C.H.W. and Timasheff, S.N., Eds.), Methods in Enzymology Vol. 114, pp. 176–187, Academic Press, Orland.
[10] Durchschlag, H. (1986) in: Thermodynamic Data for Biochemistry and Biotechnologies (Hinz, H.-J., Ed.), pp. 45–147, Springer Verlag, Berlin.
[11] Otwinowski, Z. (1993) in: Proceedings of the CCP4 Study Weekend: 'Data Collection and Processing', 28–30 January 1993 (Compiled by: Sawyer, L., Issacs, N. and Bailey, S.), pp. 56–62, SERC Daresbury Laboratory, UK.
[12] Miegel, A., Lee, L., Dauter, Z. and Maéda, Y. (1993) Adv. Exp. Med. Biol. 332, 25–32.
[13] Monteiro, P.B., Lataro, R.C., Ferro, J.A. and Reinach, F.d.C. (1994) J. Biol. Chem. 269, 10461–10466.
[14] Urbancikova, M. and Hitchcock-DeGregori, S.E. (1994) J. Biol. Chem. 269, 24310–24315.
[15] Crick, F.H.C. (1953) Acta Crystallogr. 6, 685–689.