Volume 323, number 1,2, 83–88 FEBS 12511 May 1993

E. coli expression and characterization of a mutant troponin I with the three cysteine residues substituted

Lan Kluwe, Kayo Maeda and Yuichiro Maéda

European Molecular Biology Laboratory at DESY, Notkestraße 85, D-2000 Hamburg 52, Germany

Received 25 February 1993; revised version received 1 April 1993

A TnI cDNA was cloned from rabbit fast skeletal muscle, and site-directed mutagenesis was applied to replace all the three cysteine residues, Cys-48 and Cys-64 by Ala and Cys-133 by Ser. The mutant and wild-type TnI were expressed in *E coli* and purified to homogeneity. No significant functional differences were observed between the mutant and the authentic TnI in terms of the interactions with TnT and TnC, and the ability of the reassembled Tn complex to regulate the acto-S1 ATPase activity in a calcium-dependent manner. These findings suggest that none of the cysteine residues in TnI are essential for the function of this protein and can be replaced to obtain a non-oxidizable mutant TnI which is much easier to handle and suitable as an alternative to the authentic TnI for various purposes, such as crystallization of TnI and the whole Tn, and ^{1}H NMR studies.

Calcium regulation; Troponin, Troponin I; Sulfhydryl oxidation; Protein expression in *E coli*; Site-directed mutagenesis

1. INTRODUCTION

Muscle contraction is regulated by calcium ion concentrations through Troponihn (Tn), a regulatory protein complex located on the thin filament [1]. Tn consists of three subunits: TnC, TnI and TnT. TnI is the component which inhibits the force-generating interaction between myosin and actin. TnC suppresses the inhibitory effect of TnI, and TnT is required for inducing the calcium-dependence of the TnI–C interaction [2–4]. TnC, the Ca^{2+}-binding component, has been studied most intensively. In contrast, TnI has been investigated much less, mainly due to its low solubility and the loss of its activity during storage [2–5]. Horwitz et al. reported that the three cysteine residues in TnI from rabbit fast skeletal muscle (Cys-48, Cys-64 and Cys-133) can be readily oxidized, resulting in weak interactions between subunits, and reduced activity of Tn to sensitize the actomyosin to Ca^{2+} [5]. According to them, dithiothreitol (DTT) at millimolar concentrations in a solution at pH 8.5 would be degraded in an hour [5]. We have also noticed that fresh DTT must be added almost every day during storage in order to protect TnI from oxidation, and that it is difficult to reduce the oxidized protein completely without denaturation. Oxidation of TnI also occurs in dimeric complexes of TnI–C and TnI–T as well as in the whole Tn, although at slower rates than when TnI is alone (our unpublished observations). This property of TnI is obviously inconvenient for various experiments, and for certain purposes it even becomes obstructive. For example, for crystallization trials, high protein concentrations and long incubation periods at various temperatures are required, factors which promote oxidation in TnI. For H^1 NMR studies, high protein concentrations are also needed, and sulfhydryl reagents, such as DTT, have to be removed since signals from these molecules would mask those from the protein. In order to obtain stable TnI, and therefore stable Tn, we have carried out in the present work site-directed mutagenesis on TnI cDNA from rabbit fast skeletal muscle to replace all three cysteine residues, expressed this mutant TnI in *Escherichia coli* and characterized its properties.

Correspondence address: Y. Maéda, International Institute for Advanced Research, Matsushita Electric Industrial, 6-2 Sakakigatsubo, Hohsono, Seika-cho, Kyoto 619-02, Japan. Fax: (81) (7749) 32994.

Abbreviations Tn, troponin; TnI, troponin I; TnC, troponin C; TnT, troponin T; DTT, dithiothreitol; IPTG, isopropyl-1-thio-β-D-galactopyranoside; EGTA, ethylene glycol-bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride.

2. MATERIALS AND METHODS

2.1. *Cloning*

Clones containing cDNA of TnI were screened out from a λgt10 cDNA bank of rabbit fast skeletal muscle [7] using a plasmid containing a *Pst*I segment of quail TnI cDNA [8] as a probe. The *Eco*RI fragments of the positive λgt10 were then cloned onto M13 vectors and sequenced. The most complete TnI cDNA extended to the 3'-end of the coding region but lacked 20 bp at the 5'-end which correspond to 7 amino acid residues at the N-terminus.

2.2. *Construction of the TnI expression plasmid*

The original coding sequence contained one *Nco*I and two *Bgl*I sites. For practical reasons (see below), the *Nco*I and the second *Bgl*I sites

were destroyed by site-directed mutagenesis without changing the amino acid residues. A segment covering the missing 5'-terminal sequence of the TnI coding region was constructed using a pair of synthetic oligonucleotides based on the sequence provided by J. Potter before their publication [9]. The oligonucleotides were designed so that the segment had an *NcoI* site at the starting codon and two cohesive ends, *Hin*dIII-cohesive at the flanking sequence end and *Bgl*I-cohesive at the coding region end. Codons with higher usage frequencies in *E coli* were chosen in this segment to optimize protein expression (Fig. 1B). The *Hin*dIII–*Bgl*I fragment of an M13mp18 vector containing the incomplete TnI-coding sequence was replaced with this synthesized segment. The 0 6 kb *Nco*I–*Eco*RI fragment containing the complete TnI-coding region was then re-cloned onto pTrc99C, an expression vector with a *tac* promoter [10], giving rise to plasmid pTrc-tnI for TnI expression in *E coli* (Fig. 1A).

2.3. *Mutagenesis*

Oligonucleotides used for mutagenesis were GCG GAG CAC GCC CCG CCC CT for Cys-48→Ala, CAG GA G CTG GCC AAG CAG CT for Cys-64→Ala, and CAC AAG GTG AGC ATG GAC C for Cys-133→Ser, respectively. Mutagenesis was carried out on M13mp18 templates using a Muta-Gene In Vitro Mutagenesis kit (Bio-Rad) Clones containing the designed mutations were screened out by DNA sequencing. The resultant plasmid for expression of the mutant TnI, [Ala^{48},Ala^{64},Ser^{133}]TnI, has been designated as pTrc-tnI266.

2.4. *Protein expression*

To avoid protein degradation during lysis and purification, AD202, an ompT⁻ strain, was used as the expression host [11]. Routinely, 50 ml of the bacterial cells with TnI-expression plasmids were grown overnight at 37°C in a standard medium (Merk) supplemented with 50 μg/ml ampicillin, which was then used to inoculate 2 5 l of the same medium pre-warmed in a 5 l flask. The main culture was shaken at 250 rpm and 37°C for about 2 h until OD_{600} reached between 1.0 and 1.2. Induction of protein expression was then started by adding IPTG to a final concentration of 50 μg/ml, and incubation of the culture was continued overnight under the same conditions. The cells were harvested the next day at 8,000 rpm (Sorval GSA rotor) for 5 min, washed once with 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, and stored frozen at −80°C.

2.5. *Purification*

The frozen cells from 2.5 l of culture were thawed to 4°C, resuspended with 40 ml of lysis buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 8% sucrose, 5% Triton X-100, 0.1 mM PMSF, 1 μM pepstatin and 1 μM leupepsin. Lysozyme was then added to a final concentration of 0 5 mg/ml. Lysis was carried out at 4°C for at least 30 min up to overnight. The lysed suspension was sonicated for 5 min. TnI remained in the insoluble fraction after lysis and was collected by centrifugation at 20,000 rpm (Sorval SS34 rotor) for 20 min. The pellet was washed twice with lysis buffer, and then twice with the same buffer but without Triton X-100, by repeating sonication and centrifugation [12]. The washed pellet was dissolved in 6 M urea, 10 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 0.1 mM PMSF, 1 μM pepstatin and 1 μM leupepsin, and passed through 30 ml of DEAE resin in a funnel equilibrated with the same urea solution to adsorb DNA, RNA and other contaminants. The sample was then applied to an S-Sepharose Fast Flow column (2.5 × 5 cm) and eluted with a 200–200 ml gradient of 0.1–0.3 M NaCl in the same buffer. The main elution peak at 0.15 M NaCl contained > 95% of TnI. Further purification was performed by applying the mutant TnI to a TnC-Sepharose 4B affinity column in 6 M urea solution containing 1 mM $CaCl_2$, 10 mM triethanolamine (pH 7.5) and 0.1 mM PMSF. TnI was eluted specifically by replacing 5 mM EGTA for 1 mM $CaCl_2$ in the same urea solution [13]. Wild-type TnI was purified in the same way except that all solutions were supplemented with 0.5 mM DTT. After purification, the yields of both the wild-type and the mutant TnI were more than 20 mg/l bacterial culture.

2.6. *Interaction with TnT and TnC*

TnI–C complex formations were examined on 10% polyacrylamide gels (pH 8.4) containing 6 M urea and either 1 mM $CaCl_2$ or 1 mM EGTA Electrophoresis was carried out in 80 mM glycine-Tris buffer (pH 8.4) containing 1 mM $CaCl_2$ or 1 mM EGTA at 20 mA until the dye marker (Bromophenol blue) reached the end of the gel.

To examine the TnI–T interaction, each subunit and TnI–T mixtures were analyzed using a Pharmacia Superose 12 HR 10/30 column on FPLC. The column was equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 0.5 M KCl, 1 mM DTT and 1 mM NaN_3, and elutions were carried out at a flow rate of 0 5 ml/min.

2.7. *Acto-S1 ATPase*

The Tn complexes were reassembled using authentic TnI or the mutant TnI, together with TnC and TnT from rabbit skeletal muscle. The subunits were mixed in the presence of 6 M urea, and then dialysed against 10 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT and 1 mM NaN_3 to remove urea. The reassembled Tn complexes were purified using a Mono-Q column on FPLC, and the main peaks eluted at 0 4 M KCl were collected and used for ATPase assay. Actin, S1, Tm, and the Tn subunits were prepared from rabbit skeletal muscle [14–16]. ATPase measurements were carried out in a solution containing 10 mM HEPES (pH 7.2), 30 mM KCl, 4 mM $MgCl_2$, 1 mM NaN_3, 0.5 mM DTT, 5 mM MgATP, 0.5 mg/ml F-actin, 0.1 mg/ml Tm, 0.1 mg/ml Tn, 0.05 mg/ml S1, and a calcium buffer consisting of X mM $CaCl_2$ and Y mM EGTA where X and Y are varied as X+Y= 0.1 to obtain various free calcium concentrations, [Ca^{2+}]. [Ca^{2+}] was calculated using the apparent binding constant of EGTA, pK' = 6.45 [17]. Inorganic phosphate release between 5 and 10 min after adding S1 was measured by the Malachite green method [18], and the ATPase activities were calculated as μmol Pi/min/mg S1.

2.8. *TnI oxidation*

To remove trace impurities and degraded species which might interfere with interpretation, the authentic and the mutant TnI were further purified by HPLC reverse-phase chromatography, using a Vydac C_4 column with distilled water as the aqueous solvent and acetonitrile as the organic modifier, both containing 0.1% trifluoroacetic acid. The proteins were then reduced with 1 mM DTT in the presence of 6 M urea, adjusted to a concentration of 1.5 mg/ml and then dialyzed against 10 mM triethanolamine (pH 7.4), 0 4 M KCl and 1 mM NaN_3 at 4°C for 2 days. The samples were then mixed with SDS-loading buffers with and without DTT, heated for 2 min at 100°C and applied to a 15% SDS-polyacrylamide gel [5]

3. RESULTS

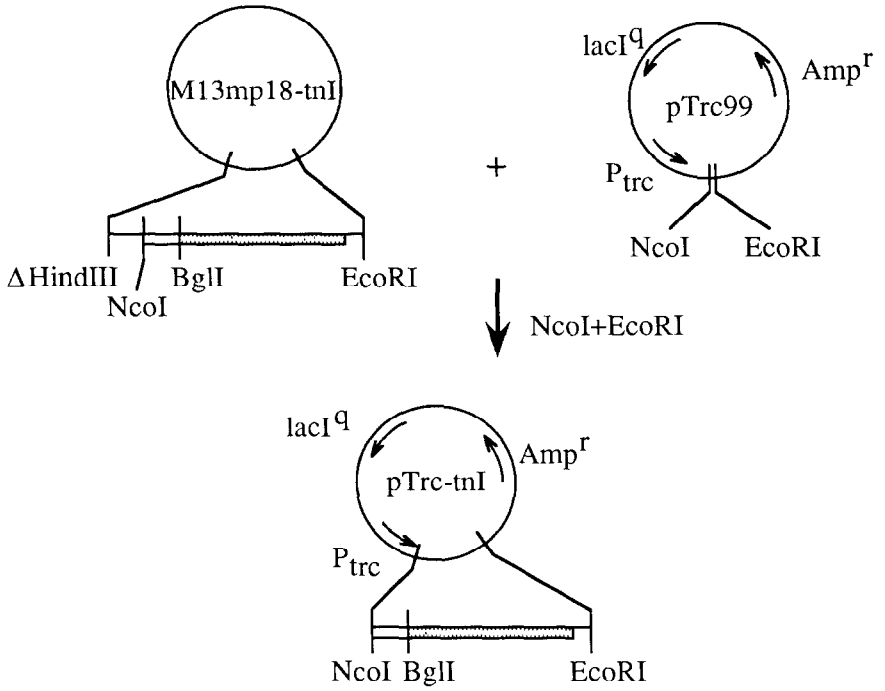
3.1. *cDNA sequence, mutagenesis, expression and purification*

The nucleotide sequence of TnI cDNA cloned in this study was identical to that provided by Dr. J. Potter before publication [9], except for T-342 and T-357, which are both C in their sequence.

Although Cys-48 and Cys-64 are replaced by isoleucine in, respectively, rabbit slow skeletal muscle [19] and crayfish muscle [20], we have changed both to alanine, since alanine is more compact than isoleucine and therefore preferred as a replacement for cysteine in mutagenesis [21]. Cys-133 was replaced with serine as in rabbit slow muscle TnI [19].

After TnC-Sepharose 4B affinity chromatography, both the mutant (Fig. 2) and wild-type TnI were purified to homogeneity. In both cases, the yield was more than 20 mg after purification from 1 l of bacterial cul-

A



B

```
                                                                    5'-AGCTCC
  1   A       G       A G
  ATGGGTGATGAAGAAAACGCAACCGTGCCATCACGGCCCGGCGGCAGCACCTGAAGAGCGTGATGCTGCAGATCGCGGCCACCGAGCTG
  M  G  D  E  E  K  R  N  R  A  I  T  A  R  R  Q  H  L  K  S  V  M  L  Q  I  A  A  T  E  L
 91                                                                          C  C
  GAGAAGGAGGAGGGCCGCCGCGAGGCCGAGAAGCAGAACTACCTGGCGGAGCACTGCCCGCCCCTGAGCCTCCCCGGTTCTATGGCCGAG
  E  K  E  E  G  R  R  E  A  E  K  Q  N  Y  L  A  E  H  C  P  P  L  S  L  P  G  S  M  A  E
181
  GTGCAGGAGCTGTGCAAGCAGCTGCACGCCAAGATCGACGCGGCCGAGGAGGAGAAGTATGACATGGAGATCAAGGTGCAGAAGAGCAGC
  V  Q  E  L  C  K  Q  L  H  A  K  I  D  A  A  E  E  E  K  Y  D  M  E  I  K  V  Q  K  S  S
271                                                                   C              C
  AAGGAGCTGGAGGACATGAACCAGAAGCTGTTCGACCTGCGGGGCAAGTTCAAGAGGCCCCCGCTGCGGCGTGTGCGCATGTCGGCTGAC
  K  E  L  E  D  M  N  Q  K  L  F  D  L  R  G  K  F  K  R  P  P  L  R  R  V  R  M  S  A  D
361
  GCCATGCTCAAGGCCCTGCTGGGCTCCAAGCACAAGGTGTGCATGGACCTGCGGGCCAACCTGAAGCAGGTGAAGAAGGAGGACACGGAG
  A  M  L  K  A  L  L  G  S  K  H  K  V  C  M  D  L  R  A  N  L  K  Q  V  K  K  E  D  T  E
451
  AAGGAGCGGGACCTGCGCGACGTGGGCGACTGGAGGAAGAACATCGAGGAGAAGTCGGGCATGGAGGGCCGCAAGAAGATGTTCGAGTCC
  K  E  R  D  L  R  D  V  G  D  W  R  K  N  I  E  E  K  S  G  M  E  G  R  K  K  M  F  E  S
541
  GAGTCCTAGGCCGCCCGACGCGT-3'
  E  S  *
```

Fig. 1. Plasmid construction, sequence and mutagenesis. (A) Open and hatched boxes indicate the synthesized 5'-terminal segment and the rest of the coding region of the cDNA cloned. (B) The wild-type TnI cDNA sequence together with the deduced amino acid sequence. The 5'-terminal sequence synthesized is underlined The bases changed for expression studies are boldfaced with the corresponding original bases indicated above.

ture. The N-terminal amino acid sequences of the purified TnIs were confirmed to be GDEEK-, consistent with the cDNA and peptide sequences [6].

3.2. *Interaction with TnC and TnT*

As described in section 2, the mutant TnI binds to TnC-Sepharose 4B column in the presence of 6 M urea in a Ca^{2+}-dependent manner as the authentic TnI [13]. Complex formation of TnI–C was also examined on polyacrylamide gels containing 6 M urea in which the TnI–C complex migrates more slowly than TnC, and TnI does not enter the gel. As shown in Fig. 3A, the mutant TnI formed a complex with TnC in the presence but not in the absence of Ca^{2+} unlike authentic TnI. These results suggested that the interaction of TnI with TnC was not substantially impaired by the mutations.

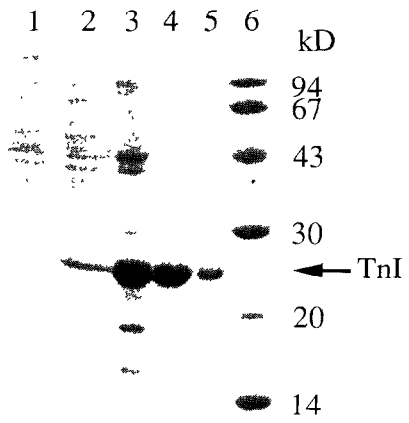


Fig. 2. Expression and purification of the mutant TnI: an SDS-PAGE pattern. Lanes: 1, cells with tnI266 plasmid without induction; 2, the same cells after induction with IPTG; 3, insoluble fraction of the lysed cells after extensive washing, 4, mutant TnI after purification; 5, authentic TnI as a control; 6, molecular weight markers

We have also examined the interaction of the mutant TnI with TnT using gel filtration. As shown in Fig. 3B, upon mixing the mutant TnI with TnT, a new peak appeared which preceeded the peaks of TnT and TnI, indicating formation of the TnI–T complex. Furthermore, on CD spectra (data not shown) from the mixture of the mutant TnI with TnT, we have observed in the near UV region (265–295 nm) the deep negative peak which is characteristic of a mixture of authentic TnI with TnT, but which is not observed with TnI or TnT alone. This negative peak was interpreted in terms of the specific interaction between TnI and TnT, causing structural changes in either or both of the subunits [5].

After reassembly of the Tn complex from TnC and TnT, both from rabbit muscle, and the mutant TnI, the complex was applied to a Mono-Q column in order to remove free subunits and heterodimers. The main peak containing all the three Tn components (see the insert of Fig. 4) was eluted at 0.4 M KCl, like the authentic Tn complex, while TnI and TnC alone were eluted at, respectively, 0.13 M and 0.48 M under the same conditions. This indicates that the Tn complex was formed with the mutant TnI.

3.3. *Regulation of the acto-S1 ATPase*

The reassembled Tn complexes with the mutant TnI were examined for their ability to regulate acto-S1 ATPase activity, using reassembled authentic Tn as a control. As shown in Fig. 4, we did not observe any significant difference between the two species. Thus, it is

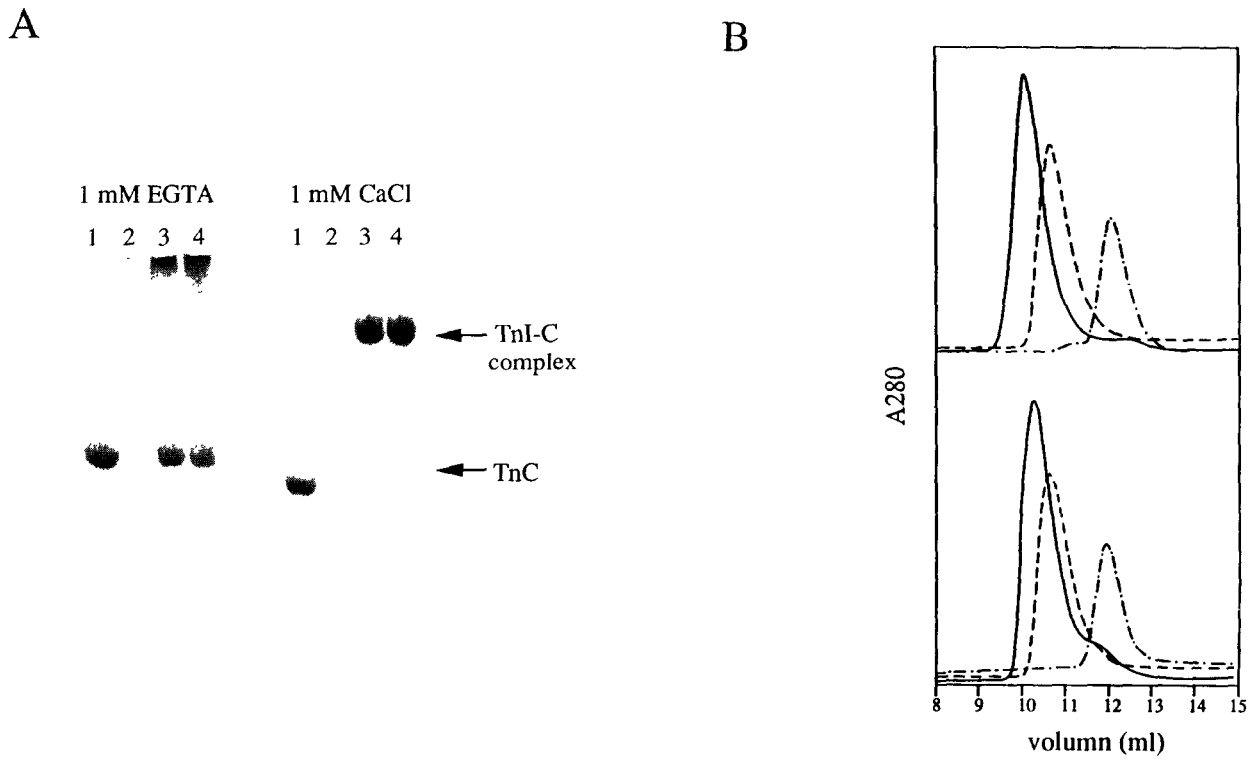


Fig. 3. Interaction with TnC and TnT. (A) PAGE in the presence of 6 M urea and either 1 mM $CaCl_2$ or 1 mM EGTA. Lanes: 1, TnC; 2, authentic TnI; 3, TnC+authentic TnI; 4, TnC+mutant TnI. (B) Gel filtration elution profiles: upper panel, authentic TnI; lower panel, mutant TnI. Solid line, TnT-I mixture; dashed line, TnT; dot-dashed line, TnI.

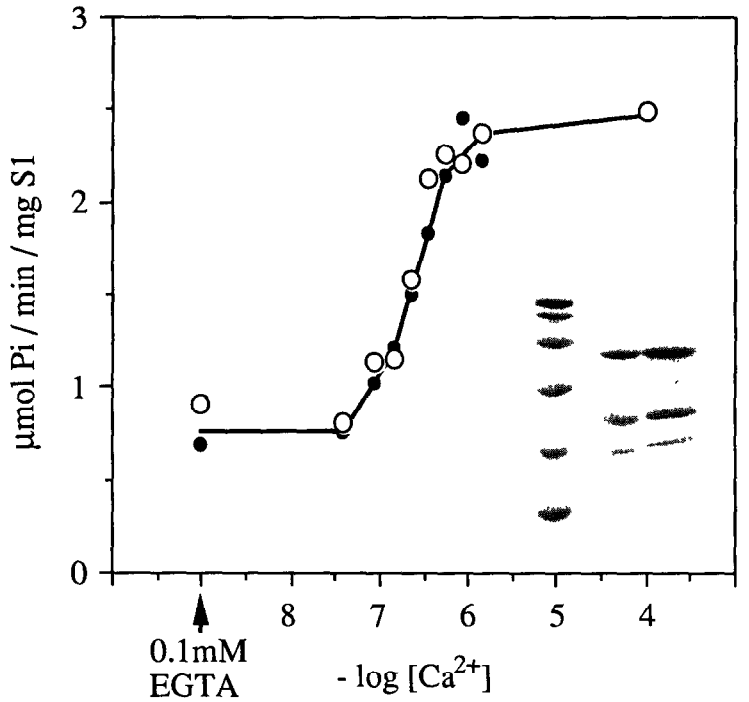


Fig. 4. Calcium-dependent regulation of the acto-S1 ATPase activity. (○) Tn complex with the authentic TnI; (●) Tn complex with mutant TnI. (Insert) PAGE pattern shows the reconstructed Tn complexes used in the assay. The lanes are (from left): molecular weight markers, Tn with authentic TnI, and Tn with the mutant TnI

unlikely that the replacement of the three cysteine residues has substantially altered the function of TnI.

3.4. *Oxidation*

On an SDS-PAGE pattern (Fig. 5), extra bands were observed in the authentic TnI under non-reducing conditions, indicating oxidation of this protein [5]. The most abundant species among them, observed as a pair of bands, both with apparent molecular weights of around 42 kDa, are likely to correspond to two species of dimers of TnI. A species migrating even faster than the monomer probably corresponds to a product with intramolecular sulfhydryl bonds. No difference was found in the mutant TnI under reducing and non-reducing conditions, which is consistent with the expectation that no oxidation occurs in this cysteine-free mutant protein.

4. DISCUSSIONS

Difficulties in handling TnI, mainly caused by the oxidation of the Cys residues, have been the major obstacle in studies of TnI and the Tn complex. The major aim of the present study was to obtain a mutant TnI which is free from oxidation while preserving all the functions of the authentic TnI. Using our *E. coli* expression system, a cysteine-free mutant TnI is easily obtained in sufficient quantity even for structural studies. Functional tests indicated that the mutant TnI, like the authentic TnI prepared from rabbit muscle and the wild-type TnI expressed in *E. coli*, interacted with TnC and TnT and formed the whole Tn complex which preserves the ability to regulate acto-S1 ATPase activity in a calcium-dependent manner. Although like authentic TnI, the mutant TnI still has low solubility at low ionic strength, possibly due to the presence of patches of basic residues, neither nitrogen gas bubbling nor addition of freshly prepared solutions of sulfhydryl reagent is required to protect the mutant TnI from further solubility decrease and rapid activity deterioration during prepa-

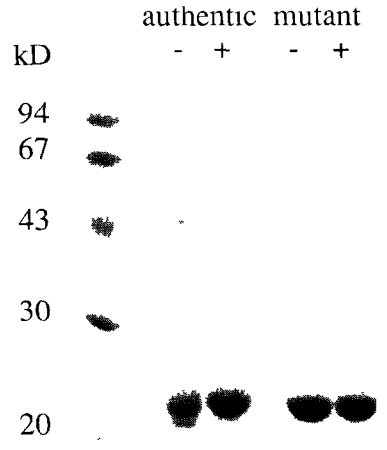


Fig. 5. TnI oxidation. An SDS-PAGE pattern with (+) and without (−) DTT added to the loading buffer.

ration and storage, providing great convenience in handling this protein. Therefore, the mutant TnI would be an attractive alternative to the authentic TnI, especially for certain purposes like crystallization and ^{1}H NMR studies.

Acknowledgements: We wish to thank Drs. R. Kellner, T Hauthaeve (EMBL, Heidelberg) and T. Kobayashi (Maryland University) for the N-terminal protein sequencing, Ms S. Weston (EMBL, Heidelberg) for oligonucleotide synthesis, Dr Y. Harada (Osaka University) for suggestions in preparing actin and S1 and measuring ATPase activity, Dr. J.H. Collins (Maryland University) for careful reading of the manuscript, and Dr I. Ohtsuki (Kyushu University) for discussion and encouragement. L.K. was a fellow of Alexander von Humboldt Foundation.

REFERENCES

[1] Ebashi, S. and Endo, M. (1968) Prog. Biophys. Mol. Biol. 18, 125–183.
[2] Ohtsuki, I., Maruyama, K and Ebashi, S (1986) Adv. Protein Chem. 38, 1–67.
[3] Leavis, P.C. and Gergely, J. (1984) CRC Crit. Rev. Biochem. 16, 235–305.
[4] Zot, A.S. and Potter, J.D. (1987) Annu Rev. Biophys. Biophys Chem 16, 535–559
[5] Horwitz, J., Bullard, B. and Mercola, D. (1979) J. Biol. Chem 254, 350–355.
[6] Wilkinson, J M. and Grand, R.J.A. (1975) Biochem. J 149, 493–496.
[7] da Cruz e Silva, E.F and Cohen, P.T.W (1987) FEBS Lett. 220, 36–42
[8] Hastings, K.E.M and Emerson Jr., C.P. (1982) Proc. Natl. Acad. Sci. USA 79, 1153–157.
[9] Sheng, Z., Pan, B.-S., Miller, T. and Potter, J D (1992) J. Biol. Chem 267, 25407–25413, with corrections to be published later.
[10] Amann, E., Ochs, B. and Abel, K.-J (1988) Gene 69, 301–315
[11] Akiyama, Y. and Ito, K. (1990) Biochem. Biophys Res. Commun 167, 711–715.
[12] Babbitt, P C., West, B.L., Buechter, D.D., Kuntz, I D. and Kenyon, G.L. (1990) Bio/Technology 8, 945–949.
[13] Syska, H., Perry, S V and Trayer, I.P. (1974) FEBS Lett 40, 253–257.
[14] Spudich, J.A. and Watt, S. (1971) J Biol. Chem. 246, 4866–4871.
[15] Margossian, S S. and Lowey, S (1982) Methods Enzymol. 85, 55–71
[16] Ebashi, S., Wakabayashi, T. and Ebashi, F. (1971) J. Biochem. 69, 441.
[17] Allen, D.G., Blinks, J.R. and Prendergast, F G. (1976). Science 195, 996–998.
[18] Ohno, T. and Kodama, T. (1991) J. Physiol. 441, 685–702.
[19] Grand, R.J.A and Wilkinson, J M (1977) Biochem. J. 167, 183–192.
[20] Kobayashi, T., Takagi, T., Konishi, K. and Cox, J.A. (1989) J. Biol. Chem. 264, 1551–1557.
[21] Bordo, D and Argos, P. (1991) J. Mol Biol. 217, 721–729