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FOR THE RECORD

Cloning and expression of chicken skeletal muscle troponin I in *Escherichia coli*: The role of rare codons on the expression level

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Contraction of skeletal muscle is regulated by intracellular Ca^{2+} levels. This regulation is mediated by the troponin–tropomyosin complex present in the actin filament. Troponin is composed of three different polypeptides: a calcium-binding component (troponin C, TnC), an inhibitory component (troponin I, TnI), and a tropomyosin-binding component (troponin T, TnT). Although the general mechanism and the role played by each component is understood (Zot & Potter, 1987), the molecular details of the conformational changes responsible for the regulation are not. The fact that the three troponin subunits can refold and reassociate in vitro (Greaser & Gergely, 1971) has allowed the use of troponin subunits produced in *Escherichia coli* to study the molecular mechanism of this regulatory complex. Expression of TnC in bacteria (Chen et al., 1988; Reinach & Karlsson, 1988; Xu & Hitchcock-DeGregori, 1988) and the analysis of site-directed mutants (Fujimori et al., 1990; Grabarek et al., 1990; Putkey et al., 1991; Sheng et al., 1991; Negele et al., 1992; Pearlstone et al., 1992; Silva et al., 1993) in conjunction with the determination of the crystal structure of TnC (Herzberg & James, 1985; Sundarlingam et al., 1985) has provided a better understanding of the calcium-induced conformational change in TnC (Silva & Reinach, 1991; Grabarek et al., 1992). This conformational change is responsible for the modulation of the inhibitory action of TnI.

We have isolated a cDNA containing the complete coding region for TnI. The nucleotide sequence of the cDNA (see Supplementary material on the Diskette Appendix) agrees with the chicken gene sequence (Nikovits et al., 1986) and with the protein sequence determined by Wilkinson and Grand (1978). Using this cDNA, a series of vectors based on the pET system were constructed in order to obtain TnI expression (see Supplementary material on the Diskette Appendix). Constructs containing 12 amino acids of S10 and 7 amino acids of the factor Xa cleavage site fused to the N-terminus of TnI (pET 501.4 and pET 508.7) expressed the fusion protein after induction (Fig. 1A). The fusion protein was purified and shown to have the predicted N-terminal sequence with the initiation methionine removed (results not shown). However, if the 19 amino acids fused to the N-terminal of TnI were removed (pET-R1), TnI could not be expressed (Fig. 1A).

After a series of frustrated attempts to express TnI, two rare codons for arginine (AGG) in positions 8 and 9 of the cDNA were shown to be responsible for the lack of expression. When these two codons were substituted for the frequently used arginine codon (CGT), a high level of TnI expression was observed (construct pET-R18 in Fig. 1).

Rare arginine codons are known to reduce translation efficiency in *E. coli* (Bonekamp & Jensen, 1988) and have been implicated in the control of translation when present in the first 25 codons after the initiation methionine (Chen & Inouye, 1990), but we are not aware of an example where they completely abolished protein accumulation as observed with this chicken TnI cDNA. Recently a rabbit cDNA has been used to express TnI in a pET plasmid (Sheng et al., 1992). The rabbit cDNA has a single AGG codon in position 9, position 8 being an asparagine. An arginine is present in position 7, but the codon used (CGC) is not a rare one. In the chicken, the DNA sequence around the two sequential Arg codons (AAG-AGGAG) is very similar to the Shine–Dalgarno sequence present in the pET vectors (AAGAAGGAG) (Studier et al., 1990),

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and this may also interfere with expression in this particular case. A detailed analysis of other regulatory mechanisms known to be involved in the initiation of protein synthesis can be found in de Smit and van Duin (1990) and Wikström et al. (1992).

Recombinant TnI was purified to homogeneity (Fig. 1B) with a final yield of 25 mg of pure protein per liter of culture. In contrast to the 19-amino acid fusion TnI, the nonfusion protein was present in inclusion granules (Fig. 1B). N-terminal sequence analysis of the purified protein confirmed its identity and showed that the initiation methionine was removed. The primary sequence of recombinant TnI is therefore identical to the muscle protein except for the lack of acetylation of the N-terminal amine found in muscle TnI (Wilkinson & Grand, 1978).

We have compared recombinant and muscle TnI with respect to three TnI properties: calcium-dependent association of TnI to TnC in the presence of 6 M urea, concentration-dependent inhibition of actomyosin ATPase, and the increase in TnC affinity for calcium upon TnI binding.

TnI binds TnC in urea gels in a calcium-dependent fashion (Head & Perry, 1974). As shown in Figure 1C, in the presence of Ca^{2+}, a stable complex between recombinant TnC and recombinant TnI can be observed in polyacrylamide gels containing 6 M urea. In the absence of Ca^{2+}, this complex is not stable and only uncomplexed TnC can be observed in the gel. No difference in behavior was observed when recombinant and muscle TnI were compared.

TnI inhibits the acto-myosin ATPase (Schaub & Perry, 1969). Figure 2A shows the inhibition of actomyosin ATPase by varying concentrations of TnI. Recombinant and muscle TnI inhibit the ATPase with a similar concentration dependence. At 1:1 molar ratio of TnI:actin (4 μM) the inhibition is close to maximum. The TnI concentration necessary for 50% inhibition was slightly lower for recombinant TnI when compared with the muscle protein, but the inhibitions reached at high TnI concentrations are not significantly different (Fig. 2A).

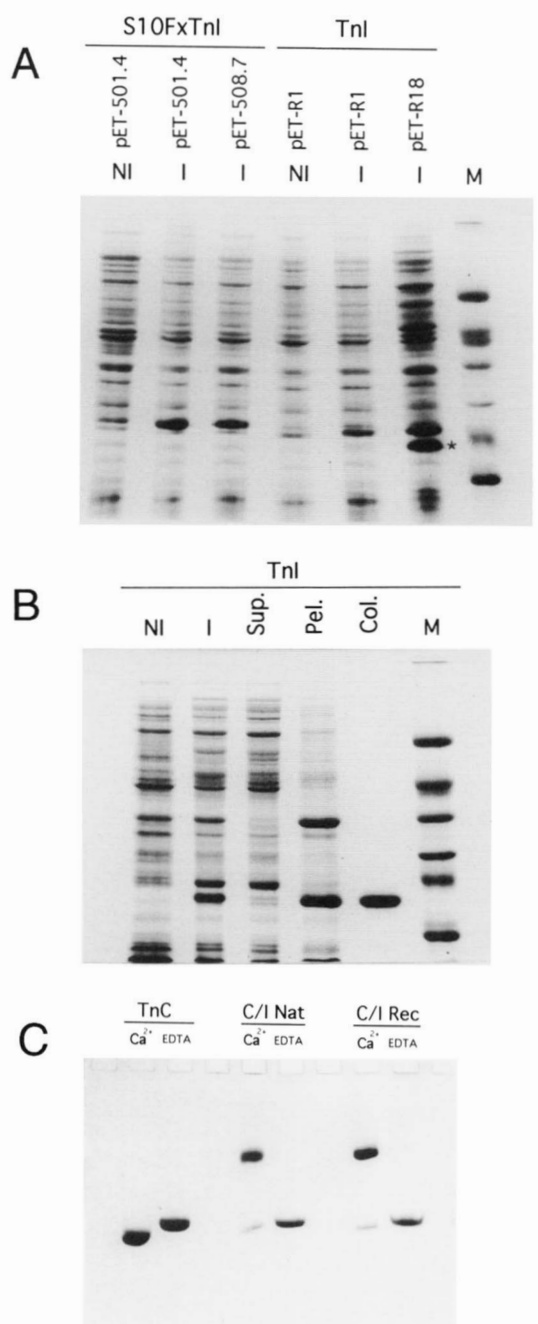

Fig. 1. TnI expression in *E. coli* (methods are described in the Supplementary material, Diskette Appendix). **A:** Expression by different plasmids. Total extracts of bacteria harboring the different expression vectors were run on sodium dodecyl sulfate (SDS)–polyacrylamide gels and stained with Coomassie blue. Samples obtained before induction (NI) and after induction (I) are shown side by side for the different vectors. Bacteria containing the plasmid pET-501.4 and pET-508.7 encoding fusion TnI expressed the fusion protein upon induction. Bacteria containing the plasmid pET-R1 encoding the nonfusion TnI showed no accumulation of TnI. An induced sample of bacteria containing the plasmid pET-R18 (nonfusion TnI after substitution of the two Arg codons) was run side by side to show the accumulation of nonfusion TnI (*). Molecular weight markers (M) of 66, 45, 36, 29, 24, and 20.1 kDa are shown. **B:** Purification of recombinant TnI. SDS–polyacrylamide gel of samples from the purification steps of recombinant TnI obtained with the plasmid pET-R18. Extracts obtained from cells before induction (NI) show no TnI, which accumulates after induction (I). After cell lysis and centrifugation, TnI is found in the inclusion granules (Pel.). No TnI is observed in the supernatant (Sup.). Pure TnI obtained after CM-Sepharose chromatography is also shown (Col.). Molecular weight markers (M) of 66, 45, 36, 29, 24, and 20.1 kDa are shown. **C:** Complex formation between TnC and TnI. Urea polyacrylamide gel of TnC (TnC), TnC/TnI complex produced with recombinant TnC and chicken muscle TnI (C/I Nat), and TnC/TnI complex obtained with recombinant TnC and recombinant TnI (C/I Rec). Each sample was run in the presence (Ca^{2+}) or absence (EDTA) of Ca^{2+}. The band corresponding to the TnC/TnI complex can be observed in the presence of Ca^{2+}. In the absence of Ca^{2+} a band corresponding to TnC is observed, and TnI does not enter the gel.

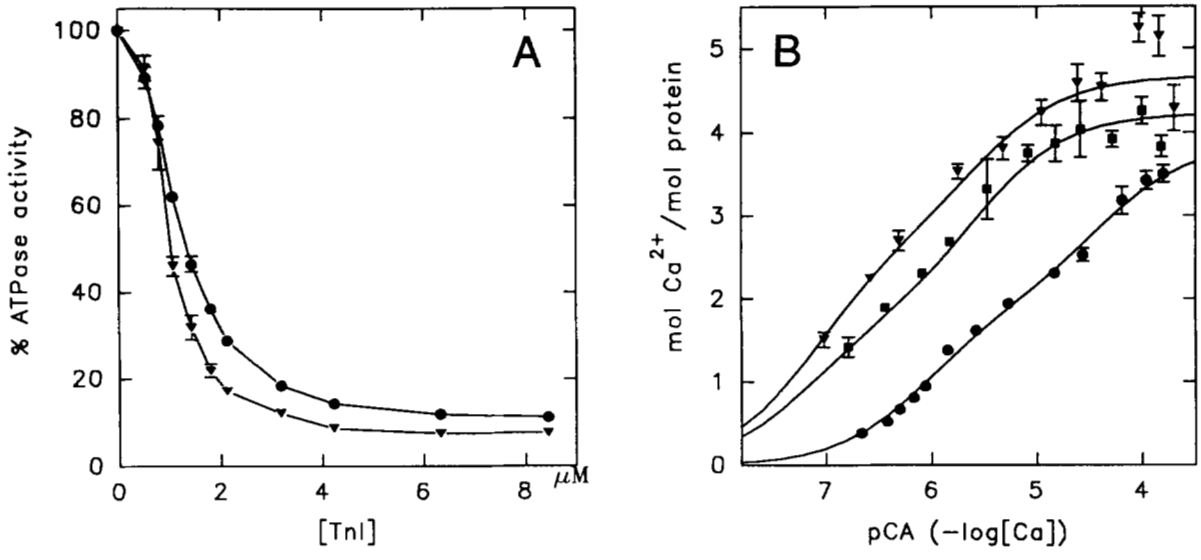

Fig. 2. Functional characterization of recombinant TnI (methods are described in the Supplementary material, Diskette Appendix). **A:** Inhibition of acto-myosin ATPase activity. ATPase activity as a function of TnI concentration is shown for chicken muscle TnI (●) and recombinant TnI (▼). Actin concentration is 4 μM. The data points are means from three independent determinations. The SEM is shown by the bars. The absolute values for the ATPase assay with no TnI were the same for both curves (0.4 μM Pi/min/mg of myosin). In some points the SEM is smaller than the symbols used. **B:** Direct calcium-binding measurements. Calcium binding to recombinant chicken TnC (●), TnC/TnI complex prepared with muscle TnI (■), and TnC/TnI complex prepared with recombinant TnI (▼) as a function of calcium concentration. The data points are means from three independent determinations. The SEM is shown by the bars. In some points the SEM is smaller than the symbols used. The lines are curves fitted to the averaged data according to the equation given on the Diskette Appendix.

We measured the binding of Ca^{2+} to TnC and to TnC/TnI complexes prepared with recombinant and muscle TnI using flow dialysis. Recombinant TnI and muscle TnI, when complexed to TnC, are capable of increasing the calcium affinity of TnC (Fig. 2B). A 10-fold increase in affinity was observed for the high affinity sites, and a 15-fold increase was observed for the low affinity sites when the complexes are compared with isolated TnC (see Table 1). An apparently larger increase in affinity was observed with recombinant TnI when compared with muscle TnI (Fig. 2B), but the dissociation constants obtained for the two complexes are not statistically different (Table 1). The dissociation constants obtained for recombinant chicken TnC are in agreement with the ones we determined by equilibrium dialysis (Fujimori et al., 1990) and flow dialysis (Silva et al., 1993). The constants reported here, in Fujimori et al. (1990), and in Silva et al. (1993) are lower than the ones reported for rabbit TnC and rabbit TnC/TnI complex (Potter & Gergely, 1975) probably due to differences in protein sequence between rabbit and chicken TnC and TnI.

We conclude that the major impediment for expression of chicken recombinant TnI in *E. coli* resides in the two sequential AGG codons present in positions 8 and 9 of the cDNA. Recombinant TnI binds TnC, inhibits actomyosin ATPase, and increases TnC affinity for Ca^{2+} in a manner similar to chicken muscle TnI.

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Table 1. *Calcium binding to TnC and TnC/TnI complexes*[a]

Protein	n_1	k_1 (× 10^{-6} M)	n_2	k_2 (× 10^{-8} M)
TnC	1.94 ± 0.07	40.11 ± 5.5	1.93 ± 0.06	90.4 ± 6.04
C/I natural	2.42 ± 0.48	2.69 ± 1.26*	1.81 ± 0.58	7.12 ± 5.39*
C/I recombinant	1.97 ± 0.18	2.78 ± 0.73*	2.70 ± 0.35*	8.02 ± 0.80*

[a] The apparent dissociation constants for the low affinity (k_1) and high affinity (k_2) calcium-binding sites and the number of sites (n_1 and n_2) are presented as average ± SEM of the individual fitted curves. * indicates $P < 0.05$ with respect to recombinant TnC.

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