Biochem. J. (1985) **225**, 549–552
Printed in Great Britain

Comparison of the structure of two cardiac troponin T isoforms

Vladimir V. RISNIK, Alexandr D. VERIN and Nikolai B. GUSEV
Department of Biochemistry, School of Biology, Moscow State University, Moscow 119899, U.S.S.R.

(*Received 2 October 1984/Accepted 20 November 1984*)

Two isoforms of troponin T have been isolated from bovine cardiac muscle. One isoform has an M_r of 31000 and a pI at about 7.1, the corresponding values for the second isoform being 33000 and 6.5. Both isoforms have identical *C*- and *N*-terminal sequences, and, according to the data from tryptic-peptide mapping, a similar structure of the central and *C*-terminal domains. The large *N*-terminal peptides of troponin T isoforms differ in the content of glutamine/glutamic acid and alanine. It is concluded that the isoform with M_r 33000 has an additional peptide enriched with glutamic acid and alanine that is inserted between the *N*-terminal pentapeptide and the cysteine located 40–60 residues from the *N*-terminus.

Many myofibrillar proteins are represented by a number of isoforms (Heywood *et al.*, 1983). As a rule, the isoforms of myofibrillar proteins are exchanged in the course of ontogenesis (Obinata *et al.*, 1980) or as a result of muscle differentiation (Dhoot *et al.*, 1978, 1979). Quite a few papers have been dedicated to the study of troponin isoforms. Van Eerd & Takahashi (1976) and Wilkinson (1980) carried out a detailed comparative analysis of the structure of troponin C isolated from cardiac fast and slow skeletal muscles. The determination of the primary structure of some troponin I isoforms formed a basis for their comparison and prediction of functionally important sites within the structure of troponin I (Wilkinson & Grand, 1978). Although it is well established that there exist several troponin T isoforms (Clarke *et al.*, 1976; Dhoot *et al.*, 1979; Putney *et al.*, 1983), until now there has been no detailed report on the structural properties of these isoforms. The only one exception is a comparison of the structure of two troponin T isoforms from chicken leg and breast muscles (Wilkinson, 1978; Wilkinson *et al.*, 1984).

Recently we described two isoforms of cardiac troponin T (Gusev *et al.*, 1983). The lack of detailed information on the structural properties of different troponin T isoforms, and, particularly, on the structure of cardiac troponin T, provides an incentive to undertake a structural comparison of two cardiac troponin T isoforms.

Abbreviation used: SDS, sodium dodecyl sulphate.

Materials and methods

The procedures used for the isolation of two isoforms of cardiac troponin T and their phosphorylation were described previously (Gusev *et al.*, 1983). The homogeneity of the isoforms was ascertained by SDS/polyacrylamide-gel electrophoresis performed by the method of Weintraub *et al.* (1975). The M_r values for troponin T isoforms were determined by 4–30%-(w/v)-polyacrylamide-gradient-gel electrophoresis as described by Reisfeld *et al.* (1962). The following basic proteins of known M_r were used as standards: cytochrome *c* (12000), bovine pancreatic ribonuclease (14300), myoglobin (17800), rabbit skeletal-muscle troponin I (20700) and troponin T (30500), dimer of troponin I (41400) and dimer of troponin T (61000). Isoelectrofocusing of troponin T was performed by the method of O'Farrell (1975).

Both isoforms of troponin T were cleaved at the single cysteine residue by the method of Jacobson *et al.* (1973). Troponin T was dissolved in 40mM-Tris/HCl buffer, pH8.0, containing 6M-urea, 0.1mM-phenylmethanesulphonyl fluoride and 1 μg of pepstatin/ml. A 2-fold molar excess of dithiothreitol was added, and after 30min incubation at 37°C, 5,5′-dithiobis-(2-nitrobenzoic acid) was added to the mixture to achieve an 8-fold molar excess over dithiothreitol. After 30min incubation at 37°C, an aqueous solution of KCN was added to the mixture to give a final concentration ten times greater than that of 5,5′-dithiobis-(2-nitrobenzoic acid). The mixture was incubated for 12–16h at 37°C and subjected to chromatography on a

Sephadex G-50 (superfine grade) column (1.6cm × 90cm) equilibrated with 10mM-HCl.

For the mapping of tryptic peptides, the single thiol group of troponin was alkylated with non-radioactive iodoacetamide, then the methionine residues were alkylated with iodo[^{14}C]acetamide (Wilkinson, 1978); finally, troponin T was subjected to trypsinolysis. The modified troponin T was digested with 3% (w/w) trypsin that had been treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK') for 6h at 37°C in 100mM-Tris/HCl, pH8.0. The resulting peptides were subjected to gel filtration on a Sephadex G-50 (fine grade) column (1.2cm × 90cm) equilibrated with 50mM-NH_4HCO_3. The changes in A_{206} and A_{280} were simultaneously monitored on a Uvicord III (LKB) apparatus. Portions were used for the determination of ^{32}P and ^{14}C radioactivity. Every third tube across the Sephadex G-50 chromatogram was evaporated and the peptides were subjected to high-voltage electrophoresis at pH6.5 in pyridine/acetic acid/water (25:1:225, by vol.).

The amino acid composition was determined in a Liquimat III (Labotron) amino acid analyser after peptide hydrolysis in 6M-HCl *in vacuo* (24h, 120°C).

Results

In a fairly recently published paper (Gusev *et al.*, 1983) we reported on two isoforms of cardiac troponin T. On the basis of SDS/polyacrylamide-gel electrophoresis, these isoforms have apparent M_r values of 38000 and 39500. Taking into account the fact that skeletal-muscle troponin T has an anomalous mobility on SDS/polyacrylamide-gel electrophoresis (Pearlstone *et al.*, 1976; Wilkinson, 1978), it seemed reasonable to redetermine the M_r of cardiac troponon T. For this purpose, we used the method of polyacrylamide-gradient-gel electrophoresis in the absence of SDS. When one performs the electrophoresis at acidic pH in the presence of 8M-urea, it is possible to overcome the difficulties arising from the strong tendency of troponin T to aggregate. By using the method of Reisfeld *et al.* (1962) and measuring the dependence of the mobility of standard proteins (see the Materials and methods section) on the logarithm of their actual M_r, we constructed a calibration curve representing a straight line with a correlation coefficient of >0.94. The thus-determined values of M_r for cardiac troponin T isoforms are equal to 31500 ± 1400 and 33100 ± 1700 respectively (means ± S.D. for four expts.) and differ only slightly from the corresponding values for skeletal-muscle troponin T (Pearlstone *et al.*, 1976; Wilkinson, 1978).

Previously published data indicate that two troponin T isoforms might differ in their pI values. To test this assumption, we determined the pI values for these proteins. The isoform with M_r 31500 produced three bands with pI6.8, 7.1 and 7.3, whereas the isoform with M_r 33000 gave two bands with pI6.4 and 6.5. These values are lower than those for rabbit skeletal-muscle troponin T (pI7.9–8.2) and are comparable with those for chicken breast troponin T (pI7.3) (Hirabayashi, 1981). The splitting of the troponin T band on isoelectrofocusing was described previously (Wilkinson, 1974; Drabikowski *et al.*, 1974; Murakami & Uchida, 1984) and could be due to the differing extents of phosphorylation or aggregation, the latter being particularly strong near the pI. The experimental results agree well with our previously published data (Gusev *et al.*, 1983) and suggest that the pI for the protein with M_r 33000 lies about 0.5 unit lower than that for the protein with M_r 31500.

Cysteine is located in the vicinity of the *N*-terminus of the both troponin T isoforms (Gusev *et al.*, 1983). After troponin T cleavage at the cysteine residue, we isolated a small *N*-terminal and large *C*-terminal peptides. The *C*-terminal peptides of both troponin T isoforms have M_r 27800 ± 1000 (means ± S.D. for four expts.). This implies that the cysteine residue is separated from the *N*-terminus of the isoform with M_r 33000 by a greater number of amino acids than in the case of the isoform with M_r 31500. The amino acid composition of the *N*-terminal peptides derived from two troponin T isoforms reveals a significant difference in the glutamine/glutamic acid and alanine content ($P<0.05$ as shown by Student's *t* test) (Table 1). At the same time we failed to detect any significant differences in the content of other amino acids. These data show that two troponin T isoforms differ at least by the structure of the *N*-terminal peptides.

Table 1. *Comparison of the amino acid composition of N-terminal peptides derived from two cardiac troponin T isoforms*

The results are presented as means ± S.D. ($n = 4$).

Amino acid	Isoform M_r ...	Composition (residues/molecule) 31500	33000
Asx		1.9 ± 0.3	2.9 ± 0.8
Ser		2.1 ± 0.3	2.1 ± 0.5
Glx		24.0 ± 2.8	29.9 ± 5.7
Gly		6.1 ± 0.7	6.1 ± 0.5
Ala		7.6 ± 0.4	12.4 ± 1.3
Val		4.7 ± 1.0	5.1 ± 0.2
Tyr		1.0 ± 0.3	1.3 ± 0.6
Lys		0.7 ± 0.3	0.8 ± 0.5
His		1.4 ± 0.7	1.5 ± 0.5
Arg		0.7 ± 0.3	0.6 ± 0.4
Total		50.2	62.7

In order to obtain more detailed information on the structure of two troponin T isoforms, we employed the peptide-mapping procedure introduced by Wilkinson (1978). Both forms of cardiac troponin T were phosphorylated with troponin T kinase at the single serine residue in the vicinity of their *N*-termini. The methionine residues of troponin T were alkylated by iodo[^{14}C]acetamide. The modified troponin T was digested with trypsin, and the peptides were applied to a Sephadex G-50 column. At first sight, the elution profiles of tryptic peptides of the both troponin T isoforms seem to be very similar (Fig. 1). At the same time, the largest ^{32}P-labelled peptide of the isoform with M_r 33000 was eluted from the column earlier than the corresponding peptide of the isoform with M_r 31500. Usually, the largest peptides derived from both troponin T isoforms were only slightly labelled by [^{14}C]acetamide. The difference in the ^{14}C radioactivity of the two largest peptides (Fig. 1) is probably due to the incomplete digestion of the troponin T isoform with M_r 31500. The well-separated largest tryptic peptide of both troponin T isoforms bore a high negative charge at pH 6.5 on paper electrophoresis and was not stained with ninhydrin. Thus these highly acidic peptides with blocked *N*-termini are phosphorylated and are the largest ones among all the tryptic peptides of cardiac troponin T. Taking these features into account and comparing the elution profiles of tryptic peptides of skeletal-muscle troponin T (Pearlstone *et al.*, 1976; Wilkinson, 1978; Wilkinson *et al.*, 1984) and cardiac troponin T, we may now conclude that the largest peptides eluted near the front of the Sephadex G-50 column are the *N*-terminal peptides, and that the larger size of the isoform with M_r 33000 is due to the increased length of this peptide. It is worthwhile to mention that, although the *N*-terminal peptides of both troponin T isoforms contain arginine and lysine residues (see Table 1), the size of the *N*-terminal tryptic peptides is rather large. This implies that the peptide bonds of the corresponding arginine and lysine residues are resistant to trypsinolysis. The *N*-terminal tryptic peptides of rabbit and chicken skeletal-muscle troponing T also contain both arginine and lysine residues (Pearlstone *et al.*, 1976; Wilkinson *et al.*, 1984).

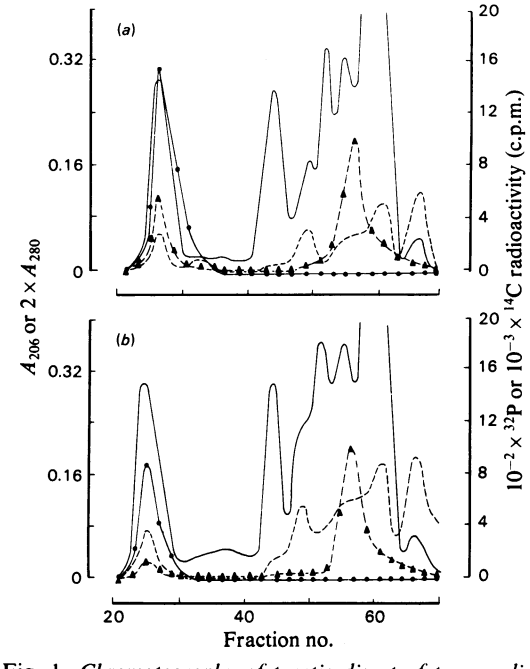

Fig. 1. *Chromatography of tryptic digest of two cardiac troponin T isoforms*

A tryptic digest of troponin T with the methionine residues alkylated with iodo[^{14}C]acetamide and a single serine residue phosphorylated with troponin T kinase in the presence of [γ-^{32}P]ATP was applied on a column (1.2cm × 90cm) of Sephadex G-50 in 50mM-NH_4HCO_3. (*a*) Cardiac troponin with M_r 31500; (*b*) cardiac troponin T with M_r 33000. ——, A_{206}; – – – –, A_{280}; ●, ^{32}P radioactivity; ▲, ^{14}C radioactivity.

Starting from fraction 40 (Fig. 1), every third fraction was evaporated and subjected to high-voltage paper electrophoresis. No obvious differences in the pattern of the peptides derived from the two troponin T isoforms were observed. The bulk of the short peptides (fractions 52–70) were either neutral or positively charged. The pattern of the radioactive peptides containing methionine was the same for both troponin T isoforms. It seems probable, therefore, that the central and *C*-terminal domains of the two proteins possess a considerable amount of sequence homology.

Discussion

A comparison of the structure of cardiac and skeletal-muscle troponin T reveals a number of common features. The composition of the *N*- and *C*-terminal peptides of all troponin T forms under study are very similar or identical (Wilkinson, 1978; Gusev *et al.*, 1983). Moreover, the highly conservative *N*-terminal peptides usually contain a phosphorylatable serine residue (Pearlstone *et al.*, 1976; Moir *et al.*, 1977). The structure of all troponin T forms is characterized by an uneven distribution of the charged residues. The *N*-terminal region is highly acidic, whereas the central, and especially the *C*-terminal, regions are enriched with positively charged amino acids. These features are common for all the so-far-known forms of troponin T.

However, despite a number of common features, troponin T isoforms differ in their pI and M_r values and in their amino acid compositions. A detailed investigation of the structure of the two forms of troponin T from chicken skeletal muscle (Wilkinson, 1978; Wilkinson *et al.*, 1984) showed that the central and *C*-terminal regions of these proteins are very similar, whereas the *N*-terminal peptides are distinguished by their glutamine/glutamic acid, alanine and histidine contents. At the same time, the *N*-terminal hexapeptides of these proteins are identical. Hence it was assumed that the two isoforms differ by a peptide that is inserted into the *N*-terminal sequence just behind the highly conservative *N*-terminal hexapeptide (Wilkinson, 1978; Wilkinson *et al.*, 1984). Both cardiac troponin T isoforms have identical *N*- and *C*-terminal sequences and reveal a great similarity in terms of the structure of the central and *C*-terminal domains. At the same time, the large *N*-terminal peptides of the both troponin T isoforms differ in their glutamine/glutamic acid and alanine contents (Table 1). Thus, in good agreement with the data of Wilkinson (1978) and Wilkinson *et al.* (1984), our results suggest that the isoform with M_r 33000 contains an additional peptide (about 15 residues long) enriched with glutamic acid and alanine. This peptide is inserted between the *N*-terminal pentapeptide and the cysteine residue located 40–60 residues from the *N*-terminus. A comparison of the differences in the structure of chicken skeletal and bovine cardiac troponin T isoforms reveals that the central and *C*-terminal domains are rather conservative, whereas the variability of the *N*-terminal domain provides for the existence of several troponin T isoforms. These isoforms can be produced by different genes (Wilkinson, 1978; Putney *et al.*, 1983) or through different splicing of a single (or of a number of similar) gene(s) (Wilkinson *et al.*, 1984).

We thank Professor S. E. Severin for helpful discussions and Mrs. R. L. Birnova for linguistic advice.

References

Clarke, F. M., Lovell, S. J., Masters, S. J. & Winzor, D. J. (1976) *Biochim. Biophys. Acta* **427**, 617–626

Dhoot, G. K., Frearson, N. & Perry, S. V. (1978) *Exp. Cell Res.* **117**, 357–370

Dhoot, G. K., Frearson, N. & Perry, S. V. (1979) *Exp. Cell Res.* **122**, 339–350

Drabikowski, W., Barylko, B., Dabrowska, R., Nowak, E. & Szpacenko, A. (1974) in *Calcium Binding Proteins* (Drabikowski, W., Strzelecka Golaszewska, H. & Carafoli, E., eds.), pp. 67–107, Elsevier, Amsterdam

Gusev, N. B., Barskaya, N. V., Verin, A. D., Duzhenkova, I. V., Khuchua, Z. A. & Zheltova, A. O. (1983) *Biochem. J.* **213**, 123–129

Heywood, S. M., Thibault, M. C. & Siegel, E. (1983) in *Cell and Muscle Motility*, vol. 3 (Dowben, R. M. & Shay, J. W., eds.), pp. 157–193, Plenum Press, New York and London

Hirabayashi, T. (1981) *Anal. Biochem.* **117**, 443–451

Jacobson, G. R., Schaffner, M. H., Stark, G. R. & Vanaman, T. C. (1973) *J. Biol. Chem.* **248**, 6583–6591

Moir, A. J. G., Cole, H. A. & Perry, S. V. (1977) *Biochem. J.* **161**, 371–382

Murakami, U. & Uchida, K. (1984) *J. Biochem (Tokyo)* **95**, 1597–1584

Obinata, T., Takano-Ohmuro, H. & Matsuda, R. (1980) *FEBS Lett.* **120**, 195–198

O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021

Pearlstone, J. R., Carpenter, M. R., Johnson, P. & Smillie, L. B. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1902–1906

Putney, S. D., Herlihy, W. C. & Schimmel, P. (1983) *Nature (London)* **302**, 718–724

Reisfeld, R. A., Lewis, U. J. & Williams, D. E. (1962) *Nature (London)* **195**, 281–283

Van Eerd, J. P. & Takahashi, K. (1976) *Biochemistry* **15**, 1171–1180

Weintraub, H., Palter, K. & van Lente, F. (1975) *Cell (Cambridge, Mass.)* **6**, 85–100

Wilkinson, J. M. (1974) *Biochim. Biophys. Acta* **359**, 379–388

Wilkinson, J. M. (1978) *Biochem. J.* **169**, 229–238

Wilkinson, J. M. (1980) *Eur. J. Biochem.* **103**, 179–188

Wilkinson, J. M. & Grand, R. (1978) *Nature (London)* **271**, 31–35

Wilkinson, J. M., Moir, A. J. G. & Waterfield, M. D. (1984) *Eur. J. Biochem.* **143**, 47–565