Effects of different enzymic treatments on the release of titin fragments from rabbit skeletal myofibrils

Purification of an 800 kDa titin polypeptide

Catherine ASTIER, Jean-Pierre LABBÉ, Claude ROUSTAN and Yves BENYAMIN*

Centre de Recherches de Biochimie Macromoléculaire LP 9008 (C.N.R.S.), Unité 249 (I.N.S.E.R.M.), Laboratoire de Motilité Cellulaire (E.P.H.E.), Université Montpellier I, B.P. 5051, Montpellier, France

In myofibrils, titin (also called connectin) molecules span from Z line to M line and constitute a third filament system containing an elastic domain in the I band. This giant protein is particularly sensitive to proteolysis *in situ*. Treatment of rabbit skeletal myofibrils with exogenous proteinases induces a release of titin

fragments, which are detected in the soluble myofibrillar fraction. The cleavage of titin occurs at specific points localized at the proximity of Z line and could lead to a concomitant release of α -actinin.

INTRODUCTION

Striated muscles are highly ordered tissues whose mechanical stability is now known to be supported by a filamentous system other than thick (myosin) or thin (actin) filament (reviewed in [1]). This third filament system is constituted by very thin structures extending from Z-line to M band [2] and is characterized by elastic properties [3]. The major component of these filaments is titin (also called connectin) [4–6], which is the largest polypeptide so far described (2800 kDa) [7,8]. The reported size of a single molecule (1 μ m) is consistent with the length of half a sarcomere. These connecting filaments are responsible for the passive tension generation in stretched myofibrils [9] and could also be involved in the control of filament assembly and the stabilization of the myosin filaments in the central position of the sarcomere [10].

The high-molecular-mass proteins such as titin or nebulin are highly sensitive to proteolysis during the myofibrillar degeneration in Duchenne muscular dystrophy [11] or during the processes that occur *post mortem* [12,13]. They are also the first targets of ionizing radiations [14]. Degradation of the parent molecule titin 1 or α -connectin generates initially titin 2 or β -connectin (molecular mass approx. 2100 kDa) [15,16]. In aged myofibrils, a 1200 kDa peptide was recently identified as the major product of titin degradation [17,18].

In the present study we have monitored the effects of limited enzymic treatments *in situ* on rabbit skeletal myofibrils and identified the high-molecular-mass polypeptides released during these processes.

MATERIALS AND METHODS

Limited proteolysis of rabbit skeletal myofibrils

Purified rabbit skeletal myofibrils were prepared at 4 °C, in the presence of 1 mM phenylmethanesulphonyl fluoride, 10 μ g/ml trypsin inhibitor, 5 μ M *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) as described [19], and stored in 50% (v/v) glycerol at –30 °C. The myofibrils were washed twice in 10 vol. of 10 mM Tris/HCl, pH 7.6, and resuspended at a final

concentration of 5–10 mg/ml in 100 mM NaCl/10 mM Tris/HCl, pH 7.8. The myofibril suspension was then treated with tosylphenylalanylchloromethane (Tos-Phe-CH₂Cl; 'TPCK')-treated trypsin from bovine pancreas (Serva) at 1:1000 (w/w) enzyme/substrate ratio, or endoproteinase Arg-C (Boehringer Mannheim) at 1:200 (w/w), or *Staphylococcus aureus* V8 proteinase (Miles) at 1:400 w/w. After each digestion time the suspension was centrifuged (5000 g, 5 min) to remove the non-solubilized fraction of myofibrils, and supernatants were immediately added to 0.5 vol. of a 3% SDS sample buffer and boiled for 1 min as described [16].

SDS/PAGE

Slab-gel electrophoresis was performed with 2–12%-(w/v)-polyacrylamide gradient gels as previously described [16]. After this run, gels were stained and destained according to the method of Laemmli [20].

Antibodies

Specific antibodies were raised against the major product of V8 proteolysis (P800) by immunizing mice (15 μ g/injection) with this polypeptide purified by gel-filtration chromatography on a Sephacryl S-500 HR column (2.5 cm $\times$ 60 cm). Polyclonal antibodies directed against α -actinin, extracted from chicken gizzard, were obtained in rabbits [21]. Monoclonal anti-titin (T12) antibodies and anti-nebulin (Nb2) antibodies were purchased from Boehringer Mannheim and Sigma respectively.

Immunoblots

Polypeptides transfer on to the nitrocellulose sheet was carried out at 60 V overnight in a 20 mM Tris/150 mM glycine/0.1% SDS/20% (v/v) methanol buffer, using a Bio-Rad transblot electrophoretic-transfer cell. Immunoreactive bands were revealed by using either an alkaline phosphatase-conjugated secondary antibody (Biosys, Compiègne, France) with NBT/BCIP (Sigma) as substrate, or peroxidase-conjugated secondary anti-

Abbreviations used: E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; V8 proteinase, *Staphylococcus aureus* V8 proteinase; Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK'); NBT, NitroBlue Tetrazolium; BCIP, 5-bromo-4-chloroindolyl phosphate.

* To whom correspondence should be sent at the following address: CRBM, Route de Mende, B.P. 5051, 34033 Montpellier Cedex, France.

Figure 1 Electrophoretic patterns of limited proteolysis supernatants from rabbit skeletal myofibrils

Myofibril suspension in 0.1 M NaCl/10 mM Tris/HCl buffer, pH 7.8, was incubated at 25 °C for various times with (a) Tos-Phe-CH₂Cl-treated trypsin (1:1000 w/w enzyme/substrate ratio), (b) endoproteinase Arg-C (1:200 w/w enzyme/substrate ratio), or (c) V8 proteinase (1:500 w/w enzyme/substrate ratio). Numbers below each lane correspond to incubation time in min. M, total myofibrillar extract; 0, supernatant from myofibril suspension kept at 25 °C for 30 min in the absence of proteinase. The arrowhead indicates the major cleavage product of titin of 1200 kDa detected in aged myofibrils (T, titin; N, nebulin; My, myosin; αA, α-actinin).

body using the enhanced chemiluminescence detection system (Amersham).

RESULTS

Release of high-molecular-mass proteolytic polypeptides

It was previously reported that mild trypsin treatment induced a selective digestion of titin, leaving other proteins almost intact [22]. In the present work, rabbit skeletal myofibrils were submitted to proteolytic enzymes such as Tos-Phe-CH₂Cl-treated trypsin, endoproteinase Arg-C or V8 proteinase. As Figure 1 shows, enzymic treatments led rapidly to a release of high-molecular-mass polypeptides detected in the supernatants of digested myofibrils. These proteolytic fragments had an electrophoretic mobility between those of titin and nebulin.

Treatment of myofibrils with Tos-Phe-CH₂Cl-treated trypsin induced a release of high-molecular-mass peptides during the incubation time. After 2 min of digestion, an early intermediate fragment, which migrated on 2–12 % gel gradient electrophoresis just below the lower band of the titin doublet (titin 2 or β-connectin), was detected in the supernatant of trypsin-treated myofibrils (Figure 1a). For longer incubation times, in addition to this latter titin fragment another slightly smaller polypeptide began to be released after 10 min of incubation with Tos-Phe-CH₂Cl-treated trypsin. This other titin-derived fragment had an electrophoretic mobility higher than the nebulin corresponding band. Myofibrils subjected to limited proteolysis with endoproteinase Arg-C also generated a delocalization of stable high-molecular-mass products after 10 min (Figure 1b). Finally, rabbit skeletal myofibrils digested with V8 proteinase generated a major stable cleavage product, apparent from 10 to at least 120 min. The electrophoretic mobility of this soluble fragment (termed P800), obtained in abundance, was similar to that of nebulin (Figure 1c). In addition, supernatants of digested myofibrils contained lower-molecular-mass protein bands. However, most of these polypeptides were not released under exogenous proteinase effects because they were also found in non-treated myofibril supernatants (Figure 1, lanes 0). We did not observe a time-dependent modification of the electrophoretic pattern of soluble fractions from non-treated myofibrils during 60 min, indicating that the protein bands present in the supernatants

could only proceed from the partial solubilization of sarcomeric proteins at the ionic strength used.

In the unsolubilized fractions, the treatment of myofibrils with V8 proteinase converted the largest form of the titin (titin 1 or α-connectin) into a polypeptide whose mobility corresponded to the lower band of the titin doublet (Figure 2). As previously described [17], we observed the appearance of an approx. 1200 kDa polypeptide after 30 min when the myofibril suspension was kept at room temperature. However, this titin fragment, resulting probably from endogenous proteolysis, was not released in the supernatant after centrifugation and remained bound to the unsolubilized myofibrillar fraction in the absence of exogenous proteinase (Figure 2). On the other hand, digestion of myofibrils with V8 proteinase rapidly induced the cleavage of this 1200 kDa titin polypeptide, which was not detected in the pellet after 5 min of enzymic treatment (results not shown). An uncharacterized fragment of ~ 500 kDa began to appear in the unsolubilized fraction of myofibrils treated with V8 proteinase.

Identification of titin proteolytic fragments

All the supernatants containing high-molecular-mass cleavage products were tested in immunoblot using a monoclonal antibody anti-titin (clone T12) which decorated the N1 line region in the sarcomere, at a position ~ 0.1 μm from the Z line [23]. This antibody recognized the high-molecular-mass fragments released after 30 min of Tos-Phe-CH₂Cl-trypsin or endoproteinase Arg-C digestion as well as the 1200 kDa peptide induced by endogenous myofibrillar proteolysis (Figure 3, lane a). The weak labelling of titin in total myofibrillar extracts resulted probably from the low efficiency of transfer over this molecular-mass range. Numerous lower-molecular-mass proteolytic fragments were also detected as a smear with the T12 anti-titin antibody in the supernatants of myofibrils incubated with Tos-Phe-CH₂Cl-treated trypsin or with endoproteinase Arg-C (Figure 3, lanes b and c). In contrast, in immunoblotting experiments the 800 kDa fragment resulting from V8 proteinase digestion of myofibrils failed to react with either the anti-nebulin antibody [23] (clone Nb2; results not shown) or the anti-titin monoclonal antibody used (clone T12). This latter recognized only two titin fragments of 170 kDa and 150 kDa in the V8-proteinase-treated myofibril supernatant.

Figure 2 Electrophoretic patterns of V8-proteinase-treated myofibrils

1 and 2, pellet and supernatant from non-treated myofibrils kept for 30 min at 25 °C; 3 and 4, pellet and supernatant from myofibrils incubated for 30 min with V8 proteinase (1:500 w/w enzyme/substrate ratio (T, titin; N, nebulin; M, myosin; A, actin).

Figure 3 Immunoblot analysis of proteinase-treated myofibrils with T12 anti-titin antibodies

a, Whole SDS myofibril extract; b, supernatants of myofibrils incubated for 30 min with Tos-Phe-CH₂Cl-treated trypsin (1:1000); c, endoproteinase Arg-C (1:200); d, V8 proteinase (1:500). The arrowhead indicates the 1200 kDa peptide of titin.

Figure 4 Elution profile of the 800 kDa polypeptide (P800) from S-500 HR Sephacryl gel-filtration column

The supernatant of myofibrils treated with V8 proteinase (1:500) for 30 min was loaded on to a S-500 HR Sephacryl column. The flow rate was 20 ml/h and 2.5 ml fractions were collected. The inset shows an SDS/polyacrylamide (2–12% gradient) gel corresponding to the fractions which are arrowed.

Figure 5 Immunoblot detection with purified anti-P800 antibodies

1, Supernatants of myofibrils treated with V8 proteinase (1:500) for 30 min; 2, whole SDS myofibril extract.

Figure 6 Immunoblot analysis with anti- α -actinin antibodies of supernatants of myofibrils V8-proteinase-treated for various times

M, whole SDS myofibril extract; 0, supernatant from myofibril suspension kept at 25 °C for 30 min in the absence of proteinase. Numbers below each lane correspond to incubation time in min.

Purification of a 800 kDa polypeptide and characterization

Since the 800 kDa polypeptide could be easily obtained and remained uncharacterized, we purified it to homogeneity by gel-filtration chromatography on a Sephacryl S-500 column (Figure 4). The 800 kDa peptide was eluted in the first peak with a protein concentration of 0.06–0.1 mg/ml, whereas α -actinin, 170 and 150 kDa titin cleavage products were eluted together just after the P800 protein as determined by immunoblotting. The second peak corresponded to the elution of low-molecular-mass polypeptides.

Polyclonal antibodies against P800 were elicited in mice using purified P800 as antigen for immunization. These antibodies were used to identify the P800 as a product of titin proteolysis, since both titin from the total myofibrillar extracts and the 800 kDa polypeptide were detected in Western-blot experiments using purified anti-P800 antibodies (Figure 5). An unidentified polypeptide which migrated just below myosin heavy chain also reacted with anti-P800 antibodies. Thus the limited degradation of myofibrillar proteins by exogenous proteinases generated a release of titin fragments.

Moreover, in all cases we observed that the release of titin proteolytic products could be concomitant with a release of α -actinin, resulting probably from Z-disc-associated protein degradation as described for calpain action [24]. The amount of removed α -actinin increased during the digestion time course, as revealed by a Western blot using anti- α -actinin polyclonal antibodies [21] (Figure 6). The α -actinin released had an un-

changed apparent molecular mass, and no proteolytic product was detected.

DISCUSSION

Proteolysis of myofibrillar proteins is the principal reason for structural changes in skeletal muscle. Several authors have reported the involvement of the Ca^{2+} -dependent proteolytic system in the metabolic turnover of myofibrillar proteins [25] or during the proteolysis *post mortem* [26,27].

In the present study we have tested the effects of limited enzymic treatments with exogenous proteinases on myofibrils *in situ*. Using mild trypsin treatment it was previously demonstrated that titin supported the tension development of muscle fibres by maintaining the order of the sarcomere [28], and it was shown that the splitting of the connecting filaments occurred in the A-I junction region [22]. We confirmed that limited enzymic treatment affected particularly this giant molecule and generated high-molecular mass fragments which were released from the sarcomeric structures. Moreover, we observed that the high-molecular-mass titin fragments released during Tos-Lys- CH_2Cl -treated-trypsin or endoproteinase Arg-C treatments were recognized by the T12 anti-titin antibody, specific to the N1 line region [23], and thus corresponded to the I band domain of the titin molecule. In supernatants from V8-proteinase-treated myofibrils, two peptides of 170 and 150 kDa containing the T12 epitope were detected, whereas the major product of titin proteolysis we have purified (a 800 kDa fragment) did not possess this epitope. In contrast, nebulin was not affected by any of the proteinases used. Immunoblots stained with anti-nebulin monoclonal antibody Nb2 did not permit us to detect the presence of nebulin proteolytic products in the supernatants of V8-treated myofibrils and no apparent modification in nebulin molecular mass was detected in the pellets (results not shown).

In addition to the titin fragments obtained in the soluble fraction of digested myofibrils, we observed a concomitant release of α -actinin, which accumulated in the supernatants with an apparent unchanged molecular mass.

As described for calpain [24], these results suggested that the action of exogenous proteolytic enzymes in releasing α -actinin could be a consequence of digestion of titin *in situ*. While titin and thin filaments interaction was previously suggested [29], the giant molecule could be associated with the Z-line and may be involved in α -actinin-actin complexes.

Thus we have shown that the titin domain localized in the proximity of the Z-line was specifically cleaved when myofibrils were incubated with exogenous proteinases. Furthermore, titin fragments containing the N1 line region and undegraded α -actinin were released during proteolytic treatments.

We thank D. Casanova for antibody preparation. We are also grateful to Dr. N. Lamb for reading this manuscript. This work was supported by a grant from the Institut Français de Recherche pour l'Exploitation de la Mer (I.F.R.E.MER) and the Association Française contre les Myopathies (A.F.M.).

REFERENCES

- Trinick, J. (1991) *Curr. Opin. Cell Biol.* **3**, 112–119
- Maruyama, K., Yoshida, T., Higuchi, H., Ohashi, K., Kimura, S. and Natori, R. (1985) *J. Cell Biol.* **101**, 2167–2172
- Itoh, Y., Suzuki, T., Kimura, S., Ohashi, K., Higuchi, H., Sawada, H., Shimizu, T., Shibata, M. and Maruyama, K. (1988) *J. Biochem.* **104**, 504–508
- Maruyama, K., Natori, R. and Nonomura, Y. (1976) *Nature (London)* **262**, 58–60
- Wang, K., McClure, J. and Tu, A. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3698–3702
- Maruyama, K., Kimura, S., Ohashi, K. and Kuwano, Y. (1981) *J. Biochem. (Tokyo)* **89**, 701–709
- Kurzban, G. P. and Wang, K. (1988) *Biochem. Biophys. Res. Commun.* **155**, 1155–1161
- Nave, R., Furst, D. O. and Weber, K. (1989) *J. Cell Biol.* **109**, 163–166
- Horowitz, R., Maruyama, K. and Podolsky, R. J. (1989) *J. Cell Biol.* **109**, 2169–2176
- Horowitz, R. and Podolsky, R. J. (1987) *J. Cell Biol.* **105**, 2217–2223
- Matsumura, K., Shimizu, T., Nonaka, I. and Mannen, T. (1989) *J. Neurol. Sci.* **93**, 147–156
- Takahashi, K., Nakamura, F. and Okamoto, M. (1979) *J. Biochem. (Tokyo)* **85**, 1539–1542
- Astier, C., Labbé, J. P., Roustan, C. and Benyamin, Y. (1991) *Comp. Biochem. Physiol.* **100B**, 465–469
- Horowitz, R., Kempner, E. S., Bisher, M. E. and Podolsky, R. J. (1986) *Nature (London)* **323**, 160–164
- Maruyama, K., Kimura, S., Yoshidomi, H., Saivada, H. and Kikuchi, M. (1984) *J. Biochem. (Tokyo)* **95**, 1423–1433
- Wang, K. (1985) in *Cell and Muscle Motility* (Shay, J. W., ed.), vol. 6, pp. 315–369, Plenum Press, New York and London
- Matsuura, T., Kimura, S., Ohtsuka, S. and Maruyama, K. (1991) *J. Biochem. (Tokyo)* **110**, 474–478
- Takahashi, K., Hattori, A., Tatsumi, R. and Takai, K. (1992) *J. Biochem. (Tokyo)* **111**, 778–782
- Nave, R., Furst, D. O. and Weber, K. (1989) *J. Cell Biol.* **109**, 2177–2187
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lebart, M. C., Mejean, C., Boyer, M., Roustan, C. and Benyamin, Y. (1990) *Biochem. Biophys. Res. Commun.* **173**, 120–126
- Yoshida, T., Higuchi, H., Kimura, S., Ohashi, K., Umazume, Y. and Maruyama, K. (1986) *Biomed. Res.* **6**, 181–186
- Furst, D. O., Osborn, M., Nave, R. and Weber, K. (1988) *J. Cell Biol.* **106**, 1563–1572
- Goll, D. E., Dayton, W. R., Singh, I. and Robson, R. M. (1991) *J. Biol. Chem.* **266**, 8501–8510
- Koohmaria, M. (1992) *Biochimie* **74**, 239–245
- Vidalenc, P., Cottin, P., Merdaci, N. and Ducastaing, A. (1983) *J. Sci. Food Agric.* **34**, 1241–1250
- Koohmaria, M., Babiker, A. S., Merkel, R. A. and Dutson, T. R. (1988) *J. Food Sci.* **53**, 1253–1257
- Higuchi, H. (1992) *J. Biochem. (Tokyo)* **111**, 291–295
- Wang, S. M. and Jeng, C. J. (1990) *J. Cell Biol.* **111**, 426a