Heterogeneity of Rabbit Muscle Creatine Kinase and Limited Proteolysis by Proteinase K

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By using sodium dodecyl sulphate/polyacrylamide-gel electrophoresis it was shown that rabbit muscle creatine kinase, both in a homogenate and purified, appears to be composed of a mixture of two peptides (mol.wts. 42100 and 40300) differing in length by about 15 amino acids. It is found that low concentrations of proteinase K from the fungus *Tritirachium album* can cleave about 38 amino acids from each chain of creatine kinase, leaving two large fragments (mol.wts. 37700 and 35500). Scission of the whole enzyme was found to be concomitant with complete loss of enzyme activity. MgADP in the presence of absence of creatine slowed the rate of proteolysis by about 50%, but the transition-state analogue complex creatine- NO_3^- -MgADP appeared to protect completely. The time course for the proteolytic inactivation in the presence of this complex, but not in its absence, was biphasic.

SDS*/polyacrylamide-gel electrophoresis is a sensitive technique for monitoring the size homogeneity of peptide chains. The present study is concerned with rabbit muscle MM-type creatine kinase (EC 2.7.3.2). No evidence for inhomogeneity has been reported to our knowledge.

Proteolytic digestion of creatine kinase has been studied by using bovine trypsin (Jacobs & Cunningham, 1968) and pig trypsin (Milner-White & Young, 1975). It was found that creatine kinase is rather resistant to the action of trypsins, so that high concentrations of the proteolytic enzymes had to be used. In addition the trypsins do not appear to cleave the enzyme preferentially at a single place in the molecule, so that unique large fragments are not readily obtained by using this enzyme.

Ebeling *et al.* (1974) have reported that proteinase K from the fungus *Tritirachium album* Limber attacks certain proteins rapidly, and they showed that it readily inactivates creatine kinase. It appears to be an endonuclease with chymotrypsin-like specificity for synthetic peptides (Moriari & Tsuzuki, 1975). In the present study we have looked in more detail at the nature and rate of the digestion process, using SDS/polyacrylamide-gel electrophoresis to separate the peptides.

NO_3^- ions greatly enhance the binding of the components of the dead-end complex creatine-MgADP to the enzyme (Milner-White & Watts, 1971). It appears that the NO_3^- ions occupy the site of the phosphoryl group transferred between the substrates in the working enzyme and mimic the

planar phosphoryl group that is expected to be a feature of the reaction mechanism. The strong binding suggests that this complex behaves as a transition-state analogue (it will be referred to below as such, although the postulated planar phosphoryl group may be an intermediate rather than a transition state). Certain other anions of suitable shape may replace NO_3^- (Milner-White & Watts, 1971; Chegwidan & Watts, 1976).

On addition of NO_3^- ions to the dead-end complex, various changes occur in the native enzyme. A spectral change in the tryptophan side chains, indicating transfer of the residues to a more polar environment (Watts, 1973), occurs; also there is a change in an attached chromophoric probe (Quiocho & Thomson, 1973); the reactivity of a thiol group is diminished by a factor of 200 (Milner-White & Watts, 1971; Quiocho & Olson, 1974). Working substrates have a similar effect on the thiol, but only decrease its reactivity by a factor of 5 (Milner-White & Kelly, 1976). E.p.r. (electron paramagnetic resonance) work on the creatine- NO_3^- -MnADP complex by Reed & Cohn (1972) has shown that the co-ordination sphere of the metal ion becomes much less accessible to solvent water. McLaughlin *et al.* (1976) have used the creatine-formate-MnADP complex for a nuclear-magnetic-resonance mapping study on the conformation of the bound ligands. McLaughlin (1974) and Price & Hunter (1976) have shown that the affinity of binding of MgADP, only when it is binding as the creatine- NO_3^- -MgADP complex, appears to be different for two sites of the dimeric enzyme.

The effect of this complex on the proteolytic digestion of the enzyme is noteworthy. It was found,

* Abbreviation: SDS, sodium dodecyl sulphate.

by using pig trypsin (Milner-White & Young, 1975), that addition of NO_3^- ions to the dead-end complex did not cause any additional effect on the nature or rate of digestion of creatine kinase; the effects found with proteinase K are presented below.

Experimental

Materials

Creatine kinase was purified from rabbit back and leg muscle and stored as previously described (Kuby *et al.*, 1954; Kelly & Milner-White, 1976). The preparations were homogeneous by the criteria of polyacrylamide-gel electrophoresis without SDS as described under 'Methods', and by gel chromatography on a Sephadex G-150 column. The specific activities were in the range 0.13–0.15 μmol of product released/min per μg of protein in the forward direction under the conditions used by Milner-White & Watts (1971) at 30°C, pH 9.0.

One sample of creatine kinase was further purified by DEAE-cellulose (DE 52; Whatman, Springfield Mill, Maidstone, Kent, U.K.) chromatography by the method of Sasa & Noda (1964).

Proteinase K from *Tritirachium album* Limber and rabbit muscle aldolase were obtained from Boehringer Corp., Lewes, Sussex, U.K.

Hen egg ovalbumin (grade V), pig stomach pepsin, horse heart cytochrome *c* and sperm-whale myoglobin were from Sigma Chemical Co., Kingston, Surrey, U.K. Bovine serum albumin was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Rabbit muscle aldolase, cross-linked by dimethyl suberimide, was prepared by Dr. J. Lumsden and Dr. J. R. Coggins by their method (Lumsden & Coggins, 1977).

N-Acetyl-L-tyrosine ethyl ester was obtained from Sigma; phenylmethanesulphonyl fluoride was from Eastman-Kodak Chemical Co., Kirkby, Liverpool, U.K.; Coomassie Brilliant Blue was from Serva Feinbiochemica, Heidelberg, West Germany; ATP and ADP were from Boehringer. All other chemicals were analytical-reagent grade from BDH Chemicals, Poole, Dorset, U.K. Bicine [*NN*-bis-(2-hydroxy-

adjusted to pH 9.0 with NaOH. In addition some experiments included ADP (1 mM) and creatine (40 mM). Extra magnesium acetate was added in amounts equimolar with ADP. In certain experiments NaNO_3 replaced sodium acetate. Digestion was initiated by adding proteinase K from a stock solution in 1 mM-HCl to give a concentration of 2 $\mu\text{g}/\text{ml}$. From the digestion mixtures samples (30 μl) were removed and added to 30 μl of phenylmethanesulphonyl fluoride (1 mM) made up freshly in 20% (v/v) ethanol. This treatment is necessary because SDS is known to stimulate the action of proteinase K (Hilz *et al.*, 1975). After 5 min at 30°C a solution (20 μl) containing SDS (5%, v/v), glycerol (50%, v/v) and 2-mercaptoethanol (5%, v/v) was added and the mixture heated for 2 min at 100°C. After cooling it was run immediately on the SDS/polyacrylamide-gel-electrophoresis system.

Creatine kinase assay. A pH-stat assembly (SBR2c/TTT1b/SBU1a from Radiometer, Copenhagen, Denmark) was used to monitor the forward reaction at 30°C and pH 9.0 (Kelly & Milner-White, 1976). The assay mixture contained finally: creatine, 40 mM; ATP, 4 mM; magnesium acetate, 5 mM; sodium acetate, 0.1 M; cysteine, 1 mM. The reaction was started by adding the creatine kinase solution and followed by addition of 5 mM-NaOH to maintain the pH.

Polyacrylamide-gel electrophoresis. Electrophoresis in 7½% polyacrylamide gels in the presence of SDS was carried out as described by Shapiro & Maizel (1969). To the reduced and detergent-treated protein samples was added 10 μl of 0.05% (w/v) Bromophenol Blue; after electrophoresis the position of the dye was marked by a thin wire. Gels were stained for protein by incubation for 1 h at 40°C in 0.1% Coomassie Brilliant Blue G 250 in methanol/acetic acid/water (5:1:4, by vol.) and destained at 40°C in methanol/acetic acid/water (1:1:8, by vol.) Gels were scanned at 600 nm in a Gilford spectrophotometer fitted with a model 252 gel scanner; the measured absorbance did not exceed 1 unit. This was quantified by cutting out the peak areas on the chart recorder papers and weighing them. The percentage of remaining whole enzyme was calculated as:

$$\frac{\text{weight of whole enzyme band}}{\text{weight of whole enzyme band} + [\text{weight of product band} \times (41\,200/36\,600)]} \times 100$$

ethyl)glycine] was recrystallized once from water; creatine was recrystallized once from 1 mM-EDTA, pH 9.0, and once from water.

Methods

Digestion conditions. The mixture was kept at 30°C and contained: creatine kinase, 1 mg/ml; magnesium acetate, 10 mM; sodium acetate, 0.1 M; Bicine, 20 mM,

Electrophoresis in the absence of SDS was by the method of Davis (1964) as modified by Hayes & Wellner (1969), with 7½% gels at pH 8.9 run at room temperature (19°C). Polyacrylamide gels containing 8 M-urea were run similarly. R_f values were in all cases expressed relative to the mobility of the Bromophenol Blue marker.

Proteinase K assay. The hydrolysis of the synthetic substrate *N*-acetyl-L-tyrosine ethyl ester was followed

Fig. 1. Scans of SDS/polyacrylamide gels of various creatine kinase preparations

Scans at 600 nm of Coomassie Blue-stained SDS/polyacrylamide gels run on 8 February 1977 with samples of creatine kinase (1.5 μ g) that were prepared from fresh muscle on the following dates: (a) 15 November 1976; (b) 1 November 1976; (c) 6 September 1976; (d) 8 November 1974. The position of the Bromophenol Blue marker is indicated.

Fig. 2. Scans of SDS/polyacrylamide gels taken during the purification procedure

Scans at 600nm of Coomassie Blue-stained SDS/polyacrylamide gels run with samples taken during a purification of creatine kinase from fresh muscle on 6 June 1977. The position of the Bromophenol Blue marker is indicated. Fractions are numbered according to the procedure of Kuby *et al.* (1954). (a) Fraction I immediately after homogenization; (b) fraction II, but before addition of any alcohol; (c) fraction III, before the second addition of alcohol; (d) fraction IV, before dialysis; (e) fraction IV, after dialysis; (f) after DEAE-cellulose purification as described in the Experimental section. This was purified from the preparation carried out on 15 November 1976 (see Fig. 1a).

by the pH-stat method used in the creatine kinase assay.

Results

Figs. 1 and 2 show SDS/polyacrylamide-gel-electrophoresis profiles of creatine kinase preparations during and after purification from muscle; those in Fig. 3 show the effect of treatment with proteinase K for various time intervals. The molecular weights of the native protein subunits were estimated to be 42100 and 40300 relative to the standard proteins bovine serum albumin, cross-linked and native rabbit muscle aldolase, sperm-whale myoglobin and horse heart cytochrome *c*, by plotting their R_F values against the logarithms of their molecular weights. The standard proteins, added to the gels at the same concentrations and volumes, gave only single bands as expected.

Under the conditions of Fig. 3 digestion of the native enzyme appears to be almost complete after 30 min. After 60 min of such digestion the gel profile shows no further change. Fig. 4 shows the degree of correlation between the amount of digestion and the loss of enzyme activity. When the data are plotted on a semilogarithmic scale a simple first-order reaction is found as expected, with a rate constant of 0.210min^{-1} . An experiment was carried out with twice the concentration of proteinase K; the rate constant in this case was 0.418min^{-1} . This demonstrates that creatine kinase-proteinase K complexes are not present in large amounts under the conditions used. The molecular weights of the digested peptides seen on the gels are estimated to be 37700 and 35500 relative to the standard proteins.

Samples of whole enzyme and of partially and completely cleaved enzyme were run on the gel-electrophoresis system without SDS at pH 8.9. A single band moving at the same position relative to the marker dye (R_F 0.08) was observed in each of the gels. Urea-containing gels were run with the native enzyme preparations, and these showed a single band (R_F 0.10 relative to the marker dye) and two much weaker bands (R_F 0.04 and 0.12), which probably correspond to the impurities seen in the SDS gels (Fig. 1).

Table 1 shows the effectiveness of substrates and substrate complexes in protecting against the action of proteinase K. Study of the rate profiles shows that the inhibition reactions are of the simple first-order kind, except with the MgADP-NO_3^- -creatine complex. A semilogarithmic plot for the digestion of the enzyme bound to this complex by proteinase K is presented in Fig. 5.

The rate of hydrolysis of the synthetic substrate *N*-acetyl-L-tyrosine ethyl ester by proteinase K was followed in the presence and absence of the substrate

Fig. 3. Proteinase K digestion of creatine kinase shown by SDS/polyacrylamide-gel scans

The digestion mixtures at 25°C contained: creatine kinase, 1 mg/ml; magnesium acetate, 10 mM; sodium acetate, 0.1 M; Bicine, 20 mM, adjusted to pH 9.0 with NaOH. The scans ($1.5\mu\text{g}$ of protein samples) of Coomassie Blue-stained SDS/polyacrylamide gels were: (a) untreated creatine kinase; (b) after treatment for 2 min with proteinase K ($2\mu\text{g/ml}$); (c) after treatment for 30 min with proteinase K ($2\mu\text{g/ml}$). The position of the Bromophenol Blue marker is indicated.

Fig. 4. Rate of digestion of creatine kinase measured by scission of the peptide chain and by loss of enzymic activity

The digestion mixtures at 25°C contained: creatine kinase, 1 mg/ml; magnesium acetate, 10 mM; sodium acetate, 0.1 M; Bicine, 20 mM, adjusted to pH 9.0 with NaOH. ▲, Enzymic activity remaining; ○, whole peptide chains remaining; both as percentages of holoenzyme value. A semilogarithmic plot gives a straight line, with a first-order rate constant of 0.210 min^{-1} .

Fig. 5. Rate of digestion of creatine kinase by proteinase K in the presence of the creatine- NO_3^- -MgADP complex (semilogarithmic plot)

The digestion mixture contained: creatine kinase, 1 mg/ml; magnesium acetate, 10 mM; Bicine, 20 mM, adjusted to pH 9.0 with NaOH; creatine, 40 mM; NaNO_3 , 0.1 M; ADP, 1 mM. ▲, Enzymic activity remaining; ○, whole peptide chains remaining; both as percentages of value for holoenzyme.

Table 1. Protection by substrates and substrate analogues against proteolysis by proteinase K

Protection is measured by the decrease in the rate constant for scission of the native peptide chain caused by substrate or substrate complexes. All digestion reactions contained: proteinase K, $2 \mu\text{g/ml}$; creatine kinase, 1 mg/ml; magnesium acetate, 10 mM; sodium acetate or sodium nitrate, 0.1 M; Bicine, 20 mM, adjusted to pH 9.0 with NaOH. Omission of sodium acetate and magnesium acetate in the absence of substrates did not affect the rate. Substrate concentrations used were: creatine, 40 mM; ADP, 1 mM. The first-order rate constant for the reaction under these conditions was 0.210 min^{-1} in the absence of added substrates.

Digestion conditions	Decrease in rate constant (%)
Sodium acetate	—
Sodium nitrate	4
Creatine+sodium acetate	6
MgADP+sodium acetate	58
MgADP+creatine+sodium acetate	58
MgADP+creatine+sodium nitrate	96

mixtures in Table 1. None of these altered the rate of the proteinase K reaction.

Discussion

Microheterogeneity

The SDS/polyacrylamide-gel-electrophoresis scans in Fig. 1 indicate the presence of two distinct bands of slightly different molecular weights. The original gels

show this feature more clearly. Other standard proteins, rabbit muscle aldolase, both native and cross-linked, pig stomach pepsin, hen egg ovalbumin, bovine serum albumin and horse heart cytochrome *c*, run simultaneously under identical conditions in the same electrophoresis apparatus, showed only one band. From their apparent differences in molecular weight (42100 and 40300) it is estimated that the bands are expected to differ by about 15 amino acids. After electrophoresis in the absence of SDS both with and without urea only one band is seen (plus small amounts of impurities probably corresponding to those in the gels of Fig. 1, in the urea-containing gels). However, since the migration of both at the running pH of 8.9 is less than one-sixth of the migration of the highly charged SDS-bound proteins in the same gels, it is not surprising that resolution is poorer in the absence of SDS.

Fig. 2 shows that the two distinct protein chains of creatine kinase can also be detected in the soluble fraction of a rabbit muscle homogenate (Fig. 2a), and are present throughout the purification procedure (Figs. 2a–2e). It is also evident that the second alcohol precipitation stage in this widely used procedure has not achieved a great increase in purity. For this reason a further DEAE-cellulose purification was carried out on one sample of creatine kinase; this shows (Fig. 2f) that all the detectable impurities can be removed by this method. Such a purification step was first used by Sasa & Noda (1964); it was also used by Fattoum *et al.* (1975).

These results indicate that the native as well as the isolated enzyme is composed of two different types of protein chain. Whether this represents a post-transcriptional modification or not remains to be determined.

Proteolysis

It is evident that proteinase K cleaves creatine kinase (peptide mol.wts. 42100 and 40300) into fragments of mol.wts. 37700 and 35500, corresponding to a loss of about 38 amino acids in each case; this occurs without rendering the large fragment susceptible to further degradation by that enzyme. It acts on creatine kinase at a much lower concentration than do other proteinases such as chymotrypsin and trypsin (Milner-White & Young, 1975).

This cleavage results in complete or almost complete loss of enzyme activity; correspondence between the amount of cleavage and the loss of enzyme activity has been demonstrated (Figs. 4 and 5). After cleavage the position of migration of the protein on polyacrylamide-gel electrophoresis without SDS is unchanged. The presence of either MgADP alone or of MgADP plus creatine lowers the rate of cleavage to the same extent (Table 1). On addition of NO_3^- ions to MgADP and creatine the digestion rate is greatly decreased. This is not merely due to the enhanced binding of MgADP in the transition-state analogue complex, because MgADP under the conditions used in Table 1 is present at nearly saturating concentrations ($K = 0.09 \text{ mM}$ for binding to enzyme alone or with creatine, at 25°C and $\text{pH } 8.0$; McLaughlin, 1974). The further protection must represent a conformational change in the enzyme specifically on the formation of the creatine- NO_3^- -MgADP complex.

Analysis of the data of Fig. 4 shows that the proteolytic reaction is a first-order one with respect to creatine kinase subunit concentration; the same is true in the presence of the substrates used in Table 1, except for the creatine- NO_3^- -MgADP complex. In this instance the time course for the reaction is seen to be biphasic. This is in accord with the findings of Price & Hunter (1976), who demonstrated a similar phenomenon with the transition-state analogue complex by using thiol-group inhibitors.

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