Biochem. J. (1981) **199**, 239–244
Printed in Great Britain

The effect of limited proteolysis on rabbit muscle creatine kinase

Nicholas C. PRICE,* Stephen MURRAY* and E. James MILNER-WHITE†

**Department of Biochemistry, University of Stirling, Stirling FK9 4LA, Scotland, U.K., and †Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.*

(*Received 27 May 1981/Accepted 18 June 1981*)

Creatine kinase from rabbit muscle is inactivated by limited proteolysis with proteinase K from *Tritirachium album*. Gel-filtration and cross-linking studies showed that the limited proteolysis did not affect the molecular weight of the enzyme under non-denaturing conditions, but did cause changes in the reactivity of the reactive thiol group on each subunit and in the ability of the enzyme to form a 'transition-state analogue' complex in the presence of magnesium acetate plus ADP plus creatine plus $NaNO_3$.

The dimeric enzyme creatine kinase (EC 2.7.3.2) from rabbit muscle is rapidly inactivated by treatment with proteinase K from *Tritirachium album* Limber (Williamson *et al.*, 1977). Inactivation was shown to be correlated with the conversion of the intact subunit (of mol.wt. 41000) into a species of mol.wt. 37000, as judged by SDS/polyacrylamide-gel electrophoresis. We have investigated this limited proteolysis in more detail, since it seemed that this approach might give information about the location of the substrate-binding site and/or catalytically important groups in the enzyme. Although the detailed structure of the enzyme is not known, there is evidence from chemical modification and other studies that histidine (Clarke & Price, 1979; Cook *et al.*, 1981), arginine (Borders & Riordan, 1975) and lysine (James & Cohn, 1974) side chains are at the active site and that a reactive cysteine side chain is near the active site (McLaughlin *et al.*, 1976; Keighren & Price, 1978).

Our results show that under non-denaturing conditions the two fragments produced by proteolysis remain associated with each other and that the ability of the enzyme to bind the substrate, ADP, is not altered. It is concluded that inactivation is probably a result of small changes in the tertiary structure of the enzyme (as manifested by a decrease in the reactivity of the cysteine side chain towards iodoacetate), and a consequent loss in the ability of the enzyme to form a 'transition-state analogue' complex (Milner-White & Watts, 1971) in the presence of MgADP plus creatine plus nitrate.

Abbreviations used: Nbs_2, 5,5'-dithiobis-(2-nitrobenzoic acid); Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; SDS, sodium dodecyl sulphate.

Experimental

Materials

Creatine kinase was isolated from rabbit skeletal muscle as described previously (Milner-White & Watts, 1971). Enzyme activity was measured by a coupled assay involving pyruvate kinase and lactate dehydrogenase under the conditions used by Price & Hunter (1976).

Proteinase K from *Tritirachium album* Limber, hexokinase from yeast, carbonic anhydrase from bovine erythrocytes, soya-bean trypsin inhibitor and the monopotassium salt of ADP were purchased from Boehringer, Lewes, Sussex, U.K. Phenylmethanesulphonyl fluoride, Nbs_2, Tricine and iodoacetic acid were purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K. Before use, iodoacetic acid was recrystallized from light petroleum (b.p. 60–80°C). Stock solutions of phenylmethanesulphonyl fluoride were made up in AnalaR-grade ethanol just before use. 8-Anilinonapthalene-1-sulphonate and trypsinogen (from bovine pancreas) were purchased from BDH Chemicals, Poole, Dorset, U.K. Sephadex G-100 and Blue Dextran 2000 were purchased from Pharmacia (G.B.), London W5 5SS, U.K. Dimethyl suberimidate dihydrochloride was purchased from Aldrich Chemical Co., Gillingham, Dorset, U.K., and stored desiccated at −18°C.

Methods

Limited proteolysis of creatine kinase was performed essentially as described by Williamson *et al.* (1977) by treatment with 0.2% (w/w) proteinase K for 30min at 20°C in 0.1M-sodium phosphate buffer, pH 8.0, containing 1mM-EDTA. After

0306-3275/81/100239-06$01.50/1

30min, the proteinase K was inactivated by addition of phenylmethanesulphonyl fluoride (final concn. 1mM). This mixture was left for 10min at 20°C and then dialysed against the appropriate buffer.

The rate of reaction of creatine kinase with iodoacetate was determined by using a back-titration method with Nbs_2 as described previously (Price & Hunter, 1976). Reactions were performed at 25°C in 50mM-Tricine (sodium salt) buffer, pH 8.0. The binding of ADP to creatine kinase was monitored by the 8-anilinonaphthalene-1-sulphonate-fluorescence-quenching method, and the data were analysed as described by McLaughlin (1974). Binding studies were performed at an enzyme concentration of 0.25mg/ml in 50mM-Tricine (sodium salt) buffer, pH 8.0, at 25°C.

Gel filtration was performed at 4°C on a Sephadex G-100 column (78cm × 1cm internal diameter) at a flow rate of approx. 1ml/h. The volume of each fraction was 1.3ml. The eluting buffer was 0.1M-sodium phosphate buffer, pH 8.0, containing 1mM-EDTA. The column was calibrated with the following proteins of known molecular weight: hexokinase (yeast), creatine kinase, bovine serum albumin, ovalbumin and myoglobin. Blue Dextran 2000 and 4-nitrophenol were used to determine the elution volumes of compounds which are completely excluded and completely included respectively. The distribution coefficient, K_d, for a given protein was calculated as described by Andrews (1970).

Cross-linking of creatine kinase by dimethyl suberimidate was performed as described previously (Bickerstaff *et al.*, 1980). Cross-linked samples were analysed by electrophoresis on 7.5% polyacrylamide gels in the presence of SDS (Weber *et al.*, 1972). Standard proteins used as molecular-weight markers were bovine serum albumin, carbonic anhydrase, trypsinogen, soya-bean trypsin inhibitor, creatine kinase and cross-linked creatine kinase (Bickerstaff *et al.*, 1980).

Results

Molecular weight of proteolysed creatine kinase

In agreement with the findings of Williamson *et al.* (1977), creatine kinase was rapidly inactivated by treatment with proteinase K. There was a concomitant conversion of the 41000-mol.wt. subunit into a 37000-mol.wt. species on SDS/polyacrylamide-gel electrophoresis. There was no evidence of further digestion by proteinase K to produce smaller fragments for periods of incubation of up to 1h. This suggests that proteinase K cleaves a single peptide bond (or possibly a limited number of adjacent peptide bonds) close to one end of the polypeptide chain of each subunit.

Under non-denaturing conditions, the molecular weight of proteolysed creatine kinase was estimated by gel filtration to be close to 80000, i.e. near the value for unmodified enzyme (82000), rather than the value (74000) that might be expected if the small fragment dissociated from the large fragment of each polypeptide chain. This finding was confirmed in an experiment in whch 1.5mg of proteolysed creatine kinase was applied to the gel-filtration column together with a small amount of unmodified enzyme (0.15mg). The fractions eluted from the column were analysed for A_{280}, which detects principally the proteolysed enzyme, and for enzyme activity, which detects only the unmodified enzyme. As shown in Fig. 1, there is a complete coincidence of the A_{280} and the enzyme activity at an elution volume corresponding to the molecular weight of unmodified creatine kinase. Essentially all the protein and all the activity applied to the column were eluted in fractions 21–29 (Fig. 1). It is estimated that species of mol.wts. 82000 and 74000 would be separated by approximately two fractions under the conditions shown in Fig. 1.

There was no evidence for elution of small fragments in fractions that would correspond to a

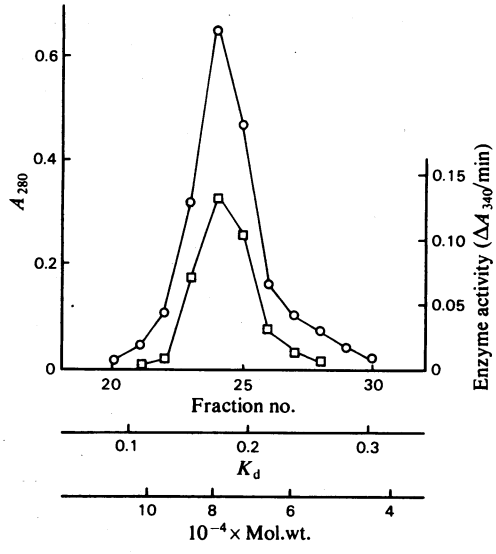

Fig. 1. *Part of elution profile for gel filtration of a mixture of unmodified and proteolysed creatine kinase on a column of Sephadex G-100*

O, A_{280} of each fraction; □, enzyme activity of 0.025ml sample of each fraction. The scale of distribution coefficient, K_d, was drawn up on the basis of the positions of elution of Blue Dextran 2000 and 4-nitrophenol. The molecular-weight scale was drawn up on the basis of positions of elution of proteins of known molecular weight as described in the Experimental section.

molecular weight of less than 10000. All such fractions were tested for A_{280}, fluorescence (excitation at 280nm and emission at 340nm), and for protein by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. These tests showed that each fraction contained no detectable protein. The detection limit was estimated as 5μg per fraction.

The conclusion from these studies was that limited proteolysis did not produce separate fragments under non-denaturing conditions. However, in view of possible problems of interpretation of gel-filtration data and the rather small differences in molecular weight involved, it was considered desirable to check this conclusion by the more direct method of cross-linking, i.e. to test whether or not the small and large fragments of each polypeptide chain could be cross-linked. The results of this type of experiment are shown in Fig. 2. Figs. 2(*a*) and 2(*b*) shows that unmodified and proteolysed enzyme show single bands on SDS/polyacrylamide-gel electrophoresis corresponding to mol.wts. of 41000 and 37000 respectively. When proteolysed enzyme is treated with 20mM-dimethyl suberimidate for 10min, the gel pattern (Fig. 2*c*) shows that the 37000-mol.wt. species is predominant, but that a second peak corresponding to a mol.wt. of 41000 has been formed. In the higher-molecular-weight region, corresponding to intersubunit cross-linking, the major band is at a mol.wt. of 74000, with a smaller peak corresponding to a mol.wt. of 82000. After 30min reaction with dimethyl suberimidate, the major peak (Fig. 2*d*) is at a position corresponding to a mol.wt. of 41000, with a shoulder corresponding to mol.wt. 37000. In the higher-molecular-weight region the major peak corresponds to a mol.wt. of 82000. Cross-linking of unmodified creatine kinase leads to peaks at positions corresponding to mol.wts. of 82000 and 41000 as previously described (Bickerstaff *et al.*, 1980). The results shown in Figs. 2(*c*) and 2(*d*) clearly demonstrate that in proteolysed creatine kinase the small fragment remains associated with the large fragment, thus allowing cross-linking to occur. The degree of resolution is not sufficient to indicate whether or not there are three distinct species in the high-molecular-weight region, corresponding to polypeptides of 82000, 78000 and 74000mol.wt. (the species of mol.wt. 78000 would arise from cross-linking of subunits of mol.wts. 41000 and 37000).

Control experiments showed that when SDS was added to proteolysed creatine kinase before dimethyl suberimidate, no cross-linking occurred and only a single peak corresponding to a mol.wt. of 37000 was observed.

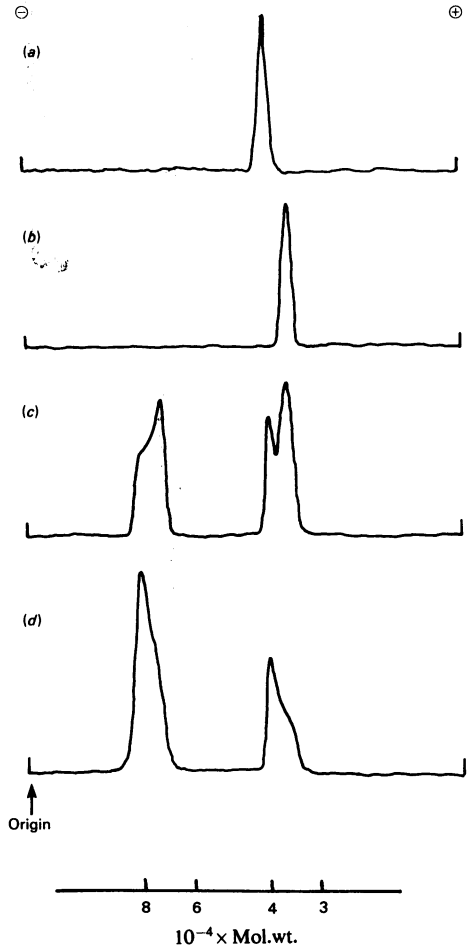

Fig. 2. *SDS/polyacrylamide-gel electrophoresis of creatine kinase samples*

The gels were stained with Coomassie Blue and after destaining scanned at 545nm: (*a*) and (*b*) represent unmodified and proteolysed enzyme respectively (2.5μg sample applied in each case); (*c*) and (*d*) represent proteolysed enzyme treated with 10mM-dimethyl suberimidate for 10min and 30min respectively (9μg sample applied in each case). The molecular-weight scale was drawn up on the basis of mobilities of proteins of known molecular weight as described in the Experimental section.

Binding of ADP to proteolysed creatine kinase

The binding of ADP to unmodified and proteolysed creatine kinase was studied by the 8-anilinonaphthalene-1-sulphonate-fluorescence-quenching method (McLaughlin, 1974), with the results shown in Table 1. These results indicate that the proteolysed enzyme can bind ADP with an affinity similar to that of unmodified enzyme. The effect of magnesium acetate or magnesium acetate plus creatine is to weaken the affinity of the enzyme for nucleotide, in agreement with results previously

Table 1. *Binding of ADP to unmodified and to proteolysed creatine kinase*
Dissociation constants were determined by the probe-fluorescence-quenching method (McLaughlin, 1974). Measurements were made at 25°C in 50mM-Tricine (sodium salt) buffer, pH 8.0.

Ligands present	Dissociation constant (μM)	
	Unmodified enzyme	Proteolysed enzyme
None	40	30
Magnesium acetate (5mM)	105	200
Magnesium acetate (5mM) + creatine (30mM)	190	180
Magnesium acetate (5mM) + creatine (30mM) + $NaNO_3$ (10mM)	3, 15*	50

* Evidence for negative co-operativity in ligand binding.

Table 2. *Reactivity of cysteine side chain in creatine kinase towards iodoacetate*
The reactions were monitored by the back-titration method using Nbs_2 (Price & Hunter, 1976). Measurements were made at 25°C in 50mM-Tricine (sodium salt) buffer, pH 8.0.

Ligands present	Second-order rate constant ($M^{-1} \cdot min^{-1}$)	
	Unmodified enzyme	Proteolysed enzyme
None	160	70
ADP (1mM)	30	35
ADP (1mM) + magnesium acetate (2mM)	250	100
ADP (1mM) + magnesium acetate (2mM) + creatine (30mM)	190	95
ADP (1mM) + magnesium acetate (2mM) + creatine (30mM) + $NaNO_3$ (10mM)	Biphasic reaction	90

obtained under comparable conditions (Keighren & Price, 1978). In the presence of magnesium acetate plus creatine plus $NaNO_3$, unmodified enzyme binds ADP in a fashion characteristic of negative co-operativity with a greatly enhanced affinity (Keighren & Price, 1978). This enhanced affinity is thought to be due to the formation of a 'transition-state analogue' complex in which the planar nitrate ion occupies the site that would be occupied by the transferable phosphoryl groups in the catalytically active complex (Milner-White & Watts, 1971; McLaughlin *et al.*, 1976). By contrast, with the proteolysed enzyme there is a much smaller increase in affinity for ADP in the presence of magnesium acetate plus creatine plus $NaNO_3$ and no evidence for any co-operativity in ligand binding. This suggests that proteolysed enzyme is unable to form such a 'transition-state analogue' complex.

Reactivity of cysteine side chain of proteolysed creatine kinase

Unmodified creatine kinase contains one reactive cysteine side chain per subunit, modification of which by reagents such as iodoacetate, iodoacetamide or Nbs_2 leads to inactivation (Price, 1979). Proteolysed enzyme also contained very nearly one (0.85) rapidly reacting cysteine side chain per subunit, as judged by the reaction with Nbs_2. The reactivity of this side chain towards iodoacetate and the effects of ligands on the reactivity are shown in Table 2. The values of the rate constants for unmodified enzyme in the absence and presence of ligands are similar to those previously reported under similar conditions (Price & Hunter, 1976). In proteolysed enzyme the reactivity of the side chain in the absence of ligands is decreased by a factor of approx. 2, compared with unmodified enzyme, and the reactivity appears to be rather less sensitive to the inclusion of ligands than is the case for unmodified enzyme. Of particular note is the finding that in the presence of ADP plus magnesium acetate plus creatine plus $NaNO_3$, the unmodified enzyme shows a slower, markedly biphasic, reaction with iodoacetate (Price & Hunter, 1976), whereas proteolysed enzyme shows no such behaviour (see Table 2).

Discussion

The results reported in the present paper throw some light on the inactivation of creatine kinase by limited proteolysis with proteinase K. The loss of activity does not result from a change in the quaternary structure of the enzyme, since gel-filtration and cross-linking studies show that, under non-denaturing conditions, the molecular weight of the enzyme is unaffected by proteolysis and the fragments from each polypeptide chain remain associated via non-covalent interactions. Similar situations have been reported in cases of limited proteolysis of other enzymes, e.g. bovine pancreatic ribonuclease (Richards & Vithayathil, 1959), rabbit skeletal-muscle phosphorylase (Raibaud & Goldberg, 1973), rabbit liver fructose 1,6-bisphosphatase (Dzugaj *et al.*, 1976), the catalytic trimer of *Escherichia coli* aspartate carbamoyltransferase (Chan & Enns, 1978) and *E. coli* glutamine synthetase (Lei *et al.*, 1979). In these cases, proteolysis is often accompanied by loss of enzyme activity, although for ribonuclease there was no change in activity and for fructose 1,6-bisphosphatase there was an 8-fold increase in activity. Limited proteolysis of the *arom* multienzyme complex of *Neurospora crassa* leads to loss of shikimate kinase activity, but not of the four other catalytic activities, without dissociation of the complex (Smith, 1981). The cross-linking method described in the present paper to show that fragments of creatine kinase remain associated after proteolysis would appear to be generally applicable. Methods that have been used in other cases have included measurements of sedimentation coefficients or mobility on electrophoresis under non-denaturing conditions. Interpretation of the results of these latter types of experiment requires assumptions to be made about the shapes of enzymes before and after proteolysis, which may not be valid in all cases. It should be pointed out that, in recent years, limited proteolysis has been recognized as an important technique in the study of large multidomain proteins (Kirschner & Bisswanger, 1976).

The results exclude the possibility that the loss of catalytic activity of creatine kinase on limited proteolysis by proteinase K is due to the dissociation of a small fragment (or fragments) of each subunit containing catalytically important side chains. Instead inactivation appears to be associated with subtle changes in the tertiary structure of the enzyme and in the ligand-induced conformational changes undergone by the enzyme. The proteolysed enzyme can still bind ADP (Table 1), although it is apparently unable to form a 'transition-state analogue' complex (Milner-White & Watts, 1971) in the presence of magnesium acetate plus ADP plus creatine plus $NaNO_3$. Studies of the reactivity of the reactive cysteine side chain towards iodoacetate (Table 2) suggest that the environment of this side chain, which is thought to be close to the active site of the enzyme (McLaughlin *et al.*, 1976), is somewhat modified in the proteolysed enzyme. The monophasic characteristics of the reaction of proteolysed enzyme with iodoacetate in the presence of magnesium acetate plus ADP plus creatine plus $NaNO_3$ are consistent with the suggestion that this enzyme derivative cannot form a 'transition-state analogue' complex.

Although the detailed structure of the enzyme and hence the precise role of the reactive cysteine side chain are not known, it has been proposed on the basis of structure-prediction methods that this side chain is at the beginning of a β-turn separating two portions of β-sheet structure (Maggio *et al.*, 1977). If this is so, the integrity of this side chain may well be important in mediating conformational changes associated with the formation of the catalytically active complex. Consistent with this proposal are the observations (i) that small modifications of the cysteine side chain lead to only a small loss of activity, whereas larger modifications lead to greater losses of activity, and (ii) that the extent of inactivation can be roughly correlated with the ability of the modified enzyme to undergo conformational changes associated with the formation of the 'transition-state analogue' complex (Smith *et al.*, 1975; der Terrossian & Kassab, 1976; Keighren & Price, 1978). In the present case it also appears that modification of the environment of the cysteine side chain by proteolysis is associated with an inability of the enzyme to form the 'transition-state analogue' complex. These results serve to emphasize the importance of the integrity of the environment of this side chain in the catalytic function of creatine kinase.

References

Andrews, P. (1970) *Methods Biochem. Anal.* **18**, 1–53

Bickerstaff, G. F., Paterson, C. & Price, N. C. (1980) *Biochim. Biophys. Acta* **621**, 305–314

Borders, C. L., Jr. & Riordan, J. F. (1975) *Biochemistry* **14**, 4699–4704

Chan, W. W.-C. & Enns, C. A. (1978) *Can. J. Biochem.* **56**, 654–658

Clarke, D. E. & Price, N. C. (1979) *Biochem. J.* **181**, 467–475

Cook, P. F., Kenyon, G. L. & Cleland, W. W. (1981) *Biochemistry* **20**, 1204–1210

der Terrossian, E. & Kassab, R. (1976) *Eur. J. Biochem.* **70**, 623–628

Dzugaj, A., Chu, D. K., El-Dorry, H. A., Horecker, B. L. & Pontremoli, S. (1976) *Biochem. Biophys. Res. Commun.* **70**, 638–646

James, T. L. & Cohn, M. (1974) *J. Biol. Chem.* **249**, 2599–2604

Keighren, M. A. & Price, N. C. (1978) *Biochem. J.* **171**, 269–272
Kirschner, K. & Bisswanger, H. (1976) *Annu. Rev. Biochem.* **45**, 143–166
Lei, M., Aebi, U., Heidner, G. E. & Eisenberg, D. (1979) *J. Biol. Chem.* **254**, 3129–3134
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Maggio, E. T., Kenyon, G. L., Markham, G. D. & Reed, G. H. (1977) *J. Biol. Chem.* **252**, 1202–1207
McLaughlin, A. C. (1974) *J. Biol. Chem.* **249**, 1445–1452
McLaughlin, A. C., Leigh, J. S., Jr. & Cohn, M. (1976) *J. Biol. Chem.* **251**, 2777–2787
Milner-White, E. J. & Watts, D. C. (1971) *Biochem. J.* **122**, 727–740
Price, N. C. (1979) *Biochem. J.* **177**, 603–612
Price, N. C. & Hunter, M. G. (1976) *Biochim. Biophys. Acta* **445**, 364–376
Raibaud, O. & Goldberg, M. E. (1973) *Biochemistry* **12**, 5154–5161
Richards, F. M. & Vithayathil, P. J. (1959) *J. Biol. Chem.* **234**, 1459–1465
Smith, D. D. S. (1981) Ph.D. Thesis, University of Glasgow
Smith, D. J., Maggio, E. T. & Kenyon, G. L. (1975) *Biochemistry* **14**, 766–771
Weber, K., Pringle, J. R. & Osborn, M. (1972) *Methods Enzymol.* **26**, 3–27
Williamson, J., Greene, J., Chérif, S. & Milner-White, E. J. (1977) *Biochem. J.* **167**, 731–737