Human 'creatine kinase conversion factor' identified as a carboxypeptidase

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The effect of partially purified 'creatine kinase conversion factor' on rabbit muscle creatine kinase is shown to be that of a carboxypeptidase, removing the C-terminal lysine residue from both subunits. These changes fully explain the three-banded electrophoretic patterns of the partially and the fully modified rabbit and human enzymes. The factor also produces a similar electrophoretic pattern with haemoglobin A; comparison with the effects of carboxypeptidases A and B permits the inference that the C-terminal residues of both α - and β -subunits are removed. Small synthetic peptides are poor or non-substrates. A low activity with hippuryl-L-lysine may be due to contamination of the preparation with carboxypeptidase N. The possibility has been excluded that the action of conversion factor on creatine kinase involves modification of the protein thiol groups. M_r , substrate-specificity, pH-activity profile and the effects of metal ions distinguish creatine kinase conversion factor from carboxypeptidases A, B and N. On the basis of this evidence it is proposed to give the conversion factor the provisional name of carboxypeptidase K.

Interest in the diagnostic role of the isoenzymes of CK has led to the discovery of various atypical forms in human plasma (Lang, 1981; Lang & Wurzburg, 1982; Bais & Edwards, 1982; Gerhard *et al.*, 1983; Bayer *et al.*, 1983). The MM isoenzyme is modified by a plasma-borne factor known as 'creatine kinase conversion factor'. This 'converts' the single band of a skeletal-muscle extract, resolved by agarose-gel electrophoresis, into a triple-banded pattern. The slowest band corresponds to the original MM isoenzyme, and the intermediate and fastest forms are thought to represent modification of one and both subunits respectively (Wevers *et al.*, 1978; Yasmineh *et al.*, 1981; Falter *et al.*, 1982; Edwards & Watts, 1983). Some enzyme activity is lost during conversion of the native MM isoenzyme *in vitro*; thiols stabilize the enzyme, but also inhibit CK-conversion-factor activity. Reversibly blocking the reactive thiol group with tetrathionate, however, protects against activity loss, but does not inhibit conversion (Edwards & Watts, 1983). The same workers excluded the possibility of CK conversion factor acting as an endopeptidase.

In the present work modification of a secondary enzyme thiol group by the conversion factor is

excluded, and the conversion factor is identified as a hitherto undescribed metal-activated carboxypeptidase. Some substrate and metal-ion requirements of the partially purified carboxypeptidase are described.

Experimental

Measurement of CK electrophoretic conversion

Incubation mixtures contained 50 mM-Tris/HCl buffer, pH 8.0, 20 μ l (1.3 units) of partially purified CK conversion factor (preparation described by Edwards & Watts, 1983), tetrathionate-treated human or rabbit CK, as previously described by Edwards & Watts (1983), to a final activity of 500 units/l (1 unit consumes 1 μ mol of phosphocreatine/min at 30°C in the assay mixture; Szasz *et al.*, 1976) and any other additions in a total volume of 100 μ l. All incubations were carried out at 37°C. At 30 min intervals samples were removed for analysis by agarose-gel electrophoresis with the use of the Corning system as described by the manufacturers (Corning, Palo Alto, CA, U.S.A.), except that 0.1% bovine serum albumin was added to the electrode buffers and the electrophoresis time was extended to 70 min. The CK loading was adjusted so that the intensity of NADPH fluorescence produced in the subsequent measurement was directly proportional to the amount of CK

Abbreviations used: CK, creatine kinase (EC 2.7.3.2); Hip- (in peptide sequences), hippuryl; ClHg-Nph-OH, 2-chloromercuri-4-nitrophenol.

activity present. The relative proportions of the three MM isoenzymes were then quantified by using the Corning 720 scanning fluorimeter/densitometer. The conversion activity was measured as the initial rate of CK subunits converted/min at 37°C (1% subunits converted/min = 1 unit).

To determine optimum pH conditions for the conversion, 50 mM-Tris/HCl buffer was used throughout and the pH varied between 7.0 and 9.5. The pH of each reaction mixture was independently checked to verify the required pH.

Large-scale conversion of CK

For the purpose of studying the possible liberation of amino acids accompanying conversion of CK, a relatively large amount of CK was needed. CK from rabbit muscle, prepared by method B of Kuby *et al.* (1954), was a gift from Mr. M. Eaton (Department of Biochemistry, Guy's Hospital Medical School). The final product [30 mg/ml; protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard] was greater than 95% pure as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The final CK preparation, after dialysis in double-distilled water adjusted to pH 9.0 with redistilled AnalaR ammonia, was stored at 4°C, with 5 mM-dithiothreitol added to prevent oxidative damage. Before use it was dialysed against three changes of 50 mM-NH₄HCO₃, pH 8.0. To 1 ml of CK solution 50 µl of partially purified conversion factor was added. The mixture was incubated at 25°C. After 24–48 h, samples were removed and the extent of conversion was determined by electrophoresis (see above). When complete conversion into the fastest band had been achieved, the incubation mixture was dialysed against two changes of 10 ml of 50 mM-NH₄HCO₃, pH 8.0. The diffusates were pooled, freeze-dried and then subjected to amino acid analysis. This was performed on a LKB 4400 amino acid analyser under standard conditions as described by the manufacturers (LKB-Produkter, Bromma, Sweden).

CK conversion by carboxypeptidases A and B

Both carboxypeptidase enzymes were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Carboxypeptidase A was prepared from bovine pancreas, and carboxypeptidase B from pig pancreas. Both enzymes were treated with di-isopropyl phosphorofluoridate by the manufacturers to eliminate trypsin and chymotrypsin activity. Incubations with CK were performed as described above except that partially purified CK conversion factor was omitted and replaced with either

carboxypeptidase A (0.2 mg/ml) or carboxypeptidase B (0.003 mg/ml).

Hydrolysis of low M_r substrates

The hydrolysis of Hip-Lys (hippuryl-L-lysine), Hip-Arg (hippuryl-L-arginine) and Hip-Phe (hippuryl-L-phenylalanine) was followed spectrophotometrically (Folk *et al.*, 1960). The same buffer system was used throughout, i.e. 25 mM-Tris/HCl, pH 7.6, containing 50 mM-NaCl and 1 mM of one of Hip-Lys, Hip-Arg or Hip-Phe. The total assay volume was 500 µl and contained 50 µl of partially purified CK conversion factor.

Preparation of haemoglobin A

Fresh human blood was obtained from a normal adult male by venepuncture and collected into a heparinized tube. The plasma was separated and removed after centrifugation (3000g for 10 min). The erythrocytes were washed twice in 150 mM-NaCl and then lysed by addition of 5 ml of distilled water/ml of packed erythrocytes. Debris was removed by centrifugation (3000g for 10 min). The haemoglobin A produced was checked by electrophoresis (see below); no other contaminating proteins were found in the haemoglobin region.

Modification of haemoglobin A by CK conversion factor and carboxypeptidases A and B

This was carried out as described for CK (above), except that CK was replaced by 0.5 mg of haemoglobin A. One of CK conversion factor (20 µl), carboxypeptidase A (20 µg) or carboxypeptidase B (2 µg) was added. Prolonged incubations of 3–18 h were necessary with CK conversion factor to convert sufficient haemoglobin A for protein detection after electrophoresis. Electrophoresis was performed as described for CK except that the electrophoresis time was 45 min, bovine serum albumin was omitted from the electrode buffer and the sample volume was 1 µl. After electrophoresis the gel was stained for protein with Amido Black 10B as described by the manufacturers.

Modification of CK by ClHg-Nph-OH

ClHg-Nph-OH was synthesized by the method of McMurray & Trentham (1969). The final product had m.p. 235–240°C (decomp.) and $\lambda_{\max}$ 405 nm (ϵ 1.74 $\times$ 10⁴ M⁻¹·cm⁻¹, assuming M_r 374) in 0.1 M-NaOH.

Incorporation of HO-Nph-Hg groups into CK was followed spectrophotometrically. Rabbit CK (6 mg/ml) in 50 mM-Tris/HCl buffer, pH 8.0, was added to a test cuvette and the same buffer to a reference cuvette. Volumes of 5 µl or less of 5 mM-ClHg-Nph-OH solution, made up in 0.1 M-NaOH, were added to both cuvettes. The course of the

incorporation of HO-Nph-Hg groups into CK was monitored at 395 nm by the decrease in absorbance. After complete incorporation, at 2 mol of HO-Nph-Hg group/mol of CK, further additions of ClHg-Nph-OH caused no change in absorbance (Quiocho & Thomson, 1973). Excess of ClHg-Nph-OH was removed by dialysis against 50 mM-Tris/HCl buffer, pH 8.0. The final product (HO-Nph-Hg-CK) was yellow and had a specific activity of 400 units/mg of protein, which was the same as the original specific activity.

The reaction of HO-Nph-Hg-CK with CK conversion factor was carried out as described for CK incubations [as above, except that CK was replaced by HO-Nph-Hg-CK (500 units/l)].

Results

Modification of creatine kinase by CK conversion factor and carboxypeptidases

The conversion of CK occurred with either human or rabbit CK in the presence of partially purified human plasma CK conversion factor. As shown in Fig. 1, rabbit muscle CK moves more electropositively than does human muscle CK; incomplete treatment with CK conversion factor gave rise to two additional bands of activity.

The rate of conversion was found to be pH-dependent (Fig. 2). The partially purified conversion factor showed an optimum rate at pH 8.0 in the presence of 500 units of CK (specific activity 400 units/mg of protein)/l. Analysis of the amino acids released from CK after treatment with partially purified CK conversion factor showed that lysine was the major amino acid liberated,

equivalent to 1.8 mol/mol of CK dimer. Also found were glycine and serine, equivalent to 0.7 and 0.4 mol/mol of CK dimer respectively.

CK conversion was also achieved with pancreatic carboxypeptidase A or B with both human and rabbit CK, yielding the same three-banded patterns. Carboxypeptidase B was particularly active over a wide pH range, whereas carboxypeptidase A activity progressively increased with increase in pH (Fig. 2).

No difference in the rate of CK conversion was found when the modified HO-Nph-Hg-CK was used as substrate for CK conversion factor. HO-Nph-Hg-CK sub-banding was identical with that of native CK.

Substrate specificity of CK conversion factor

A three-banded pattern similar to that for CK was also obtained by treating a solution of haemoglobin A with the partially purified conversion factor at pH 8.0. Under the same conditions carboxypeptidase B produced an electropositive shift in haemoglobin A to a position equivalent to that of the intermediate band observed after treatment with the partially purified CK conversion factor.

Fig. 1. Relative electrophoretic positions of tetrathionate-treated human and rabbit muscle CK (lanes 1 and 3 respectively) and tetrathionate-treated human and rabbit muscle CK after partial treatment with human plasma CK conversion factor (lanes 2 and 4 respectively)

For experimental details see the text.

Fig. 2. Effect of pH on the relative rate of conversion of tetrathionate-treated human CK by partially purified CK conversion factor (●), carboxypeptidase A (■) and carboxypeptidase B (▲)

For experimental details see the text. The results are expressed in terms of the relative rate of electrophoretic conversion of CK compared with the rate at optimum pH for each preparation tested. The maximum activities were found to be: partially purified CK conversion factor, 2.9 units/mg of protein (pH 8.0); carboxypeptidase A, 32.8 units/mg of protein (pH 9.5); carboxypeptidase B, 7330 units/mg of protein (pH 8.5). The concentration of CK was 500 units/l.

Fig. 3. Effect of various metal ions on the rate of electrophoretic conversion of CK by partially purified CK conversion factor

For experimental details see the text. In all cases rate of CK conversion was compared with control incubations where no additional metal salts were added. The compounds tested were CoCl_2 (●), MnCl_2 (○), $\text{Ni}(\text{NO}_3)_2$ (▼), CuSO_4 (▲), CdSO_4 (△), FeSO_4 (■) and ZnSO_4 (□).

Treatment with carboxypeptidase A produced a similar result to that seen with carboxypeptidase B, except that with prolonged treatment a partial reversion to the original band position was observed.

Under the standard conditions used, there was no detectable hydrolysis of Hip-Arg or Hip-Phe by the partially purified CK conversion factor, and only a slow hydrolysis of Hip-Lys ($\Delta A/\text{min} = 0.004$). Addition of Hip-Lys, Hip-Arg or Hip-Phe at concentrations up to 0.1 mM to the assay incubation mixture had no effect on the rate of conversion of CK.

Effects of metal ions and chelating agents on CK conversion factor

The effect of various metal ions on the rate of CK conversion by the partially purified CK conversion factor was tested (Fig. 3). Co^{2+} and Mn^{2+} were found to be activators, whereas Fe^{2+} and Zn^{2+} were inhibitors. Ni^{2+} had little effect at 1 mM or less, and Cu^{2+} and Cd^{2+} had little effect at

Table 1. Relative rate of Hip-Lys hydrolysis by partially purified CK conversion factor in the presence of additional metal ions

The metal salts were added to incubation mixtures in the cuvette and the rate of hydrolysis was monitored spectrophotometrically as described in the Experimental section. The rate of Hip-Lys hydrolysis was compared with that in control assays that contained no additional metal ions.

Metal salt added	Relative Hip-Lys hydrolysis (%)	
	1 mM-Metal salt	10 mM-Metal salt
None	100	100
CoCl_2	122	260
$\text{Ni}(\text{NO}_3)_2$	110	580
MnCl_2	72	35
CdSO_4	0	0
HgCl_2	0	0
CuSO_4	0	0
FeSO_4	0	0
ZnSO_4	0	0

0.1 mM or less; at 10-fold greater concentrations than these, a total loss of CK activity occurred.

Hg^{2+} was also tested, but inactivated CK at all concentrations above 1 μM . At 1 μM electrophoretic changes were noted, but these were due to a direct effect of Hg^{2+} on CK. At 0.1 μM and below Hg^{2+} had no effect on CK activity or conversion.

EDTA at 1 mM concentration had previously been found to inhibit CK conversion completely (Edwards & Watts, 1983). A similar complete inhibition was observed with 1 mM-penicillamine. The chelating properties of penicillamine depend on the integrity of both thiol and amino groups. Hence, as was expected, *N*-acetylpenicillamine at 1 mM concentration was less effective, with the activity being decreased to 84% of the uninhibited rate; this compares with a decrease to 70% in the presence of 1 mM-2-mercaptoethanol.

Effect of metal ions on Hip-Lys hydrolysis

The effect of various metal ions on the rate of hydrolysis of Hip-Lys by the partially purified CK conversion factor was tested (Table 1). Both Co^{2+} and Ni^{2+} were found to be activators, Mn^{2+} was found to be a slight inhibitor, and Cd^{2+} , Hg^{2+} , Cu^{2+} , Fe^{2+} and Zn^{2+} were all strong inhibitors.

Discussion

Analysis by agarose-gel electrophoresis revealed three bands of muscle-type CK activity in plasma, but only one in an aqueous muscle extract. The difference is due to the presence in plasma of a proteinaceous heat-labile factor (Wevers *et al.*, 1978). We find that a similar electrophoretic conversion of CK can be achieved by incubation with pan-

creatic carboxypeptidase enzymes, particularly carboxypeptidase B. This suggested that the removal of a basic C-terminal amino acid would produce the observed electrophoretic conversion. Olson & Kuby (1964) have determined the C-terminal amino acid to be lysine. As predicted, after treatment with the plasma factor, we found that lysine was the major free amino acid hydrolysed from CK, equivalent to approx. 1 mol/mol of monomer CK. The progressive removal of two positive charges per CK dimer is consistent with the electrophoretic changes observed to produce three pseudo-isoenzymes of MM-CK.

Plasma CK conversion factor showed distinct differences from other known carboxypeptidases. The M_r has been determined as 190000 (Falter *et al.*, 1981), which is significantly larger than those of the pancreatic carboxypeptidases A and B, at 34400 and 34000 respectively (Hartsuck & Lipscomb, 1971; Folk, 1971). Carboxypeptidase N is a larger protein, of M_r 280000, and has been isolated from human plasma (Plummer & Hurwitz, 1978). It is responsible for the inactivation of bradykinin and kallidin in plasma and may be involved in the control of other physiologically active peptidases (Plummer & Erdős, 1981).

The partially purified CK conversion factor did not hydrolyse Hip-Phe or Hip-Arg. A slow hydrolysis of Hip-Lys was observed, which may be explained by a slight contamination by carboxypeptidase N, which hydrolyses Hip-Lys at a greater rate than it does Hip-Arg but does not hydrolyse Hip-Phe (Erdős *et al.*, 1964). Carboxypeptidase B also hydrolyses Hip-Lys and Hip-Arg, but not Hip-Phe; carboxypeptidase A, on the other hand, hydrolyses Hip-Phe, but not Hip-Lys or Hip-Arg (Appel, 1974). Hence CK conversion factor shows a different specificity towards these small peptides from any of the other carboxypeptidases.

With protein substrates the activity of carboxypeptidase A towards a C-terminal basic amino acid may be increased at higher pH values, as shown in the present study and by Olson & Kuby (1964). However, the pH profile of the activity of carboxypeptidase A upon CK conversion is clearly different from those of the carboxypeptidase B and the CK conversion factor (Fig. 2). The activity of partially purified CK conversion factor is optimal at pH 8.0 and, unlike that of carboxypeptidases A and B, decreases sharply above this pH. Carboxypeptidase N, also in this buffer, has a pH optimum at 7.0 or below (Erdős *et al.*, 1964). The partially purified CK conversion factor was also able to convert haemoglobin A into two faster electrophoretic bands, presumably by the hydrolysis of arginine and histidine residues from the α - and β -chains respectively. Neither carboxypeptidase A nor carboxypeptidase B could reproduce this

result. In both cases only one additional band, equivalent to the loss of one basic amino acid, was observed. This may be explained by C-terminal hydrolysis of arginine from the α -chain by carboxypeptidase B and the hydrolysis of histidine (and then tyrosine) from the β -chain by carboxypeptidase A. The combined action of carboxypeptidases A and B on haemoglobin A produces a more extensive hydrolysis of the C-terminal residues of the α - and β -chains (Brunori *et al.*, 1964), the results of which could not be readily interpreted by the current method of analysis.

Further evidence for the contamination of CK conversion factor by carboxypeptidase N (as mentioned above) and the occurrence of CK conversion factor as a distinct carboxypeptidase was the effect of metal ions on the rate of hydrolysis of Hip-Lys. As shown by Table 1, both Co^{2+} and Ni^{2+} were activators whereas Mn^{2+} , Cd^{2+} , Hg^{2+} , Cu^{2+} , Fe^{2+} and Zn^{2+} were inhibitors. These results are fully consistent with observations reported for the hydrolysis of Hip-Lys by carboxypeptidase N (Erdős & Sloane, 1962; Erdős *et al.*, 1967). By contrast, CK conversion factor with CK as substrate is activated by Mn^{2+} and relatively unaffected by Cu^{2+} , Cd^{2+} and Ni^{2+} . Although the action of carboxypeptidase N on CK is not known, a totally different profile of metal-ion activation would not be predicted.

The carboxypeptidases show other differences in their behaviour towards metal ions (reviewed by Appel, 1974). Co^{2+} activates CK conversion factor, in common with the effect on carboxypeptidases A, B and N. However, activation of CK conversion factor by Mn^{2+} is in common with only carboxypeptidase A. Zn^{2+} and Fe^{2+} were found to be inhibitors of CK conversion factor. Zn^{2+} is an activator of carboxypeptidases A and B, but an inhibitor of carboxypeptidase N. Fe^{2+} activates carboxypeptidase A, and has no effect on carboxypeptidases B and N. Neither Cd^{2+} , nor Cu^{2+} nor Ni^{2+} , below 0.1 mM, had a significant effect on CK conversion factor. At similar concentrations Cd^{2+} is an activator of carboxypeptidase A and an inhibitor of carboxypeptidases B and N. Cu^{2+} is an inhibitor of carboxypeptidase A, and Ni^{2+} is an activator of carboxypeptidases A and N.

The stabilizing effect of EDTA on MM-CK subbanding in serum samples was observed by Chapelle *et al.* (1981). We were able to show that this effect was due to inhibition of the CK conversion factor (Edwards & Watts, 1983). The same effect is produced by incubation with another chelating agent, penicillamine. Less effective were 2-mercaptoethanol and *N*-acetylpenicillamine, which could have been active as chelators or reducing agents. EDTA has been shown to inhibit carboxypeptidases A and N

(Appel, 1974), but not carboxypeptidase B (Folk *et al.*, 1960). 2-Mercaptoethanol is a strong inhibitor of carboxypeptidase N (Erdős *et al.*, 1967), much more than with CK conversion factor.

As reported previously (Edwards & Watts, 1983), blocking the reactive thiol group of CK with tetrathionate protects the enzyme against activity loss, but does not affect conversion. Similarly, conversion was not affected when the secondary thiol group, accessible to modification by mercurials without activity loss, was blocked by ClHg-NpH-OH. Hence there is no evidence for involvement of CK thiol groups in the CK conversion process.

From the present results we conclude that CK conversion factor is an exopeptidase active towards C-terminal basic amino acid residues in proteins, but not in the low- M_r substrates Hip-Lys and Hip-Arg. The CK conversion factor is larger than either carboxypeptidase A or B, yet smaller than carboxypeptidase N. In common with other carboxypeptidases, CK conversion factor was found to be activated by Co^{2+} ions; however, the effects of other metal ions were not identical with the pattern of activation and inhibition observed for other carboxypeptidases. The effect of chelating agents was inactivation of CK conversion factor, presumably by interaction with metal ions essential for activity, possibly integral to the structure of the protein. CK conversion factor appears to possess all the properties of a hitherto undescribed carboxypeptidase with a wider specificity for protein substrates than simply CK; accordingly we propose that this should provisionally be called carboxypeptidase K (for kinase as the first found substrate), EC 3.4.12.x, but recognize that, until it becomes possible to characterize a more highly purified enzyme preparation, the possibility that carboxypeptidase N is responsible for the observed results cannot be entirely excluded.

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