Brain Adenosine 5'-Triphosphate–Creatine Phosphotransferase

PURIFICATION, THIOL GROUP REACTIVITY AND THE AMINO ACID SEQUENCE AROUND THE REACTIVE THIOL GROUPS

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1. The purification of creatine kinase (ATP–creatine phosphotransferase, EC 2.7.3.2) from ox brain by a method that is quicker, simpler and gives much higher yields than other published procedures is described. 2. Stoichiometric inhibition studies with iodoacetate showed that the enzyme, like that from muscle, has two reactive thiol groups that are essential for enzyme activity. 3. The amino acid sequence around the essential thiol groups was determined and found to be virtually identical with that in creatine kinases from rabbit and ox muscle, and very similar to that found in arginine kinase; the evolutionary significance of this is discussed. 4. The identification of DNS-amino acids on thin layers of silica gel was found to have, in many cases, distinct advantages over that on polyamide layers.

The guanidinophosphotransferases form a closely related group of enzymes with similar properties and function. They catalyse the reversible transfer of the terminal phosphoryl group of ATP to a guanidine derivative in the presence of Mg^{2+} . They all contain one thiol group/unit of molecular weight, which is approx. 40 000 (Mahowald & Kuby, 1960; Watts, Rabin & Crook, 1961; Virden & Watts, 1966; Kassab, Pradel, Der Terrossian & Thoai, 1967), that is involved in the enzymic activity. Their molecular weights are either approx. 40 000 (arginine kinase) or approx. 80 000 (creatine kinase and the less commonly occurring members of the group). Those with a molecular weight of 80 000 appear to be dimeric and the subunits are probably identical (Eppenberger, Eppenberger, Richterich & Aebi, 1964; Thomson, Eveleigh, Laws & Miles, 1968a; Pradel, Kassab, Conlay & Thoai, 1968). Vertebrates have at least two creatine kinase (ATP–creatine phosphotransferase, EC 2.7.3.2) isoenzymes, the most intensively studied being the brain type (BB) and muscle type (MM). The subunits of these can hybridize to form an MB isoenzyme sometimes found in heart muscle (Eppenberger, Dawson & Kaplan, 1967). The BB isoenzyme appears to be the more primitive one, since in the early stages of ontogeny it is the only one found, even in muscle tissue. In muscle it slowly disappears and is replaced first by MB isoenzyme and finally by MM isoenzyme (Eppenberger *et al.* 1964).

The amino acid sequences around the essential thiol groups of rabbit MM isoenzyme and bovine MM isoenzyme have been reported by Thomson, Eveleigh & Miles (1964) and Thomson *et al.* (1968a) respectively; they were identical. Atherton, Laws,

Miles & Thomson (1970a) gave a preliminary account of the sequence around the essential thiol group of ox BB isoenzyme, and this was largely identical with the MM isoenzyme. These studies confirmed the conclusion reached by Eppenberger *et al.* (1967), Thomson *et al.* (1968a) and Atherton & Thomson (1969) that creatine kinases from the same tissue of different species are more alike than those from different tissues of the same species. Der Terrossian, Pradel, Kassab & Thoai (1969) found that the amino acid sequence around the essential thiol group of lobster arginine kinase is very similar to the sequences mentioned above, showing that this part of the sequence of guanidinophosphotransferases has been substantially conserved throughout their evolution.

In the present paper we report on the number and reactivity of the thiol groups of ox brain creatine kinase and the complete amino acid sequence of the tryptic peptide containing the essential thiol group of the enzyme. In conjunction with this work we have developed a method for preparing large quantities of ox brain creatine kinase that is quicker, simpler and gives much higher yields than other published procedures (Wood, 1963; Keutel, Jacobs, Okabe, Yue & Kuby, 1968). We also give further details, not previously published in full, of the sequence of the corresponding peptide from rabbit muscle creatine kinase.

MATERIALS

All chemicals were of the highest purity commercially available. Urea, iodoacetate [recrystallized from light petroleum (b.p. 60–80°C) before use], phenyl isothiocyanate (redistilled before use), DNS chloride and $(NH_4)_2SO_4$,

(especially low in heavy metals) were obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.). Dithiothreitol was from Calbiochem (Los Angeles, Calif., U.S.A.) and dithioerythritol, tris and crystalline ATP (disodium salt) were from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Iodo[2-¹⁴C]acetate was from the Radiochemical Centre (Amersham, Bucks., U.K.). Trypsin, chymotrypsin, carboxypeptidase A and leucine aminopeptidase were from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.) and subtilisin was Nagarse from Miles Laboratories Inc. (Kankakee, Ill., U.S.A.) (dialysed before use). Other chemicals were from BDH Chemicals Ltd., Hopkin and Williams Ltd. (Chadwell Heath, Essex, U.K.) and May and Baker Ltd. (Dagenham, Essex, U.K.).

Dowex resins were from Bio-Rad Laboratories (Richmond, Calif., U.S.A.), Sephadex gels (fine grade used for chromatography) were from Pharmacia Fine Chemicals (Uppsala, Sweden) and Whatman DE 52 DEAE-cellulose was from H. Reeve Angel and Co. (London E.C.4, U.K.), who also supplied the paper for electrophoresis. Camlab (Cambridge, U.K.) supplied Merck silica gel G. Visking dialysis tubing was from Hudes Merchandising Corp. (London W.1, U.K.).

The pH-stat was made by Radiometer (Copenhagen, Denmark), the amino acid analyser by Technicon Instrument Co. Ltd. (Chertsey, Surrey, U.K.) and the flow cell by Nuclear Enterprises (G.B.) Ltd. (Edinburgh, U.K.). Radioautography was carried out with Kodak X-ray Film.

Ox brains were kindly provided by the Agricultural Research Council Institute for Animal Diseases (Compton, Berks., U.K.). They were removed from the animals immediately after slaughter, washed free of superficial blood with cold running water and transported to the laboratory frozen with solid CO₂. They were stored at -15°C but were thawed overnight in the cold-room before use.

EXPERIMENTAL AND RESULTS

Isolation of ox brain creatine kinase

For amino acid sequence and kinetic studies with ox brain creatine kinase we needed to prepare 500mg batches of high-specific-activity enzyme. The method of Wood (1963) gave comparatively low yields and specific activities that were further decreased when the method was scaled up. The method of Keutel *et al.* (1968) takes longer and is more complicated. It gives high-specific-activity material but yields are low, over 6kg of brain being needed to prepare 500mg of the enzyme.

The following is a method, based on that of Wood (1963), that we have used extensively (it was used to prepare the enzyme for the experiments by Thomson *et al.* 1968a). It gives creatine kinase with specific activities greater than 100 Kuby units/mg in yields two to three times those obtained by Keutel *et al.* (1968) and is also simpler.

All steps in the isolation procedure were carried out in a cold-room at 2°C or in a refrigerated centrifuge at 0°C. All materials and apparatus were pre-equilibrated to cold-room temperature,

Table 1. *Isolation of ox brain creatine kinase*

This is the preparation referred to under the heading 'dithiothreitol' in Table 2; 1.65kg of brain tissue was used.

Fraction	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (Kuby units/ mg)	Total activity (Kuby units)	Purification	Recovery (%)	Recovery from column (%)
I Homogenate	2580	10.75	27700	3.2	89000	1.0	100	—
II Acetone extract	4460	0.99	4430	12.9	57100	4.0	64	—
III Magnesium acetate extract	164	9.7	1590	24.7	39300	7.7	44	(100)
IV DE 52 DEAE-cellulose peak (main part)	10.1*	26.6	269	99	26600	31.0	34.5	78
DE 52 DEAE-cellulose peak (trailing edge)	4.1*	17.8	73	55	4000	17.2		

* After concentration.

except acetone, which was stored at -15°C . Protein in the potassium chloride extract was determined by using the biuret reagent (Gornall, Bardawill & David, 1949), which was standardized with ovalbumin. Other protein concentrations were calculated from E_{280} values (Noda, Kuby & Lardy, 1954). A typical preparation is described below and is summarized in Table 1.

Fraction I. Five whole brains (about 2kg) were cut into strips approx. 1 cm square. Batches (200g) of these were homogenized in 400ml of 10mM-potassium chloride–1mM-disodium EDTA.

Fraction II. After being stirred for 30min, the homogenate was adjusted to pH 9 with 5M-ammonia and transferred to 5-litre conical flasks. An equal volume of acetone at -15°C was added slowly from reservoirs fitted with capillary stems dipping into the homogenate, which was continually stirred with care being taken to avoid excessive frothing. Stirring was continued for an additional 16h and insoluble material was then removed by centrifuging for 10min at 16000g.

Fraction III. By using the capillary-stemmed reservoirs, 2M-magnesium sulphate, pH 8.5, was slowly added to the acetone extract to make it 20mM with respect to magnesium sulphate. A volume of acetone equal to the volume of 2M-magnesium sulphate was then added as before and stirring was continued for another 1h. The precipitate was then recovered by centrifuging at 16000g for 10min and finely dispersed in and thoroughly extracted with two successive volumes of 70mM-magnesium acetate, pH 9, containing 0.32mg of dithioerythritol/ml, equal to 4 and 2% of the initial potassium chloride–EDTA extract volume. The combined extracts, recovered by centrifuging at 16000g for 20min, were dialysed against three changes of 2 litres of 10mM-tris, containing 0.08mg of dithioerythritol/ml, adjusted to pH 7.7 with concentrated hydrochloric acid. The resulting

cloudy solution, which remained opalescent even after centrifugation at 35000g, was chromatographed on a Whatman DE 52 DEAE-cellulose column as described below.

Fraction IV: column chromatography (Fig. 1). Whatman DE52 DEAE-cellulose was prepared and equilibrated with 10mM-tris–HCl buffer, pH 7.7, as recommended by the manufacturer. A glass column (4cm $\times$ 35cm) was filled with the absorbent, which was allowed to settle with the flow rate restricted to 54ml/h by an LKB Perpex pump connected to the column outlet. The column was then equilibrated, at the same flow rate, with the same buffer containing 0.08mg of dithioerythritol/ml. At all subsequent stages tris buffers, made up as above, contained 0.08mg of dithioerythritol/ml.

Before the enzyme extract was loaded on the column, the outlet was connected to an LKB Uvi-cord I ultraviolet monitor, the Perpex pump was transferred to the column-inlet line and the flow rate was adjusted to 102ml/h. The enzyme solution was then pumped on to the column, followed successively by 100ml of 10mM-tris–HCl buffer, pH 7.7 and then the same buffer made 100mM with respect to sodium chloride. When the extinction at 254nm of the first peak, which contained no creatine kinase activity, began to fall, the enzyme activity was eluted with a linear gradient of 0.1–0.35M-sodium chloride in a total volume of 2 litres of buffer; 6min fractions were collected by using a BTL Chromafac and Chromatimer. The creatine kinase activity of samples diluted 100-fold with 10mM-tris–HCl buffer, pH 7.7, by means of a Griffin Diluspence, were determined by an automated procedure based on the manual phosphate assay method of Kuby, Noda & Lardy (1954) but with a Technicon Auto-Analyzer. The results were semi-quantitative but enabled the distribution of the enzyme in all the column fractions to be determined rapidly.

Those fractions containing most of the enzyme

Fig. 1. Purification of ox brain creatine kinase from a magnesium acetate extract prepared as described in the text. A column (4cm $\times$ 35cm) of Whatman DE 52 ion-exchange DEAE-cellulose was used. Creatine kinase was eluted with a gradient of NaCl in 10mM-tris–HCl buffer, pH 7.7, containing 0.08mg of dithioerythritol/ml. The flow rate was 102ml/h. Other conditions are as given in the text. —, Transmission at 254 nm; ----, concn. of NaCl; Δ , concn. of protein; $\circ$, creatine kinase activity.

activity were pooled and solid ammonium sulphate was added slowly with stirring to 70% saturation, with care being taken to maintain the pH at 7.7. Stirring was continued for 1 h more and the precipitated enzyme was recovered by spinning for 20 min at 16000g. It was resuspended in a small volume of 20mm-tris-HCl buffer, pH 7.7, and equilibrated by dialysis against the same buffer. When necessary enzyme solutions were concentrated as follows. A dialysis bag containing the dilute enzyme was placed in a polythene bottle containing dry Sephadex G-25 (coarse grade). The contents of the bottle were continually mixed by slow mechanical rotation. In this way creatine kinase concentrations of 50 mg/ml or higher could readily be achieved in a few hours. Before being labelled with iodo[2-¹⁴C]acetate the concentrated enzyme solution was equilibrated with 10mm-tris-HCl buffer, pH 7.7, containing only 1 µg of dithioerythritol/ml.

This procedure required 4 days to produce high yields of creatine kinase with specific activities greater than 100 units/ml. Enzyme not used immediately was stored at -15°C as a suspension in ammonium sulphate solution. The results of one such preparation are shown in Table 1.

Creatine kinase so prepared was homogeneous by analytical ultracentrifugation. This was kindly carried out by Dr M. P. Esnouf (Nuffield Department of Clinical Biochemistry, University of Oxford) on a Spinco model E analytical ultracentrifuge. The conditions were as follows: rotor speed 59780 rev./min, 10mm-tris-HCl buffer, pH 8.6, 20°C. Photographs were taken at 16 min intervals after full rotor speed had been reached.

Early difficulties in the preparation of the brain enzyme, mainly due to the instability of the enzyme in the absence of thiol agents, were largely overcome by incorporation of dithiothreitol in the preparation

(Thomson *et al.* 1968a). Increased yields over those reported by Wood (1963) and Keutel *et al.* (1968) (twofold and fourfold in terms of Kuby units/g respectively) were achieved by thorough maceration of the brain tissue, by omitting centrifugation of the homogenate before the addition of acetone and by raising the dithiothreitol concentration in the magnesium acetate used for extraction from 0.1 to 0.4 mg/ml. The results of these modifications are shown in Table 1 and also in Table 2 under the heading 'dithiothreitol'. Later, with dithioerythritol, which being non-hygroscopic is more convenient to handle, a further improvement in yield was obtained (Table 2).

The recovery of enzyme activity from the DEAE-cellulose columns varied between 74 and 85%. The wider spread of overall enzyme recoveries (Table 2) resulted from difficulties in achieving efficient extraction of the magnesium sulphate precipitate, which was difficult to disperse thoroughly in magnesium acetate.

Thiol groups of creatine kinase

Determination of thiol groups. This was carried out by the method of Ellman (1959) with 5,5'-dithiobis-(2-nitrobenzoate). To maintain the enzyme thiol groups in their reduced form, enzyme solutions (which were normally stored in the presence of 0.1 mg of dithioerythritol/ml) were dialysed against 10mm-tris, adjusted to pH 8.0 with concentrated hydrochloric acid, containing 1 µg of dithioerythritol/ml. The diffusate was used as the reference solution in the spectrophotometer. Total thiol groups were determined as above in the presence of 6 M-guanidinium chloride.

Thomson *et al.* (1968a) reported values of 1.32 and 7.5 for reactive and total thiol groups respectively in

Table 2. *Comparison of brain creatine kinase preparations: recoveries and specific activities*

Reference ...	Wood (1963)	Keutel <i>et al.</i> (1968)	This paper (dithiothreitol)	This paper (dithioerythritol)	This paper (dithioerythritol*)
Weight of brain used (kg)	0.45	6.4	1.65	approx. 2	
No. of preparations	1	1	1	4	
Initial homogenate (units/g of brain)	12.7	23.8	55.5	—	
Magnesium acetate extract (units/g of brain)	10.2	—	24.5	21-39 (30.2)†	
Final preparation (units/g of brain)	7.1	10.8	16.6	19-32 (25.4)†	14-32 (20.5)†
Specific activity of final preparation (units/mg of protein)	49-73	117‡	99	81-112 (97)†	98-112 (106)†

* Middle fractions of DE52 DEAE-cellulose column active peak only.

† Values in parentheses are the mean values from the four preparations.

‡ These authors also prepared crystalline creatine kinase of higher specific activity in low yields.

Table 3. *Thiol groups in creatine kinase isoenzymes*

Thiol groups of ox brain creatine kinase were determined by the method of Ellman (1959) in the presence of 1 μ g of dithioerythritol/ml (see the text). The numbers of determinations are shown in parentheses. Values for the brain isoenzymes are expressed as means $\pm$ s.d. and were obtained from five different preparations.

Source	Thiol groups (mol/81 000 g)		Reference
	Reactive	Total	
Ox brain	1.94 $\pm$ 0.17 (14)	9.38 $\pm$ 0.55 (16)	This paper
Ox muscle	1.47 (3)	7.32 (3)	Thomson <i>et al.</i> (1968a)
Rabbit muscle	1.60 (3)	7.70 (3)	Bayley & Thomson (1967)

Fig. 2. Inhibition of ox brain creatine kinase by iodoacetate. The creatine kinase concentration was 47 mg/ml in a total volume of 80 μ l of 10 mM-tris-HCl buffer, pH 7.7, containing 1 μ g of dithioerythritol/ml. Incubation was for 2 h at 30°C. The reaction was stopped by diluting 40-fold with buffer containing 0.1 mg of dithioerythritol/ml.

ox brain creatine kinase, although it was appreciated that, because of the instability of the enzyme, these values were probably low. With the improved purification method described above, and particularly as a result of incorporating dithioerythritol in the solutions, values of 1.94 and 9.38 for reactive and total thiol groups respectively were obtained (Table 3). It was noticeable that, in contrast with the muscle enzymes, modification of the reactive thiol groups of the brain enzyme with 5,5'-dithiobis-(2-nitrobenzoate) led to a progressive further release of the thiophenol at approx. 1% of the rate for the rapidly reacting thiol groups (see the Discussion section).

Inhibition by iodoacetate. At high enzyme concentrations (approx. 50 mg/ml) rabbit muscle creatine kinase is completely inhibited by 2 equivalents of iodoacetate or iodoacetamide (Mahowald, Noltmann & Kubly, 1962). Stoichiometric inhibition curves of the brain enzyme also indicate virtually complete inhibition under similar experimental conditions, although a small but variable enzymic activity frequently remains even at higher

molar ratios of iodoacetate to creatine kinase (Fig. 2). The stability of partially alkylated creatine kinase varies with the degree of alkylation, however (Dawson, Eppenberger & Kaplan, 1967; Atherton, Laws & Thomson, 1970b), and this, together with possible conformational changes induced by alkylation and affecting thiol reactivity (Kumudavalli, Moreland & Watts, 1970; Atherton *et al.* 1970b), may be responsible for the non-linearity observed (Fig. 2). It is therefore likely that, as with creatine kinase, the brain enzyme has two reactive thiol groups, which are essential for enzyme activity.

Amino acid sequence studies of ox brain creatine kinase

Labelling of the enzyme. Much of this work was done on the following preparation. A 311 mg portion of ox brain creatine kinase (60 mg/ml) was labelled with 2 molar equivalents of iodo[2-¹⁴C]acetate (7 μ Ci/ μ mol) at 30°C and pH 9.0 for 1 h. Solid urea was added to bring the solution to 8 M and alkylation was continued for another 3 h with a twofold excess of unlabelled iodoacetic acid over all thiol groups. This was to avoid oxidation of any remaining thiol groups and to prevent disulphide exchange reactions. The reaction was terminated with 0.1 ml of 2-mercaptoethanol and the solution was exhaustively dialysed against sodium hydroxide solution, pH 9.5. This preparation was mixed with 521 mg of creatine kinase that had been carboxymethylated with unlabelled iodoacetic acid (Crestfield, Moore & Stein, 1963) to act as carrier.

Enzymic hydrolysis of peptides. (a) Trypsin. Carboxymethylated creatine kinase was hydrolysed in a pH-stat for 1 h with 2% (w/w) trypsin at 30°C and pH 8.0. The titrant was sodium hydroxide. (b) Chymotrypsin. Peptide T5 (1 μ mol/ml) was digested with 0.1% (w/v) chymotrypsin for 1.5 h at 30°C in 0.1 M-ammonium hydrogen carbonate. The reaction was stopped with acetic acid and the solution was dried *in vacuo*. It was dissolved in electrophoresis buffer (see below). (c) Carboxypeptidase A. Peptide (40 nmol) was dissolved in 0.2 ml

of 0.1M-ammonium hydrogen carbonate, 1.2 μ l of carboxypeptidase solution (50mg/ml) was added and the mixture was incubated for 4h at 37°C.

Fig. 3. Chromatography of tryptic peptides on Dowex 50. The tryptic digest of approx. 800mg of carboxymethylated ox brain creatine kinase was made 0.3M with respect to pyridine. Then 20ml of 0.3M-pyridine-formate buffer, pH3.2, was added and the pH adjusted to 3.1 with 2M-HCl. The solution was loaded on to a column (2 cm $\times$ 100 cm) of Dowex 50 (X2) that had been equilibrated with 0.3M-pyridine-formate buffer, pH3.2, at 38°C. The flow rate was 60ml/h and peptides were eluted with a linear gradient of 1 litre of 0.3M-pyridine-formate buffer, pH3.2, in series with 1 litre of 4.25M-pyridine-acetate buffer, pH5.6 (see Konigsberg & Hill, 1962); 15min fractions were collected, the eluate being monitored with a scintillation plastic flow cell. The radioactivity in each peak is shown as a percentage of the total loaded on the column; 89% of the radioactivity loaded on the column was recovered.

Fig. 4. Chromatography of peptide T5 on a column (2.5cm $\times$ 40cm) of Sephadex G-50. The column was equilibrated and eluted with 10mm-ammonia. The flow rate was 50ml/h. Eluates were monitored as in Fig. 3. All the radioactivity loaded was recovered in peaks T5-1 and T5-2, the proportion in each peak being shown.

(d) Leucine aminopeptidase. Peptide (60nmol) was dissolved in 14 μ l of 0.1M-ammonium hydrogen carbonate, 27 μ l of water and 5 μ l of 70mM-magnesium acetate; 4 μ l of leucine aminopeptidase was added (to give a final concentration of 0.43 μ g/ml) and the solution was incubated overnight at 40°C.

Peptide purification. (a) Ion-exchange chromatography (Fig. 3). For part of the work, tryptic peptides were purified by chromatography on successive columns of Dowex 50 (X2) and Dowex 1 (X2) (Thomson *et al.* 1964). However, recent batches of Dowex 1 have been unsatisfactory as they often adsorbed peptides irreversibly. Thus we now rely on other methods (see below) to complete the purification. (b) Gel filtration. Tryptic peptides were purified on successive columns of Sephadex G-50 and G-25 (Fig. 4; Sephadex G-25 conditions as for Sephadex G-50). Peptides eluted from electrophoretograms were often contaminated with amino acids such as glycine and serine. These were removed on a column (1.5cm $\times$ 30cm) of Sephadex G-15 eluted with 10mm-ammonia. (c)

Fig. 5. High-voltage electrophoresis at pH2 of a chymotryptic digest of 0.7 μ mol of peptide T5. The digest was dissolved in pH2 buffer (formic acid-acetic acid-water, 1:4:45, by vol.) and subjected to electrophoresis for 35min at 50V/cm. Peptide C1 was radioactive; peptides C2, C3, C4 and C5 stained purple and peptide C6 yellow with ninhydrin. Peptides C2, C3 and C4 stained red with α -nitrosonaphthol.

Fig. 6. High-voltage electrophoresis at pH 6.5 of chymotryptic peptides of peptide T5. Peptides C2, C3 and C4 were cut from the electrophoretogram of Fig. 5. They were sewn on to new sheets of paper and subjected to electrophoresis for 35 min at 40 V/cm. The buffer was pyridine-acetic acid-water (25:1:225, by vol.). Peptides C2', C3' and C4' were all non-radioactive and stained purple with ninhydrin and red with α -nitrosonaphthol.

High-voltage electrophoresis. Peptides were dried *in vacuo*, dissolved in buffer, banded on Whatman 3MM paper (0.25–0.5 mg/cm) and subjected to electrophoresis at pH 2 by using a Michl tank cooled with white spirit. In many cases the relevant zones were cut out and sewn on to new sheets of paper for electrophoresis at pH 6.5 (Fig. 6). Peptides were located by radioautography and by staining of trial strips with ninhydrin (0.2% in acetone) and α -nitrosonaphthol (Smith, 1969). They were eluted by the method of Edstrom (1968) with 10 mM-ammonia or pH 2 electrophoresis buffer. This method of elution gave greater yields of peptides than the usual chromatographic methods. Chymotryptic peptides resulting from hydrolysis of peptide T5 were similarly purified by successive electrophoresis at pH 2 and 6.5 (Figs. 5 and 6).

Amino acid analysis. Peptides were hydrolysed *in vacuo* for 16 h at 105°C with 6.5 M-hydrochloric acid (constant-boiling). The hydrochloric acid was then removed rapidly under vacuum and the hydrolysate dissolved in 0.2 ml of sodium citrate buffer, pH 2.0 (50 mM in citrate, 0.2 M in Na⁺). Analyses were carried out with a Technicon single-column automatic amino acid analyser equipped with an automatic sample loader and programming valve (Thomson & Eveleigh, 1969). An automatic data-handling system and a computer program were used, in part, to evaluate the chromatograms (Thomson, Gibbons, Eveleigh & Miles, 1968b).

N-terminal determination and sequence analysis. The DNS-Edman method of Gray (1967a,b) was used. DNS-amino acids were identified by uni-dimensional t.l.c. (solvents allowed to ascend 10 cm) on 20 cm × 20 cm layers of silica gel G (see the Discussion section). Normally two successive chromatograms were run to identify N-terminal residues positively. The solvents found to be most useful were benzene-pyridine-acetic acid (16:4:1, by vol.) (Deyl & Rosmus, 1965) and chloroform-2-methylbutan-2-ol-acetic acid (140:60:1, by vol.) (Morse & Horecker, 1966). The latter solvent was

especially useful for separating leucine and isoleucine, since isoleucine runs at an intermediate solvent front about 1 cm in front of leucine.

Carboxymethylcysteine N-terminal groups were identified by the loss of radioactivity from the aqueous layer after extraction with butyl acetate in the DNS-Edman procedure.

Identification of acidic and amide residues. These were identified by the electrophoretic mobilities of the parent peptides at pH 6.5 and by digestion of the peptides with carboxypeptidase A or leucine aminopeptidase followed by quantitative amino acid analysis.

RESULTS

Fig. 3 shows a typical separation on Dowex 50 of the tryptic peptides from ¹⁴C-labelled ox brain creatine kinase. Two main peaks, peptides T2 and T5, were always obtained and their ratio varied with the conditions of tryptic digestion.

Peptide T2 was eluted from Sephadex G-50 as a single sharp peak, whereas peptide T5 gave two peaks, peptides T5-1 and T5-2 (Fig. 4), probably through aggregation, especially since the first peak eluted was broader than the second. Peptides T2, T5-1 and T5-2 all gave single peaks on Sephadex G-25. Leading and trailing edges of peaks were normally discarded in the interests of purity.

When subjected to electrophoresis at pH 2, peptides T2, T5-1 and T5-2 all gave single radioactive bands well separated from inactive ninhydrin-positive bands. Electrophoresis at pH 6.5 resolved a further non-radioactive band from peptide T2. All three peptides were virtually uncharged at this pH. The mobilities of peptides T5-1 and T5-2 were identical at both pH values. Peptide T2 was completely eluted from paper with 10 mM-ammonia, but only 60 and 35% of peptides T5-1 and T5-2 respectively were eluted with this solvent, presumably because of their limited solubility, since a further 18% of both peptides T5-1 and T5-2 could

Table 4. *Amino acid analyses of tryptic peptides*

The method is described in the text. Values rounded off to the nearest integer are given in parentheses. Abbreviation: CM-Cys, *S*-carboxymethylcysteine.

Peptide ...	Amino acid composition (mol of residue/mol of peptide)		
	T2	T5-1	T5-2
CM-Cys	0.8 (1)	0.8 (1)	0.8 (1)
Asp	1.1 (1)	2.6 (3)	3.0 (3)
Thr	1.7 (2)	1.8 (2)	1.8 (2)
Ser	1.1 (1)	1.4 (1)	0.9 (1)
Glu	—	1.2 (1)	1.0 (1)
Pro	1.0 (1)	1.6 (2)	2.1 (2)
Gly	1.9 (2)	3.2 (3)	3.1 (3)
Met	—	1.1 (1)	1.4 (1)
Ile	1.0 (1)	0.9 (1)	1.0 (1)
Leu	2.9 (3)	3.5 (4)	4.0 (4)
Tyr	—	1.8 (2)	1.7 (2)
Phe	—	1.0 (1)	1.0 (1)
His	—	1.2 (1)	1.3 (1)
Arg	0.9 (1)	1.0 (1)	1.0 (1)

Table 5. *Chymotryptic digestion of peptide T5: elution of peptides from paper*

Peptide T5 (0.7 μ mol) was digested and subjected to electrophoresis as described in the text. Peptides were eluted with 10mM-ammonia; subsequent elution with pH2 buffer (formic acid-acetic acid-water, 1:4:45, by vol.) failed to increase these yields.

Peptide	Amount eluted (μ mol)
C1	0.55
C2	0.15
C3	0.22
C4	0.03
C5	0.13
C6	0.50

be eluted with pH2 buffer. The final pure preparations of peptides T2 and T5 contained respectively 11.6 and 27.5% of the radioactivity in the tryptic digest. These recoveries are very good when compared with the yields of these peptides in Fig. 3. Their amino acid compositions are shown in Table 4, which shows that the peptides were identical.

Since only part of the amino acid sequence could be determined on the large peptide T5 this was hydrolysed by chymotrypsin and the resulting peptides (C1-C6) were purified by electrophoresis at pH2 and 6.5 (Figs. 5 and 6).

Table 5 shows that, as with the tryptic peptides, non-aromatic peptides, as distinct from aromatic ones, were eluted from paper in high yields. The amino acid analyses of these peptides are shown in Table 6.

The results of amino acid sequence studies are shown in Fig. 7.

Amino acid sequence studies of rabbit muscle creatine kinase. When Thomson *et al.* (1964) published the amino acid sequence of the essential thiol-containing tryptic peptide, T5, of rabbit muscle creatine kinase they were unable to determine the sequence of its chymotryptic peptide C4 beyond saying that it was

Although the complete sequence has been reported (Eveleigh & Thomson, 1965), the results on which this was based were not given, but are shown in Fig. 8. The complete sequence of peptide T5 from the rabbit muscle enzyme is given in Fig. 9.

DISCUSSION

The isolation procedure for brain creatine kinase described in this paper has already been discussed in detail; some comments on the DNS-Edman procedure may be useful. Many workers have replaced electrophoretic analysis of DNS-amino acids (Gray, 1967a) by two-dimensional t.l.c. on polyamide layers (Woods & Wang, 1967). However, we have found that the latter method has several disadvantages when compared with unidimensional t.l.c. on silica gel. These are: (a) it takes longer to apply spots, (b) solvent movement is slower and (c) polyamide sheets are expensive and supplies are difficult to obtain (even though they can be re-used a limited number of times). The method we use has the following advantages: (a) the whole process of spotting and running a plate takes no more than 45 min, (b) several samples together with standards can be run on one plate and for small peptides a positive *N*-terminal identification is often possible after one run and (c) the ease with which leucine and isoleucine can be resolved (see above). The methyl acetate-propan-2-ol-ammonia solvent of Seiler & Wiechmann (1964) is useful for confirming the presence of ϵ -DNS-lysine. T.l.c. of polyamide layers can very usefully supplement t.l.c. on silica gel if, for instance, *N*-terminal arginine is suspected or *N*-terminal proline is to be confirmed.

There is no doubt that the thiol groups of brain creatine kinase are more reactive than those of the muscle isoenzymes. Thus in the absence of added thiol compounds to stabilize the enzyme reactive thiol values of less than 1.0 mol/mol are regularly obtained by the Ellman (1959) method (A. R. Thomson, unpublished work). The greater lability of the brain enzyme is further emphasized by its behaviour during column chromatography, since in the absence of dithioerythritol the specific activity of the active column fractions declines rapidly. Incorporation of dithioerythritol in the

Table 6. *Amino acid analyses of chymotryptic peptides*

The method is described in the text. Values rounded off to the nearest integer are given in parentheses. Abbreviation: CM-Cys, *S*-carboxymethylcysteine.

Peptide ...	Amino acid composition (mol of residue/mol of peptide)					
	C1	C2	C3	C4	C5	C6
CM-Cys	0.9 (1)	—	—	—	—	—
Asp	1.2 (1)	1.0 (1)	—	2.1 (2)	1.0 (1)	—
Thr	0.9 (1)	—	—	—	—	1.1 (1)
Ser	1.1 (1)	—	—	—	—	—
Glu	—	1.3 (1)	—	0.9 (1)	—	—
Pro	1.1 (1)	—	—	1.0 (1)	1.2 (1)	—
Gly	—	—	1.0 (1)	1.4 (1)	—	1.9 (2)
Met	—	—	—	0.5* (1)	0.5* (1)	—
Ile	0.8 (1)	—	—	—	—	—
Leu	2.0 (2)	—	1.0 (1)	1.3 (1)	—	1.0 (1)
Tyr	—	1.0 (1)	1.0 (1)	1.6 (2)	—	—
Phe	—	0.9 (1)	—	0.6 (1)	—	—
His	—	—	—	1.2 (1)	0.8 (1)	—
Arg	—	—	—	—	—	1.0 (1)

* These values are probably low because of oxidation.

T5	Asn-Tyr-Glu-Phe-Met(Pro,Asn,His,Leu,Gly,Tyr,Ile,Leu,Thr,CM-Cys,Pro,Ser,Asn,Leu,Gly,Thr,Gly,Leu,Arg)
T2	Ile,Leu,Thr(CM-Cys,Pro,Ser,Asn,Leu,Gly,Thr,Gly,Leu,Arg)
C2	Asn-Tyr-Glu-Phe
C5	Met-Pro-Asn-His
C3	Leu-Gly-Tyr
C4	Asn-Tyr(Glu,Phe,Met,Pro,Asn,His,Leu,Gly,Tyr)
C1	Ile-Leu-Thr-CM-Cys-Pro-Ser-Asn-Leu
C6	Gly-Thr-Gly-Leu-Arg

Fig. 7. Amino acid sequences of peptide T5 from ox brain creatine kinase. All sequences shown were determined by the DNS-Edman method. Abbreviation: CM-Cys, *S*-carboxymethylcysteine.

buffers yielded preparations that were more stable and had higher specific activities. The slower secondary reaction with 5,5'-dithiobis-(2-nitrobenzoate) (see above), suggesting reaction of less accessible thiol groups, may be interpreted as indicating a structural role for the reactive thiol groups, but, equally, may be the consequence of introducing a bulky charged aromatic group into the molecule at a highly sensitive part of its structure. Since there is no significant change in the molecular weight of brain creatine kinase on alkylation of its two reactive thiol groups with iodoacetamide [J. F. Laws & A. R. Thomson (unpublished work); the same applies to the muscle enzyme (Thomson *et al.* 1968a)] it is unlikely that the essential thiol groups are

primarily concerned in the maintenance of a dimeric structure.

The creatine kinase isoenzymes from brain and muscle tissue differ significantly in electrophoretic mobility, amino acid composition and kinetic properties. It would be expected, however, that features such as the chemical environment of the reactive thiol groups, which are important for structural and functional integrity of the molecule, would show minimum alteration. Thus the amino acid sequence in the region of the essential thiol groups of rabbit and ox muscle creatine kinases are identical (Fig. 9) (Thomson *et al.* 1968a). In addition, as the present work shows, the ox brain enzyme contains a sequence (phenylalanine to

arginine; Fig. 9) that is the same as that for the muscle enzymes except for two replacements, one the very conservative replacement of valine by isoleucine and the other the non-conservative replacement of glutamic acid by proline. The residues on the *N*-terminal side of phenylalanine are different in the two isoenzymes. The unusual Cys-Pro feature of the muscle enzymes has also been found in brain creatine kinase (see also below).

Thomson *et al.* (1964) showed that the two essential thiol groups of rabbit muscle creatine kinase were identical in that they both formed part of a unique tryptic peptide. The results with brain

creatine kinase are not as clear-cut, since full alkylation of the essential thiol groups was difficult to achieve, but nevertheless suggest that they are also identical. Thus the two major radioactive peptides T2 and T5 accounted for 16.8 and 52.1% respectively of the radioactivity in the tryptic digest, and subsequent purification showed that they contained no other contaminating radioactive peptides; purified peptides T2 and T5 contained respectively 11.6 and 27.5% of the radioactivity in the tryptic digest; they had identical specific radioactivities, and peptide T2 was shown to be a hydrolysis product of peptide T5. This evidence, together with the results of peptide 'mapping' (Thomson *et al.* 1968a; Atherton & Thomson, 1969), therefore strongly suggests that the two essential thiol groups are identical and that ox brain creatine kinase itself consists of two identical subunits each with a single catalytic site and one reactive and essential thiol group.

Arginine kinase of invertebrate muscle fulfils the same role as creatine kinase in vertebrates and is generally regarded as its evolutionary precursor, a view supported in general terms by its amino acid composition (Virden & Watts, 1966; Der Terrossian, Kassab, Pradel & Thoai, 1966; Thomson *et al.* 1968a) and by mechanistic studies (Smith & Morrison, 1969). For example, lobster arginine kinase has five thiol groups/molecule of mol. wt. 37000 (Virden & Watts, 1966), brain creatine kinase has ten thiol groups (Table 3) and ten half-cystine residues (B. J. Miles & A. R. Thomson, unpublished work) in a molecule of mol. wt. approx. 81000 (the muscle enzyme has eight thiol groups/molecule), and both enzymes require essential thiol groups for enzyme activity (Elodi & Szorenyi, 1956). They also show other points of considerable

C4	Met(Glx,Asx,His)Leu-Gly-Tyr
A1	(Met,Glx,Asx)
A2	(His,Leu)
A3	(Leu,Gly,Tyr)
S1	(Glx,Asx)
S2	(His,Leu)
S3	(Gly,Tyr)
C4	Met-Glu-Asn-His-Leu-Gly-Tyr

Fig. 8. Sequence of peptide C4 of rabbit muscle creatine kinase. →, Edman degradation; ←, carboxypeptidase A. Peptides A1–A3 were from acid hydrolysis (conc. HCl at 40°C for 72 h); peptides S1–S3 were from digestion with subtilisin. Complete hydrolysis with leucine aminopeptidase released glutamic acid and asparagine. Dilute acid hydrolysis (30mm-HCl at 105°C for 8 h) gave a full yield of aspartic acid but no methionine, and hence these residues are not adjacent.

(1) Creatine kinase (rabbit muscle)

Ala-Gly-Pro-His-Phe-Met-Glu-Asn-His-Leu-Gly-Tyr-Val-Leu-Thr-Cys-Pro-Ser-Asn-Leu-Gly-Thr-Gly-Leu-Arg

(2) Creatine kinase (ox muscle)

(Ala,Gly,Pro,His,Phe,Met,Glx,Asx,His,Leu,Gly,Tyr)Val-Leu-Thr-Cys-Pro-Ser-Asn-Leu-Gly-Thr-Gly-Leu-Arg

(3) Creatine kinase (ox brain)

Asn-Tyr-Glu-Phe-Met-Pro-Asn-His-Leu-Gly-Tyr-Ile-Leu-Thr-Cys-Pro-Ser-Asn-Leu-Gly-Thr-Gly-Leu-Arg

(4) Arginine kinase (lobster)

Glu-Thr-Cys-Pro-Thr-Ser-Asn-Leu-Gly-Thr-Val-Arg

Fig. 9. Amino acid sequences around the reactive cysteine residues of creatine kinases and arginine kinase. References: (1) Thomson *et al.* (1964); Mahowald (1965); Eveleigh & Thomson (1965); this paper; (2) Thomson *et al.* (1968a); (3) this paper; (4) Der Terrossian *et al.* (1969).

similarity in mechanism (Virden & Watts, 1966; Smith & Morrison, 1969). Whether or not the essential thiol groups of both enzymes are involved in substrate binding (O'Sullivan, Diefenbach & Cohn, 1966), catalysis (Watts & Rabin, 1962) or maintenance of structure (Kumudavalli *et al.* 1970; Atherton *et al.* 1970a), their modification leads to inactivation.

Der Terrossian *et al.* (1969) have further emphasized the homology between arginine kinase and creatine kinase. They reported the isolation of a tryptic peptide from lobster arginine kinase containing the essential thiol group of the enzyme. This peptide had a sequence very similar to that of the corresponding tryptic peptide of muscle and brain creatine kinases, including the Cys-Pro sequence (Fig. 9). This conservation of structure over a considerable period of evolution also argues for an important functional role for the reactive thiol groups of these enzymes. Arginine kinase is reputed to be the most primitive guanidinophosphotransferase, and it is reasonable to believe that the dimeric enzymes of this group evolved from it by gene duplication and divergent evolution (Watts & Watts, 1968). The closer similarity of brain creatine kinase to arginine kinase in terms of thiol groups when compared with the muscle enzymes may be a reflexion of the more primitive nature of brain creatine kinase (Eppenberger *et al.* 1964) and possibly of a closer evolutionary relationship. Further amino acid sequence studies on the guanidinophosphotransferases would be of great value in unravelling evolutionary pathways.

Very little information on the amino acid sequences is available for phosphotransferases in which groups other than guanidine acts as the phosphoryl group acceptor. Myokinase has two reactive thiol groups/molecule that behave in a similar manner to those of creatine kinase (Mahowald *et al.* 1962). It is noteworthy, however, that these two reactive thiol groups are not identical, and are not related in sequence to those of creatine kinase (Weeds & Noda, 1968). There is also evidence that they may be concerned more with maintenance of the conformation of the active site than with substrate binding or catalysis (Kress, Bono & Noda, 1966; Kress & Noda, 1967).

In view of the complicated kinetics of the phosphotransferases, and the difficulty of interpreting substrate protection experiments (Adams *et al.* 1970), it seems likely that three-dimensional structures will be required before the role of the thiol groups in these enzymes will be understood.

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