The Amino Acid Sequence of the Peptide Containing the Thiol Group of Creatine Kinase from Normal and Dystrophic Chicken Breast Muscle

COMPARISON OF SOME OF THE IMMUNOLOGICAL PROPERTIES OF THE ANTIBODIES DEVELOPED IN RABBITS AGAINST THESE ENZYMES

By BUDDHA P. ROY*

Molecular Enzymology Laboratory, Department of Biochemistry, Medical School, University of Bristol, Bristol BS1 8TD, and Department of Pharmacology, Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K.

(Received 14 November 1973)

The major ^{14}C -labelled peptides from creatine kinase from normal and dystrophic chicken muscle obtained by carboxymethylating the reactive thiol groups with iodo[2- ^{14}C]acetic acid and digestion with trypsin were purified by ion-exchange chromatography on Dowex-50 (X2) and by paper electrophoresis. The chromatographic characteristics of the ^{14}C -labelled peptides, their electrophoretic mobilities at pH 6.5, and their amino acid compositions were identical for the two enzymes. The sequence of amino acids around the essential thiol groups of creatine kinase from normal and dystrophic chicken muscle was shown to be Ile-Leu-Thr-CmCys-Pro-Ser-Asn-Leu-Gly-Thr-Gly-Leu-Arg (CmCys, carboxymethylcysteine). This sequence is almost identical with that for the creatine kinases in human and ox muscle and bovine brain and is very similar to that of arginine kinase from lobster muscle. Antibodies to the enzymes were raised in rabbits and their reaction with the creatine kinase from normal and dystrophic muscles in interfacial, immunodiffusion and immunoelectrophoretic experiments was studied. The cross-reaction between normal muscle creatine kinase and antisera against the dystrophic muscle enzyme (or vice versa) observed by immunodiffusion and by immunoelectrophoretic experiments further suggests that the enzymes from normal and dystrophic chicken muscle are similar in structure. The results of the present study, the identical amino acid sequence of the peptides containing the reactive thiol group from both the normal and dystrophic chicken muscle enzymes and the immunological similarities of the two enzymes are in accord with the similarity of the two enzymes observed by Roy *et al.* (1970).

In recent years the attention of several groups of workers has been focused on the enzyme creatine kinase because of its possible role in normal muscle contraction. The concentration of enzyme is decreased in muscle and greatly increased in the serum of dystrophic patients (Pearce *et al.*, 1964). Serum creatine kinase activities many hundred times that of the normal have been observed in mothers who are carriers of the Duchenne type of dystrophy. Creatine kinase has approx. mol.wt. 81000 (Kuby *et al.*, 1954; Eppenberger *et al.*, 1967; Kumudavalli *et al.*, 1970) and has two reactive thiol groups (Mahowald & Kuby, 1960; Watts *et al.*, 1961; Eppenberger *et al.*, 1967), except in dogfish, where there are four free (Simonarson & Watts, 1972) and a total of about eight thiol groups

per molecule of enzyme. Three electrophoretically distinct isoenzymes, a slow-moving muscle type, a fast-moving brain type and an intermediate hybrid form, have been reported (Eppenberger *et al.*, 1964; Dawson & Kaplan, 1965; Kendrick-Jones & Perry, 1967). The enzyme consists of two subunits, i.e. the muscle type referred to as MM, the brain type known as BB and the intermediate or hybrid form referred to as MB (Dance & Watts, 1962). In dystrophy the muscle creatine kinase pattern reverts to the foetal type, which is electrophoretically similar to the brain type (Miyazaki *et al.*, 1965), but a hybrid form has also been detected in human dystrophic muscle (Jacobs *et al.*, 1969).

Recently the recognition of hereditary myopathies in animals such as mice, chickens, hamsters etc. has provided further opportunity to study this disease (Michelson *et al.*, 1955; Homburger *et al.*, 1963; Rigdon, 1966; Asmundson & Julian, 1956). Hooton & Watts (1966a) reported the presence of an aberrant form of creatine kinase in dystrophic mouse muscle.

* Present address: Protein and Polypeptide Hormone Laboratory, Department of Physiology, Faculty of Medicine, University of Manitoba, Medical College, 770 Bannatyne Avenue, Winnipeg, Manitoba R3E 0W3, Canada.

Comparison of the normal muscle enzyme with dystrophic muscle enzyme made it apparent that the latter had only half the specific activity of the former and contained only one reactive thiol group; moreover a single difference was also found on peptide 'maps' and was attributed to the loss of one reactive cysteine residue. From the difference in the electrophoretic mobilities of the peptides, Hooton & Watts (1966a) suggested that the missing cysteine residue is possibly replaced by glutamic acid.

On these grounds the hypothesis was formed that dystrophic tissue contained a modified creatine kinase under genetic control, this being a possible primary cause of the disease. We may ask whether this defect in dystrophic mouse muscle applies to all animals affected by the disease and is therefore a feature of muscular dystrophy in general. It was decided to investigate this problem in further detail by using New Hampshire chickens with hereditary myopathy. The earlier part of this work has been presented (Roy *et al.*, 1969, 1970; Roy, 1971, 1972) and no significant differences in creatine kinases from normal and dystrophic chicken muscle were observed by various criteria. The present paper is concerned with the amino acid sequence around the reactive thiol groups of normal and dystrophic muscle creatine kinase and serological comparison of the two enzymes by immunodiffusion, immunoelectrophoretic and interfacial tests.

Experimental

Materials

Trypsin was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Carboxypeptidase B (di-isopropylphosphorofluoride-treated) was from Sigma Chemical Co., St. Louis, Mo., U.S.A. Thermolysin was a gift from Dr. J. Williams (University of Bristol, U.K.). Dowex resins were purchased from Bio-Rad AG, Bromley, Kent, U.K. Iodo-[2-¹⁴C]acetic acid was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., diluted two- to four-fold with unlabelled iodoacetic acid and was recrystallized from light petroleum (b.p. 60–80°C). The final specific radioactivity was 8–10 mCi/mmol. Dystrophic and control chickens were kindly supplied by Dr. D. W. Peterson, Department of Poultry Husbandry, University of California, Davis, Calif., U.S.A. All chickens were male and were at least 6 months old.

Creatine kinases from normal and dystrophic muscle

Preparation and assay. Enzymes were prepared from chicken breast muscle by the method of Roy *et al.* (1970), modified from that of Eppenberger *et al.* (1967).

Enzymes were assayed either by the phosphate method of Kuby *et al.* (1954) or spectrophotometrically by the method of Tanzer & Gilvarg (1959). Protein was determined either with the biuret reagent (Gornall *et al.*, 1949), with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as the standard, or from E_{280} values by using E_{280} 8.7 (Kuby *et al.*, 1954).

Treatment of creatine kinase from normal and dystrophic muscle with iodo[2-¹⁴C]acetic acid. Purified enzymes with specific activities of approx. 70 units/mg were labelled with iodo[¹⁴C]acetic acid by the method of Crestfield *et al.* (1963). For the carboxymethylation an enzyme concentration of 50 mg/ml was used. The free thiol groups were first allowed to react with 2 mol of iodo[¹⁴C]acetic acid/mol of enzyme (a mol.wt. of 81000 was used) for 45 min at 30°C with 0.1 M-glycine buffer, adjusted to pH 9.0 with 0.1 M-NaOH solution. A sample from this mixture was exhaustively dialysed against the same buffer. The protein content of the samples was determined from their extinction at 280 nm (Kuby *et al.*, 1954) and by the biuret method (Gornall *et al.*, 1949). The radioactivity in the sample was measured by using a gas flow counter.

From the radioactivity counts of the sample, after making corrections for the efficiency of the instrument (which was 60%) and from the specific radioactivity of the iodo[2-¹⁴C]acetic acid, the extent of iodoacetate incorporation (mol/mol of enzyme) was found to be 1.95 for the normal muscle enzyme and 1.70 for the dystrophic muscle enzyme. No significant enzyme activity remained in the original mixture and solid urea was added to bring the final concentration in the solution to 8 M. The 'buried' thiol groups were then blocked with unlabelled iodoacetic acid by adding a fourfold molar excess of iodoacetic acid to prevent oxidation of any of the thiol groups and to avoid disulphide-exchange reactions. The reaction proceeded for 3 h at 30°C and was terminated by adding 0.1 ml of 0.5 M-2-mercaptoethanol. Urea and other reagents were removed by exhaustive dialysis against dilute aq. NH₃ soln. at pH 9.0; occasional turbidity (0.1% of the total protein) that appeared after dialysis was removed by centrifugation. Under these conditions it is likely that the reaction of the iodoacetic acid is restricted to thiol groups.

Enzyme digestion

The ¹⁴C-labelled carboxymethylated enzyme was digested with trypsin at an enzyme/substrate ratio of 1:50 (w/w) and was either incubated at 37°C in 0.1 M-NH₄HCO₃ for 2 h or titrated at 37°C in a pH-stat with 0.01 M-NaOH at pH 8.0 for 1½–2 h until the uptake of alkali stopped.

Digestion of the purified peptides with thermolysin was carried out in 0.05M-ammonium acetate–0.005M-CaCl₂ buffer, pH 8.5, for 2½ h at 60°C.

For the determination of C-terminal residues the peptides were digested with carboxypeptidase B in 0.05M-(NH₄)₂CO₃ at an enzyme/peptide ratio of 1:50 (w/w) and incubated at 37°C. Samples were taken at different intervals, the amino acids liberated were qualitatively detected by high-voltage paper electrophoresis at pH 2.1 and the composition was confirmed on the amino acid analyser (Ambler, 1967).

Peptide purification

Initial fractionation of the tryptic digest was performed by ion-exchange chromatography on a column of Dowex-50 (X2); the general procedure recommended by Konisberg & Hill (1962) was used. The resin was packed on a 2.5 cm × 100 cm column, water jacketed at 37°C, and was equilibrated with the starting buffer, 0.3M-pyridine formate, pH 3.2. The protein hydrolysate was made 0.3M with respect to pyridine, and then loaded on to the column, and the peptides were eluted with a linear buffer gradient of 1 litre each of 0.3M-pyridine formate (pH 3.2) and 4.5M-pyridine acetate (pH 5.6). Fractions (10 ml) were collected at an approximate flow rate of 50 ml/h. The effluent was monitored by counting radioactivity of samples in a gas-flow counter (Chase, 1960) or was monitored continuously by using a scintillation flow-through cell (Nuclear Enterprises, Edinburgh, U.K.).

Peptides were further purified by high-voltage electrophoresis at pH 6.5, 2.1 or 3.5 by using a Michl (1951) electrophoretic tank and the method of Ambler (1963). The peptides were eluted from the paper with aq. 1% (w/w) NH₃ and dried under vacuum. The cadmium acetate–ninhydrin reagent (Heilmann *et al.*, 1957) was used to stain the guide strip. The radioactive peptide was first detected by radioautography and was later counted for radioactivity. The number of amide groups in peptides was deduced from the electrophoretic mobility of the peptide (Offord, 1966). The mobilities of peptides at pH 6.5 are given with respect to aspartic acid and the sign indicates the nature of the charge,

Amino acid analysis and sequence determination

Peptides were hydrolysed with 0.2 ml of 6M-HCl containing 1 µl of 2-mercaptoethanol in sealed evacuated tubes at 105°C overnight. The hydrolysates were analysed for amino acids by using a Technicon TSM1 amino acid analyser.

Peptide sequencing was carried out by the dansyl–Edman procedure (Gray & Hartley, 1963; Gray, 1967; Hartley, 1970), except for very small amounts of material, where losses in each step of sequencing

were quite high, when the solid-phase automatic sequencer (Laursen, 1971; Laursen *et al.*, 1972) was used at the Max-Planck Institute for Molecular Genetics, Berlin, by kind permission of Professor H. Wittmann. For the latter method, the peptides were treated with 0.50 ml of 20% (v/v) hydrazine at 105°C for 30 min, to deguanidate the arginine residues to yield ornithine residues, the δ-amino groups of which were used to attach the peptide to aminopolystyrene resin in the presence of a coupling agent, *p*-phenyldi-isothiocyanate, and the samples were evaporated to dryness (Laursen, 1971; Laursen *et al.*, 1972). The dried samples (approx. 100 nmol) were redissolved in 0.05 ml of *N*-methylmorpholine–water (1:1, v/v), the solution was adjusted to pH 9.5 with trifluoroacetic acid, and 1 mg (5 µmol) of *p*-phenyldi-isothiocyanate in 0.1 ml of dimethylformamide was added and the tube was heated to 45°C for 30 min. Each peptide was then transferred to a test tube containing 35 mg of peptide resin previously swollen in 0.25 ml of dimethylformamide, which had been stirred for 75 min with 0.1 ml of 50% (w/v) methyl thiocyanate in methyl cyanide and 0.1 ml of sequencing buffer (Laursen, 1971). This buffer consisted of a mixture of *N*-methylmorpholinium trifluoroacetate at pH 8.1 and pyridine (2:3), prepared by adding 3.75 ml of trifluoroacetic acid and 28 ml of *N*-methylmorpholine to 200 ml of water. The resulting solution was 0.25M in the conjugate acid and 1M in *N*-methylmorpholine. The mixture was stirred for another 75 min and, in order to remove excess of reagents, methanol (8 ml) was added to it. The mixture was centrifuged and the solvent was removed by pipette; the process was repeated three times. The resin, to which the peptide was now attached, was dried under vacuum and the residue (about 50 mg) mixed with glass beads (950 mg) to prevent channelling of solvents. The mixture was packed carefully into a degradation reaction column for sequencing.

The peptide degradation was continuously carried out in an automatic sequencer as described by Laursen *et al.* (1972). The radioactive phenylthiohydantoins released from the peptides were identified by the isotope-dilution method (Laursen, 1971; Laursen *et al.*, 1972). Mixtures of unlabelled phenylthiohydantoins corresponding to the amino acid composition of the peptide were added to the radioactive phenylthiohydantoins released and were separated on silica-gel-coated aluminium sheets, identified as u.v.-absorbing spots and counted for radioactivity in scintillation vials (Laursen *et al.*, 1972).

Nomenclature

Tryptic peptides from creatine kinase from normal and dystrophic muscle are designated by the letters N and D respectively. The peptides obtained by

further degradation of tryptic peptides by thermolysin are indicated by T.

Production of antibodies

Antibodies to chicken creatine kinases (normal and dystrophic) were produced in young adult rabbits. A dilution of the enzyme (40mg/100ml) in NaCl (0.9g/100ml) for injection was emulsified with an equal volume of Freund's complete adjuvant, i.e. 4–5mg of heat-killed *Mycobacterium butyricum* mixed in 8.5ml of mineral oil and 1.5ml of Arlacel (Honeywill Atlas Ltd., Carshalton, Surrey, U.K.); the emulsification was carried out until the mixture became milky. A 1ml portion of the emulsion, containing either of the enzymes from normal or dystrophic animals, in Freund's adjuvant was injected into each rabbit subcutaneously below the neck from a sterile syringe and needle. Injections were given once a week over a period of 4 weeks, and thereafter continued for another 2 weeks when necessary. The rabbits were bled from an ear vein after this had been stimulated by rubbing the surface with toluene. The blood was left for 2h at room temperature, and the serum was separated by centrifugation at 3500rev./min for 30min and stored in the deep-freeze (-20°C) until it was used.

Experiments to demonstrate the presence of antibody

Interfacial or ring test. The ring test was carried out at pH 7.4 and 0.9% NaCl was used for dilution of antisera and antigens (i.e. the enzymes). The serum from the immunized rabbits was tested undiluted as well as in dilutions of 1:2, 1:4 and 1:8 (v/v).

Antigens, i.e. the enzymes, were layered carefully into the test tubes above a solution of antibody (or vice versa) by pipetting 0.2ml of the latter solution. These tubes were left for 30min at room temperature before inspection for formation of precipitation rings at interfaces.

Immunodiffusion in agar gel. The antigen used was creatine kinase (from normal and dystrophic muscle) in 0.01M-phosphate-buffered saline (0.9% NaCl), pH 7.4, at a concentration of 1–4mg/ml. Antiserum from rabbits immunized against both creatine kinases was used both diluted and undiluted.

Agar-gel plates were made from 1% agar in 0.1M-sodium phosphate buffer, pH 7.4, as described by Ouchterlony (1949). A pattern of one central well and the peripheral wells was punched in each plate, and the distance between the central well and the peripheral wells varied from 2 to 5mm.

The central well was loaded with antibody solution

Fig. 1. Elution profile of a tryptic digest of creatine kinase from (a) dystrophic and (b) normal chicken breast muscle on a Dowex-50 (X2) column

Of the total radioactivity added to the column, the percentage in each peak for normal (N) and dystrophic (D) peptides is shown; 10ml fractions were collected. For details see the text.

and the peripheral well with antigen solution or vice versa. The plates were then covered and incubated at 37°C in a humid atmosphere for up to 72h (Plate 1).

Immunoelectrophoresis. Slides were thoroughly cleaned, placed on a Shandon tray and coated with a 1% solution of agar in barbitone buffer, pH8.6 (barbitone 7.36g, barbitone sodium 41.2g, water 4 litres; 20ml of 5% (w/v) thymol in propan-2-ol as preservative was added to the mixture). The agar gel set after 10min and immediately the surface was levelled by means of a scraper. The pattern was cut out of the agar with a cutter and trough and the wells were aspirated with a hypodermic needle on a vacuum line. The wells of the slides prepared for immunoelectrophoresis were filled with the enzymes (2–5µl at enzyme concentrations of 4mg/ml). Electrophoresis was carried out with a current of 5 mA at 200V for 3h. The troughs were then filled with antisera and the tray was left overnight for precipitation lines to develop. The slide was thoroughly washed in 0.9% NaCl and later with water and then dried in an incubator (37°C) overnight. This was followed by staining with 0.1% Naphthalene Black for 5–10min, washing with methanol–acetic acid (9:1, v/v) for approx. 15min and drying.

Results

Peptide analysis

The elution profile of the tryptic digest for creatine kinase from normal and dystrophic chicken muscles is shown in Fig. 1. Although they are not exactly identical, there are similarities between the elution profiles; in particular, the major radioactive peptides (normal and dystrophic) were eluted almost at the same places in the column. The total recoveries of the peptides from the Dowex-50 (X2) column,

calculated on the basis of radioactivity, are 96% for the normal and 98% for the dystrophic muscle proteins, of which about 67% of the total radioactivity is present in the normal muscle (N₂, Fig. 1) creatine kinase peptide and 72% in the dystrophic muscle peptide (D₃, Fig. 1). From 357mg of the normal creatine kinase, 0.53µmol of purified peptide (N₂), and from 120mg of dystrophic muscle creatine kinase 0.23µmol of purified peptide (D₃), were obtained after the peptides (N₂ and D₃) from the column were purified electrophoretically at pH6.5, 2.1 and 3.5.

The peptides N₂ and D₃ stained pink with cadmium–ninhydrin, exhibited identical electrophoretic mobilities (0.02 at pH6.5) and were apparently identical in their amino acid composition (Table 1). Seven steps of the dansyl–Edman degradation showed the *N*-terminal sequence of the normal peptide (N₂) to be Ile-Leu-Thr-CmCys*–Pro-Ser-Asx. To obtain further information, the peptide N₂ was subjected to thermolysin digestion and three fractions were obtained at pH6.5, the mobilities of which are shown in Table 2. The mixture of neutral peptides was further purified at pH2.1 and the two fractions obtained were sequenced (Table 2).

Asparagine was confirmed from the mobility of the peptide N₂T_{3.1} at pH6.5. There is no overlap of sequences between peptides N₂T_{3.1} and N₂T_{3.2}, but as the original peptide (N₂) is a product of tryptic digestion it is reasonable to assume that the arginine residue is at the *C*-terminus of peptide N₂. Carboxymethylcysteine as *N*-terminal residue was confirmed by the loss of radioactivity in the remaining peptide after the next step of the dansyl–Edman procedure. The complete sequence of peptide N₂ was therefore Ile-Leu-Thr-CmCys–Pro-Ser-Asn-Leu-Gly-Thr-Gly-Leu-Arg.

* Abbreviation: CmCys, carboxymethylcysteine.

Table 1. Amino acid composition of tryptic peptides from normal and dystrophic muscle creatine kinases labelled with iodo[¹⁴C]acetic acid

The methods used are described in the text.				
Amino acid residue	Dystrophic muscle peptide (D ₃)		Normal muscle peptide (N ₂)	
	Residue content (mol/mol of peptide)	Residue to nearest whole no.	Residue content (mol/mol of peptide)	Residue to nearest whole no.
Arg	0.9	1	1.05	1
Leu	2.8	3	2.65	3
Ile	0.8	1	0.9	1
Thr	1.7	2	1.9	2
CmCys	0.8	1	0.9	1
Asp	0.9	1	1.2	1
Pro	0.8	1	0.9	1
Ser	0.8	1	0.8	1
Gly	1.8	2	1.9	2
Lys	Trace		Trace	

Table 2. *Thermolytic peptides derived from ¹⁴C-labelled tryptic peptides (N₂ and D₃) of creatine kinase from normal and dystrophic chicken muscle*

—————→ Sequence carried out by the dansyl method; ←———— sequence by carboxypeptidase B.			
Peptide	Electrophoretic mobility at pH 6.5 (m)	Composition	Sequence
N ₂ T ₁	+0.65	Leu (0.9), Arg (1.0)	Leu-Arg ————→
N ₂ T ₂	+0.38	Leu (0.9), Thr (0.9) Gly (1.7), Arg (0.9)	Gly-Thr-Gly- ————→ Leu-Arg ← ————→
N ₂ T _{3,1}	-0.40	Ile (1.1), Leu (0.9) Thr (0.8)	Ile-Leu-Thr-CmCys ————→
		CmCys (0.9), Pro (1.0) Ser (0.9), Asp (0.9)	Pro-Ser-Asn ————→
N ₂ T _{3,2}	0	Gly (1.9), Thr (0.7)	Gly-Thr-Gly ————→
D ₃ T ₁	+0.67	Leu (0.9), Arg (0.9)	Leu-Arg ————→
D ₃ T ₂	+0.37	Thr (0.9), Gly (1.8) Leu (1.9), Arg (0.9)	Leu-Gly-Thr-Gly- ————→ Leu-Arg ← ————→
D ₃ T ₃	-0.41	Ile (1.1), Leu (1.0) Thr (1.9), CmCys (0.7) Pro (1.0), Ser (0.8) Asp (1.1)	Ile-Leu-Thr-CmCys ————→ Pro-Ser-Asn ————→

The thermolytic fragments of the dystrophic muscle peptide D₃ were purified at pH6.5. The mobilities of the three peptides were similar to those of the normal peptide N₂. The neutral peptides were further purified at pH2.1, which gave one strong ninhydrin-positive band and one very faint one. The sequences shown in Table 2 are identical with those of the normal muscle material with the exception of the peptide Gly-Thr-Gly. The faint second spot could not be obtained in sufficient amount to permit sequencing by standard methods. The peptide was therefore sequenced in the automated sequencer

as described in the Experimental section. No phenylthiohydantoin were released even after the degradation had been repeated three times more beyond the arginine residue, and the following sequence fitted exactly with the amino acid composition of peptide D₃ (Table 1): Ile-Leu-Thr-CmCys-Pro-Ser-Asn-Leu-Gly-Thr-Gly-Leu-Arg.

No differences in the amino acid sequence of the peptides containing the reactive thiol group of normal (N₂) and dystrophic (D₃) chicken muscle creatine kinase were found. The amino acid sequence around the essential thiol groups of chicken muscle

EXPLANATION OF PLATE I

Two-dimensional immunodiffusion experiments with rabbit antiserum against creatine kinase from normal and dystrophic chicken muscle

The antisera against normal (AN) and dystrophic (AD) creatine kinases were placed in the centre wells. The peripheral wells (1, 2 and 3) contained purified creatine kinase from normal chicken muscle in concentrations of 1, 2 and 4 mg/ml respectively and the remaining wells (4, 5 and 6) contained creatine kinase from dystrophic chicken muscle in concentrations of 1, 2 and 4 mg/ml respectively. Immunodiffusion was carried out for 48 h at 37°C. Before staining with Naphthalene Black the plate was washed in NaCl (0.9%) for 15 h.

creatine kinase (this paper) is the same as in the rabbit, human and ox muscle enzymes (Table 3), except for the replacement of valine by isoleucine, thus conserving the neutral amino acids as found in the brain enzyme (Atherton *et al.*, 1970). By comparing the peptides from similar regions of other muscle creatine kinases (Table 3) it seems likely that cleavage of a Tyr-Ile bond has taken place in chicken muscle creatine kinase, possibly because of chymotryptic activity in the trypsin used. Further sequencing of chicken enzyme may resolve this point.

Demonstration of antibody in serum from immunized rabbits

Interfacial or ring test. Rabbits produced antibodies to the enzymes. A cross-reaction between normal muscle enzyme and antiserum against dystrophic muscle enzymes (or vice versa) was noted.

Immunodiffusion. Precipitation areas developed between the peripheral wells and the central wells. These were first seen after about 24h and were best developed between 48 and 72h, after which they gradually faded away. Usually not more than one precipitation band was seen, except in one experiment where two areas were noticed; in this experiment the antigen was the normal muscle enzyme placed in one of the peripheral wells and anti-(dystrophic muscle enzyme) serum was in the central well. This double arc was not observed when the experiment was repeated with dystrophic muscle enzyme either when anti-(dystrophic muscle enzyme) or anti-(normal muscle enzyme) serum was used. The results of the immunodiffusion experiments (Plate 1) clearly demonstrated the presence of antibodies against both enzymes and showed cross-reaction, which suggests the conformational similarities of the two enzymes.

Immunoelectrophoresis. When the slides were developed, precipitation arcs were noticed with normal and dystrophic muscle enzyme and a cross-reaction between them was noted.

Discussion

The creatine kinases isolated from normal and dystrophic chicken muscle showed similarities in the following parameters, as reported by Roy *et al.* (1970): (1) specific activity; (2) free and total thiol groups (5,5'-dithiobis-2-nitrobenzoic acid-titratable); (3) sedimentation, electrophoretic and kinetic properties; (4) peptide 'maps'; (5) thermal stability.

The results of the present study show identity in the amino acid sequence surrounding the reactive thiol group from the normal and the dystrophic chicken muscle enzyme and similarities in the immunological properties of the two enzymes. In their work with mouse creatine kinase, Hooton & Watts (1966a) isolated the enzyme from the

Table 3. *Comparative acid sequences around the reactive thiol residues of creatine kinases and arginine kinase*

References: (1) Mahowald (1965); Atherton *et al.* (1970); (2) Palmieri *et al.* (1971); Thomson *et al.* (1968); (3) this paper; (4) Atherton *et al.* (1970); (5) der Terossian *et al.* (1969).

(1) Creatine kinase (rabbit muscle)	Ala-Gly-Pro-His-Phe-Met-Glu-Met-Glu-Asn-His-Leu-Gly-Tyr-Val-Leu-Thr-Cys-Pro-Ser-Asn-Leu-Gly-Thr-Gly-Leu-Arg
(2) Creatine kinase (human and ox muscle)	Val-Leu-Thr-Cys-Pro-Ser-Asn-Leu-Gly-Thr-Gly-Leu-Arg
(3) Creatine kinase (chicken muscle)	Ile-Leu-Thr-Cys-Pro-Ser-Asn-Leu-Gly-Thr-Gly-Leu-Arg
(4) 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
(5) Arginine kinase (lobster)	Glu-Thr-Cys-Pro-Thr-Ser-Asn-Leu-Gly-Thr-Val-Arg

dystrophic mouse muscle, preparing it in exactly the same manner as the normal muscle enzyme. They found that the enzyme from dystrophic muscle possessed half the specific activity of the normal enzyme and that the former contained 1.2, whereas the latter had 2, reactive thiol groups/molecule of enzyme. Thomson *et al.* (1968) reported that they had found 1.32 mol of thiol group/mol of enzyme from normal ox brain when no thiol-protecting agent (e.g. dithioerythritol) was used during the purification. They also observed that the instability of this enzyme was specially marked during column chromatography and this resulted in a very poor yield of enzyme with low activity. However, much higher yields and enzymes with higher specific activities were obtained when dithioerythritol was used throughout the process of purification; also the number of essential thiol groups was increased from 1.32 to 1.94 per molecule of enzyme (Atherton *et al.*, 1970). The lack of use of any thiol-protecting agent in the preparation of the mouse enzyme (Hooton & Watts, 1966a) might explain why they obtained less activity and a lower number of reactive thiol groups from the dystrophic muscle enzyme. This fits in with the suggestion by Hooton & Watts (1966b) that the thiol groups of the dystrophic enzyme are more prone to oxidation as an alternative explanation for the missing thiol group in the dystrophic mouse enzyme.

Hooton & Watts (1966a) reported that the tryptic peptide containing one of the reactive thiol groups of the normal mouse muscle enzyme was missing from peptide 'maps' of the dystrophic muscle enzyme, a new peptide being observed. The electrophoretic and chromatographic characteristics of the dystrophic muscle peptide (i.e. the one from which the cysteine residue was missing) were thought to be consistent with the replacements of the cysteine residue by glutamic acid (Hooton & Watts, 1966a), but it is difficult to draw such a conclusion without knowledge of the amino acid composition of the peptide. Further, the replacement by a glutamic acid residue of cysteine would require at least two (and more likely three) nucleotide base changes, which seems rather unlikely to occur (Morgan *et al.*, 1966).

No significant differences were observed in the K_m values for normal and dystrophic chicken (Roy *et al.*, 1970) or mouse (Hooton & Watts, 1966a) enzymes. However, only 50% inactivation with dystrophic mouse enzyme (Roy *et al.*, 1970) was observed when their free thiol groups were blocked with iodoacetate, compared with 100% inactivation for the normal enzymes of both species.

A distinct difference in the inhibition reactions of chicken brain-type and muscle-type enzymes was observed by Hooton (1968), who found that no more than 75% of the enzyme activity of the brain type

could be inactivated by reaction with a 100-fold molar excess of iodoacetate or iodoacetamide. He suggested that possibly this was due to conformational changes in the subunit leading to masking of one of the active thiol groups and resulting in incomplete inactivation. That the rabbit muscle creatine kinase was almost completely insensitive to iodoacetamide in the presence of creatine, Mg^{2+} and ADP was noted by O'Sullivan *et al.* (1966). However, Kumudavalli *et al.* (1970), using human muscle enzyme, reported inhibition (60%) by iodoacetamide in the presence of creatine, Mg^{2+} and ADP. With bovine brain enzyme, Atherton *et al.* (1970) also did not find complete inhibition under certain conditions of enzyme concentration and temperature. Roy *et al.* (1970) suggested that if the thiol groups in the two subunits were not identical and if the reactivity of one of them was modified by subunit interaction, the incompleteness of the inhibition of the chicken enzyme could be explained, and this would also be applicable to the mouse enzyme.

In the present experiments, the extent of iodoacetate incorporation was almost 2 mol/mol of enzyme (for both the normal and dystrophic chicken muscle), which strongly suggests that there are two thiol groups per dimer of the enzymes, assuming that the reaction with iodoacetic acid is restricted to thiol groups. The identical amino acid sequence around the reactive thiol groups of the enzyme from normal and dystrophic chicken muscle and the serological similarities of the two enzymes further suggest that the chemical environment of these thiol groups is very similar in the two enzymes.

It should, however, be noted that muscular dystrophy is a term used for a wide range of muscle defects which differ in detail, and the findings with one species should therefore be extrapolated to another species with extreme caution. However, the results of the present study make it unlikely that a defective creatine kinase (involving the reactive thiol groups) is the primary cause of muscular dystrophy, but the possibility that mouse dystrophy is a different kind of myopathy from that of chicken (Hooton & Watts, 1966a) and that modifications in other parts of the creatine kinase molecule may cause a dysfunction of the muscle cannot be ruled out.

I am indebted to Dr. J. Williams (Bristol) for his encouragement and advice throughout the course of this work, to Professor H. Wittmann (Berlin) for allowing me to use the automated amino acid sequencer and to Mr. W. Krauss for his expert technical assistance. Grateful thanks are due to Professor J. W. Thompson, Dr. J. L. Putman and Dr. C. M. Nicholls for their support at different stages of the work. The co-operation of Dr. Janet Strangeways, Dr. K. Dakshinamurti, Dr. I. Graham and the help of Dr. Gentle and Dr. D. Arnstee with the immunological work is thankfully acknowledged.

Thanks are also due to Mrs. J. Fowler for carrying out the amino acid analyses. The financial assistance (personal grant and expenses) of the Muscular Dystrophy Group of Great Britain and the short-term Fellowship provided by the European Molecular Biology Organization (EMBO) to visit the Max-Planck Institute for Molecular Genetics (Berlin) are gratefully acknowledged.

References

- Ambler, R. P. (1963) *Biochem. J.* **89**, 349–378
 Ambler, R. P. (1967) *Methods Enzymol.* **11**, 155–166
 Asmundson, V. S. & Julian, L. M. (1956) *J. Hered.* **47**, 248–252
 Atherton, R. S., Laws, J. F., Miles, B. L. & Thomson, A. R. (1970) *Biochem. J.* **120**, 589–600
 Chase, G. D. (1960) in *Principles of Radioisotope Methodology*, pp. 128–130, Burgess Publishing Company, Minneapolis, Minnesota
 Crestfield, A. M., Moore, S. & Stein, W. H. (1963) *J. Biol. Chem.* **238**, 622–627
 Dance, N. & Watts, D. C. (1962) *Biochem. J.* **84**, 114–115
 Dawson, D. M. & Kaplan, N. O. (1965) *J. Biol. Chem.* **240**, 3285–3221
 der Terossian, E., Pradel, L. A., Kassab, R. & Thoai, N. V. (1969) *Eur. J. Biochem.* **11**, 482–490
 Eppenberger, H. M., Eppenberger, M., Richterich, R. & Aepli, H. (1964) *Develop. Biol.* **10**, 1–16
 Eppenberger, H. M., Dawson, D. M. & Kaplan, N. O. (1967) *J. Biol. Chem.* **242**, 204–209
 Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 752–766
 Gray, W. R. (1967) *Methods Enzymol.* **11**, 139–151
 Gray, W. R. & Hartley, B. S. (1963) *Biochem. J.* **89**, 379–380
 Hartley, B. S. (1970) *Biochem. J.* **119**, 805–822
 Heilmann, J., Barollier, J. & Watzke, E. (1957) *Hoppe-Seyler's Z. Physiol. Chem.* **309**, 219–220
 Homburger, F., Baker, J. R., Nixon, G. W. & Whitney, R. (1963) *Medna Exp.* **6**, 339–345
 Hooton, B. T. (1968) *Biochemistry* **7**, 2063–2071
 Hooton, B. T. & Watts, D. C. (1966a) *Biochem. J.* **100**, 637–646
 Hooton, B. T. & Watts, D. C. (1966b) *Clin. Chim. Acta* **16**, 173–176
 Jacobs, H. K., Okabe, K., Yue, R., Deutel, H., Ziter, F., Palmieri, R. & Kubby, S. A. (1969) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **23**, 495
 Kendrick-Jones, J. & Perry, S. V. (1967) *Nature (London)* **208**, 1068–1070
 Konisberg, W. & Hill, R. J. (1962) *J. Biol. Chem.* **237**, 2547–2561
 Kubby, S. A., Noda, L. & Lardy, H. A. (1954) *J. Biol. Chem.* **209**, 191–201
 Kumudavalli, I., Moreland, B. H. & Watts, D. C. (1970) *Biochem. J.* **117**, 513–523
 Laursen, R. A. (1971) *Eur. J. Biochem.* **20**, 89–102
 Laursen, R. A., Hor, M. J. & Bonner, A. G. (1972) *FEBS Lett.* **21**, 67–70
 Mahowald, T. A. (1965) *Biochemistry* **4**, 732–740
 Mahowald, T. A. & Kubby, S. A. (1960) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **19**, 46
 Michelson, A. H., Russell, E. S. & Harman, P. J. (1955) *Proc. Nat. Acad. Sci. U.S.A.* **41**, 1079–1084
 Michl, H. (1951) *Montash. Chem.* **82**, 489–493
 Miyazaki, K., Toyada, R., Tomino, H., Yoshimatsa, K. S., Katsunama, N. & Fujino, A. (1965) *Mod. Med. (Osaka)* **20**, 202
 Morgan, A. R., Wells, R. & Khorana, H. G. (1966) *Proc. Nat. Acad. Sci. U.S.A.* **56**, 1899–1906
 Offord, R. E. (1966) *Nature (London)* **211**, 591–593
 O'Sullivan, W. J., Diefenbach, H. & Cohn, M. (1966) *Biochemistry* **5**, 2666–2773
 Ouchterlony, O. (1949) *Acta Pathol. Microbiol. Scand.* **26**, 507–515
 Palmieri, R., Keutel, H., Jacobs, H., Okabe, K., Yue, R., Ziter, F., Tyler, F. & Kubby, S. A. (1971) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **30**, 1255
 Pearce, J. M. S., Pennington, R. J. & Walton, J. N. (1964) *J. Neurol. Neurosurg. Psy.* **27**, 1–4
 Rigdon, R. H. (1966) *Ann. N.Y. Acad. Sci.* **138**, 28–48
 Roy, B. P. (1971) *Abstr. FEBS Meet. 7th* p. 967
 Roy, B. P. (1972) *6th Symposium on Current Research in Muscular Dystrophy*
 Roy, B. P., Laws, J. F. & Thompson, A. R. (1969) *Abstr. FEBS Meet. 6th* p. 849
 Roy, B. P., Laws, J. F. & Thompson, A. R. (1970) *Biochem. J.* **120**, 177–185
 Simonarson, B. & Watts, D. C. (1972) *Biochem. J.* **128**, 1241–1253
 Tanzer, M. L. & Gilvarg (1959) *J. Biol. Chem.* **234**, 3201–3204
 Thomson, A. R., Eveleigh, J. W., Laws, J. F. & Miles, B. J. (1968) in *Biochemical Evolution and Homologous Enzymes* (Thoai, N. V. & Roche, J., eds.), pp. 255–275, Gordon and Breach, New York
 Watts, D. C., Rabin, B. R. & Crook, E. N. (1961) *Biochem. J.* **82**, 412–417