Adenosine 5'-Triphosphate-Creatine Phosphotransferase from Dystrophic Mouse Skeletal Muscle

A GENETIC LESION ASSOCIATED WITH THE CATALYTIC-SITE THIOL GROUP

By B. T. HOOTON AND D. C. WATTS*

Department of Biochemistry, University College London, Gower Street, London, W.C. 1

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1. A column procedure for the purification of creatine kinase from normal and dystrophic mouse muscle is described. 2. The native enzymes are indistinguishable by various physical criteria and have mol.wt. about 80 000. 3. The purified enzyme from dystrophic muscle is only half as active as the normal, contains only one thiol group readily alkylated by iodoacetamide instead of two and has one less free thiol group/mol. 4. Michaelis constants for MgATP and creatine are the same for both preparations. 5. The inhibitor constant for ADP at pH 9.0 is different in the two enzymes and this may account for the different degrees of inhibition observed *in vitro* with the drug Laevadosin. 6. The enzyme from dystrophic muscle is protected by an equilibrium mixture of substrates against inhibition by iodoacetamide to a greater extent than the normal enzyme. 7. 'Fingerprinting' suggests one peptide difference between creatine kinases from normal and dystrophic muscle. 8. The possibility that this finding represents the primary lesion in dystrophy is discussed.

The hereditary myopathies are a group of diseases characterized by a progressive loss of muscular function accompanied by numerous biochemical disorders and in some cases early death (Cooper & Miller, 1962; Pennington, 1963). An important feature of this disease is the liberation of several enzymes from muscle into the blood stream, in particular ATP-creatine phosphotransferase (EC 2.7.3.2), which catalyses the reaction:

The primary cause of the hereditary myopathies is unknown. Although much evidence has been accumulated about changes in the levels of activity of muscle enzymes no serious attempt has hitherto been made to characterize any of the enzymes themselves. It seemed a strong possibility that an enzyme lesion could be a primary cause of hereditary muscular dystrophy and the unique role of creatine kinase in mediating energy production for muscular contraction made it an obvious choice for study.

The present investigation into the structure of creatine kinase from dystrophic muscle was made possible by the availability of a strain of mice (Bar Harbor strain 129) that possess a dystrophy showing some degree of similarity to the human Duchenne

type. The dystrophy is progressive, affecting the lower limbs first, causing similar biochemical lesions and death after about 12 weeks. A major difference in mice is that inheritance is autosomal rather than sex-linked; also, creatine kinase is either not liberated into the blood stream or only in small amounts. However, it was considered that a full investigation of the mouse enzyme would provide useful information about the feasibility of characterizing human muscle enzymes by using even smaller amounts of material than that available from dystrophic mice.

The results suggest that there has been a replacement of one of the essential catalytic thiol groups in the creatine kinase of dystrophic mice by another amino acid. This is supported by qualitative enzyme differences as well as by quantitative differences. The possible importance of a creatine kinase with two fully active catalytic sites in muscle contraction is discussed as well as the possibility that loss of thiol group could be the primary lesion in muscular dystrophy.

MATERIALS AND METHODS

Materials

Buffers. Quartz-redistilled water was used throughout. The pH was measured on a Radiometer Titrator type TTT1c fitted with a scale expander. Borate buffer, pH 8.2 and 10.001, was prepared by titrating 4.326 g. of boric acid in

* Present address: Department of Biochemistry and Chemistry, Guy's Hospital Medical School, London, S.E. 1.

water to pH 8.2 with saturated NaOH at 20°, making up to 5l. and rechecking the pH. Borate buffer, pH 9.0 and I0-028, was prepared as described in *Documenta Geigy* (1956, p. 105). Phosphate buffer, pH 6.4 and I0.1 at 25°, and phosphate buffer, pH 8.0 and I0.05 at 25°, were prepared as described by Datta & Grzybowski (1961).

Chemicals. All reagents were of A.R. grade except where stated. MgSO₄ and creatine were recrystallized from mm-EDTA solution. Trypsin for 'fingerprinting' was salt-free and obtained from Worthington Biochemicals Corp. (Freehold, N.J., U.S.A.). Laevadosin ('for injection'; Calmic Ltd., Crewe, Cheshire) was a gift from Professor J. N. Cummings. 5,5'-Dithiobis-(2-nitrobenzoic acid) was obtained from the Aldrich Chemical Co. Inc. (Milwaukee, Wis., U.S.A.).

Guanidine hydrochloride was prepared from guanidine carbonate by reaction with a slight excess of HCl. The product was evaporated to dryness on a rotary film evaporator, dissolved in a minimum volume of mm-EDTA and crystallized by cooling at -10°. Recrystallization with mm-EDTA was found necessary to achieve a suitably pure product.

Methods

Protein estimation. Unless otherwise stated this was carried out by the method of Gornall, Bardawill & David (1949), with bovine plasma albumin, fraction V (Armour Pharmaceutical Laboratories, Eastbourne, Sussex), as a standard.

Enzyme assay. The phosphorylation of creatine by ATP at pH 9.0 at 37° was followed by titrating the H⁺ ions liberated in the reaction by employing a Radiometer TTT1c titration assembly. The stoichiometry of H⁺ released to phosphate transferred is known to be unity (Mahowald, Noltmann & Kuby, 1962). The standard assay mixture (final volume 5 ml.) was set up in a thermostatically controlled reaction vessel fitted with a magnetic stirrer and contained ATP and MgSO₄ (4 mm each), creatine (40 mm) and, to diminish loss of enzyme activity (Cho, Haslett & Jenden, 1960), mercaptoacetic acid (1 mm) (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.). Approx. 0.06 N-NaOH was used as a titrant and was standardized before use against 15.67 mm-potassium hydrogen phthalate.

With a 4:1 reduction gearbox between the Titrigraph and the 0.5 ml. burette assembly this concentration of alkali gave approx. 0.08 μ equiv. of NaOH/1% of the chart width. The reaction was always started by the addition of enzyme in less than 0.1 ml. to the reaction mixture, which had previously been titrated to pH 9.00 with 2 N-NaOH from a micrometer syringe. In the course of an assay the volume of titrant NaOH added to the reaction volume was never more than 0.01 ml. This dilution was ignored in the determination of initial velocities. Progress curves gave straight lines over 5 min. in the range 0-1 μ g. of creatine kinase/ml. of reaction medium and the initial velocities were proportional to enzyme concentration. The blank titration rate obtained by omitting either the enzyme or ATP was found to be approx. 0.3% of the chart width/min. It was concluded that this was due to uptake of atmospheric CO₂ rather than spontaneous hydrolysis of ATP.

RESULTS

Purification of creatine kinase

Normal (Dy?) and dystrophic (dydy) mice (strain 129 obtained from Rosco B. Jackson Memorial Laboratory, Bar Harbor, Maine, U.S.A.) were obtained by breeding from heterozygotes (Dydy). The offspring of mice that did not produce dystrophic progeny in experimental crosses were assumed to be normal homozygous (DyDy).

For each enzyme preparation batches of six normal and six dystrophic mice were treated in an exactly similar manner. The mice were killed by cervical dislocation, and the back leg and pelvic muscle were rapidly removed and placed in ice-cold 10 mm-potassium chloride. In one experiment the enzyme was purified by the procedure B of Kuby, Noda & Lardy (1954). This procedure was also used to prepare a standard preparation of purified rabbit creatine kinase for comparative purposes. With the small amounts of muscle available (approx. 10 g.) this fractionation method was found to be generally

Table 1. *Specific activities of several preparations of creatine kinases from normal and dystrophic mouse muscle*

Specific activity is defined as μ equiv. of H⁺ released/min./mg. of protein under the conditions described in the text.

Initial treatment of animals and extraction media	Specific activity	
	Enzyme from normal muscle	Enzyme from dystrophic muscle
Sample I: muscle stored for 8 weeks at -30° before extraction in 10 mm-KCl	170	80
Sample II: fresh muscle extracted with 10 mm-KCl	160	80
Sample III: fresh muscle extracted with 10 mm-KCl in mm-EDTA, pH 7.2	240	110
Sample IV: prepared by the method B of Kuby <i>et al.</i> (1954)	80	40

unsuitable and resulted in heavy losses of material and serious inactivation. In view of this a new purification procedure using column chromatography was developed. Preliminary treatment of the muscle varied slightly (Table 1). The muscle was homogenized with 50 ml. of 10 mM-potassium chloride and centrifuged at 34 000g for 10 min. at 4°. The sediment was re-extracted twice more in the same way with further 50 ml. lots of 10 mM-potassium chloride. The combined supernatants were then dialysed against two 1 l. changes of borate buffer, pH 8.2 and 1.0-0.01, at 4° under reduced pressure (550 mm. Hg) until the volume was reduced to 6 ml. The concentrated extract was frozen (-15°) overnight and then thawed and dialysed for a further 20 hr. against 2 l. of the same buffer. The precipitate was removed by centrifugation and the supernatant fractionated on a DEAE-cellulose column.

DEAE-cellulose was well washed with mM EDTA, pH 7.0, and packed into a column (2.5 cm. × 70 cm.). The column was equilibrated with approx. 3 l. of borate buffer, pH 8.2 and 1.0-0.01, at 4°. Elution was carried out by using a sodium chloride gradient, which was achieved by running 20 mM-sodium chloride in borate buffer, pH 8.2 and 1.0-0.01, into 1.5 l. of the same buffer in a magnetically stirred constant-volume mixing chamber. The mixing chamber was connected to the top of the column; 5 ml. fractions were collected at a rate of 20 ml./hr. Effluent was monitored with a Büchler automatic effluent analyser. When necessary, protein con-

centration in the effluent was measured at 280 mμ. Fig. 1 shows that the elution profiles of the enzymes from normal and dystrophic muscle are substantially the same. The fractions showing the highest specific activity were combined and concentrated by dialysis against borate buffer, pH 9.0 and 1.0-0.0058, as described above. The purified concentrated enzyme represented a yield of 50-60% of that applied to the column. The specific activities of several preparations are shown in Table 1. Specific activity is defined as the rate of H⁺ liberation/min./mg. of enzyme protein in the presence of creatine (40 mM), ATP and MgSO₄ (4 mM each) and mercaptoacetic acid (1 mM) at 37° and pH 9.00 (Mahowald *et al.* 1962).

Purity and characterization of the creatine kinases

Starch-gel electrophoresis. Preparations were analysed by horizontal starch-gel electrophoresis with the discontinuous buffer system of Poulik (1957) modified as described by Virden & Watts (1964). The enzymes from normal and dystrophic muscle showed identical electrophoretic mobilities, which were the same as that found for the purified rabbit enzyme. Staining with Amido Black revealed in both a single strong protein band, associated with enzyme activity, and a faint, more electropositive, component running just in front of it. A similar pattern was obtained with purified rabbit creatine kinase.

Fig. 1. Purification of creatine kinases from normal and dystrophic mouse skeletal muscle on DEAE-cellulose columns. The experiments were carried out as described in the text. (a) Dystrophic muscle extract; (b) normal muscle extract. —, $E_{280\text{ m}\mu}$; ---, ionic strength of the eluent; ●, total activity units/fraction (arbitrary units); ○, specific activity.

With a continuous phosphate buffer system at pH 6.0 (Gammack, Huehns, Shooter & Gerald, 1960) the electrophoretic mobility of the enzyme was small, but again only one major component was revealed and the enzymes from both normal and dystrophic muscle showed the same electrophoretic mobility.

Sucrose-density-gradient centrifugation. This was carried out in the SW 39 rotor of a Spinco model L2 preparative ultracentrifuge. Linear 5–20% (w/v) sucrose gradients (4.4 ml.) were prepared and used as described by Virden, Watts, Watts, Gammack & Raper (1966). Fig. 2 shows that the creatine kinases of normal and dystrophic mouse skeletal muscle sediment to the same extent as the purified rabbit enzyme. From this it was inferred that both mouse enzymes have the same mol.wt. (81 000) as that reported for the rabbit kinase (Noda, Kuby & Lardy, 1954). The symmetry of the protein peaks suggests that the enzyme from dystrophic muscle is only slightly less homogeneous than the normal mouse enzyme.

Sedimentation analysis. A single moving boundary was apparent in the schlieren patterns

Fig. 2. Analysis of creatine kinases (sample I) from rabbit, normal mouse and dystrophic mouse muscle on linear sucrose gradients. The conditions were as described in the text. (a) Enzyme from normal mouse muscle; (b) enzyme from dystrophic mouse muscle; (c) enzyme from rabbit muscle. —, $E_{280 \text{ m}\mu}$; $\circ$, activity/fraction (in arbitrary units).

obtained during sedimentation of the creatine kinases from normal and dystrophic muscle in a Spinco model E analytical ultracentrifuge. Both enzymes gave $S_{\text{obs}} 3.7\text{S}$ and there was no evidence of heterogeneity.

'Fingerprinting'. The procedure was essentially that of Ingram (1958). Creatine kinase (1 mg./ml.) was denatured by heating (at 90° for 2 min.) and digested with trypsin (0.02 mg./ml.) at pH 8.5 and 35° in the Radiometer pH-stat without buffer. The final volume was 4 ml. and the pH was maintained with 0.05N-ammonia. The digestion was continued for 2 hr. and the ammonia titres were the same for creatine kinases from normal and dystrophic muscle. The digested enzyme was freeze-dried once, dissolved in deionized water and samples (1–2 mg.) of both enzymes were applied side by side to Whatmann 3MM paper. Electrophoresis (200 v/cm. width of paper for 45 min.) was carried out in pyridine-acetic acid-water (10:0.4:90, by vol.) buffer, pH 6.4 (Michl, 1951). The paper was dried (for 2 hr. at room temperature) and either stained directly or the strips containing the peptides were cut out (1.5 cm. wide) and sewn on to another sheet of Whatmann 3MM paper for descending chromatography in pyridine-3-methylbutan-1-ol-water (7:7:6, by vol.) for 16 hr. side by side in the same tank (Baglioni, 1961). The peptide spots were detected with a cadmium-ninhydrin stain based on that of Barrolier (1955).

An electrophoretogram achieved by running digests of creatine kinase from normal and dystrophic muscle simultaneously side by side affords a useful additional criterion of the relative purity of the two preparations. Fig. 3 shows that the appearances of the two digests (equivalent to 1 mg. each of creatine kinase) are almost identical, indicating a comparable degree of purity. Comparison of the 'fingerprints' of normal mouse and rabbit creatine kinases (Fig. 4) reveals a general similarity of pattern but with a number of clear-cut differences. Between the enzymes of normal and dystrophic mice only one clear peptide difference could be resolved. This peptide, which has the same electrophoretic and chromatographic properties as glutamic acid under the conditions used, can also be seen in the one-dimensional electrophoretogram (Fig. 3).

Kinetic properties of creatine kinases of normal and dystrophic mouse muscle

Michaelis constants. The K_m for creatine was determined by measuring enzyme initial velocities with the ATP-magnesium sulphate concentration held constant at 4 mM and varying the creatine concentration between 3 and 30 mM. For ATP the creatine concentration was held at 40 mM and the

Fig. 3. Electrophoretograms of tryptic digests of creatine kinases (sample III) from normal and dystrophic mouse muscle at pH 6.4. The conditions are as described in the text. A, Digest of enzyme from normal mouse muscle; B, digest of enzyme from dystrophic mouse muscle (equivalent to a loading of 1 mg. of native enzyme each); M₁, aspartic acid; M₂, glutamic acid; M₃ and M₃, neutral amino acids; M₄, histidine; M₅, arginine; M₆, lysine; B₂ is the peptide only found in the digest of enzyme from dystrophic muscle that corresponds to M₂. The low intensity of B₂ is thought to be because it is formed from only one of the two sub-units of creatine kinase from dystrophic muscle.

Fig. 4. Tracings of 'fingerprints' of tryptic digests of creatine kinases (sample III). The method was as described in the text. (a) Digest of enzyme from normal mouse muscle; (b) digest of enzyme from dystrophic mouse muscle; (c) digest of enzyme from rabbit muscle.

Table 2. Comparison of the kinetic constants of creatine kinases (sample I) from normal and dystrophic and rabbit muscle at 37° and pH 9.0

	Constant (moles l. ⁻¹)		
	Enzyme from normal mouse muscle	Enzyme from dystrophic mouse muscle	Enzyme from rabbit muscle (Kuby <i>et al.</i> 1954)
K_m (ATP)	9×10^{-4}	8×10^{-4}	5×10^{-4}
K_m (creatine)	1.8×10^{-2}	1.7×10^{-2}	1.6×10^{-2}
K_i (ADP)*	1.5×10^{-3}	2.2×10^{-3}	—

* The ADP concentrations used were 1 mM and 2 mM.

the Lineweaver & Burk (1934) plots are listed in Table 2.

Inhibition by Laevadosin and ADP. The drug Laevadosin, which appears to be a mixture of purine and pyrimidine nucleosides, GMP and ATP, together with sodium metabisulphite and *p*-chloro-*m*-cresol, has been used in the therapy of progressive muscular dystrophy. Fig. 5 shows the effect of adding increasing quantities of the drug to a standard assay mixture. The normal creatine kinase was inhibited to a greater extent than the enzyme from dystrophic muscle. An attempt was made to check these effects by using the standard phosphate assay procedure to assay for labile phosphocreatine formed in the reaction (Watts, Rabin & Crook, 1961). This revealed that the Laevadosin contained a large amount of free phosphate, suggesting that perhaps ADP, formed as a hydrolysis product of ATP, was the active in-

Bioch. 1966, 100

ATP concentration varied between 0.25 and 2.5 mM. The Mg²⁺/ATP ratio was maintained at 1:1 in all experiments. The Michaelis constants derived from

21

Fig. 5. Inhibition of creatine kinases (sample II) from dystrophic and normal mouse muscle by Laevadosin at 37°. Inhibition was measured by determining the initial velocity of H^+ production in the presence of increasing amounts of Laevadosin in a standard assay mixture containing ATP (4 mM), $MgSO_4$ (4 mM), creatine (40 mM) and mercaptoacetate (1 mM) at pH 9.0. ●, Enzyme from normal muscle; ○, enzyme from dystrophic muscle.

Fig. 6. Inhibition of creatine kinases (sample I) from normal and dystrophic mouse muscle by iodoacetamide in the absence and presence of an equilibrium mixture of substrates at 25°. The reaction mixture (final volume 0.5 ml.) contained iodoacetamide (0.2 mM), KNO_3 (120 mM), borate buffer, pH 9.0 and I 0.005, and in the absence of substrates either (Δ) enzyme from normal muscle (2.5 μM) or (○) enzyme from dystrophic muscle (2.5 μM). In the experiments on substrate protection ATP and $MgSO_4$ (each 0.66 M) and creatine (8.3 mM) were added to the reaction mixture and pre-incubated (5 min.) with either (▲) enzyme from normal muscle (2.5 μM) or (●) enzyme from dystrophic muscle (2.5 μM). Samples were removed at time-intervals and added to 10 mM-mercaptoacetate, pH 9.0, before assay for residual enzyme activity in a standard assay mixture.

inhibitor. Accordingly, the effect of several fixed ADP concentrations on the initial velocity of enzyme catalysis was investigated under the conditions described above for determining the Michaelis

Fig. 7. Progress curves for the reaction of creatine kinases (sample I) from normal and dystrophic mouse muscle with iodoacetamide at 25°. The reaction was continuously followed by monitoring the production of I^- ions by using a silver-silver iodide electrode. The 4 ml. reaction volume contained KI (1 μM), Tween 80 (0.02%, v/v), borate buffer, pH 9.0 and I 0.012, iodoacetamide (0.1 mM) and either (○) enzyme from normal muscle (5.3 μM) or (●) enzyme from dystrophic muscle (4 μM). The reaction was started by the addition of iodoacetamide.

constant for creatine. The Lineweaver-Burk plots obtained were of the non-competitive type for both enzyme preparations and the inhibition constants for ADP are listed in Table 2.

Inhibition by iodoacetamide. Creatine kinases from normal and dystrophic mouse muscle were incubated with 0.2 mM-iodoacetamide at 25° and at time-intervals samples were transferred to 10 mM-mercaptoacetate, pH 9.0, which effectively prevented any further reaction with the enzyme thiol groups, and then assayed for enzyme activity. Fig. 6 shows that, on the basis of percentage activity loss, both the enzymes from normal and dystrophic muscle were inactivated at the same rate. However, if substrates were added to the reaction medium before the addition of iodoacetamide, so that the transphosphorylation went to equilibrium, the progress curves for activity loss obtained were different for the two preparations (Fig. 6).

Progress curves for the alkylation of the enzymes from normal and dystrophic muscle by iodoacetamide, obtained by following the iodide produced in the reaction with a silver-silver iodide electrode (Watts *et al.* 1961), reveal that the normal enzyme contains two reactive thiol groups/mol. whereas that from the dystrophic muscle has one (Fig. 7). Table 3 lists the apparent second-order velocity constants for these reactions.

Reaction with 5,5'-dithiobis-(2-nitrobenzoic acid). The reaction of this thiol reagent with both enzymes was investigated in the absence and presence of guanidine hydrochloride in the manner described by Ellman (1959). With the native enzymes the stoichiometry was found to agree with that ob-

Table 3. *Apparent second-order velocity constants for the reaction of iodoacetamide with the thiol groups of creatine kinases from normal and dystrophic mouse and rabbit muscle at 25°*

Method of measurement	K (l. mole ⁻¹ min. ⁻¹)		
	Enzyme from normal mouse muscle	Enzyme from dystrophic mouse muscle	Enzyme from rabbit muscle (D. C. Watts, unpublished work)
By loss of enzyme activity (Fig. 6)	520	550	600 (at 20°)
By loss of enzyme activity in the presence of an equilibrium mixture of substrates (Fig. 6)	220	115	
By the rate of iodide liberation (Fig. 7)	440	440	815
By the rate of iodide liberation in the presence of an equilibrium mixture of substrates	—	—	349
Percentage protection			
By loss of enzyme activity in the presence of an equilibrium mixture of substrates	57.7	79.1	—
By the rate of iodide liberation in the presence of an equilibrium mixture of substrates	—	—	57.2

Table 4. *Number of thiol groups in creatine kinases from normal and dystrophic mouse and rabbit muscle*

Conditions for the reaction with iodoacetamide are given in Fig. 7. The reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (0.66 mM) was carried out in phosphate buffer, pH 8.0 and 1.0 M. The extent of reaction was calculated assuming a molar extinction coefficient of 13 600 for the 2-nitro-5-thiobenzoate ion measured at 420 m μ . For the determination of the total thiol groups present 6 M-guanidine hydrochloride was incorporated into the reaction mixture.

	Enzyme from normal mouse muscle	Enzyme from dystrophic mouse muscle	Difference (normal—dystrophic)	Enzyme from rabbit muscle
Active-site thiol groups:				
(1) by DTNB	2.1	1.2	0.9	—
(2) by iodoacetamide	1.8	0.9	0.9	2
				(D. C. Watts, unpublished work)
Total thiol groups: by DTNB	6.8	5.5	1.3	6.7
				(Thomson, Eveleigh & Miles, 1964)

tained with iodoacetamide (Table 4). In the presence of guanidine hydrochloride an additional four to five thiol groups/mol. reacted for both enzymes (Table 4).

DISCUSSION

The study of mouse creatine kinase was made difficult by the small amounts of muscle available and by the problem of obtaining enzyme preparations of comparable purity from dystrophic and normal skeletal muscle, tissues that show considerable biochemical dissimilarities (Cooper & Miller, 1962; Pennington, 1963). The column fractionation procedure finally adopted gave good yields and specific activities with the normal mouse enzyme similar to those quoted by Mahowald *et al.* (1962)

for the rabbit enzyme (Table 1). When the enzymes from normal and dystrophic muscle were prepared in an exactly similar manner with the same batches of reagents the creatine kinase from dystrophic muscle was always found to possess a specific activity half that of the normal (Table 1), even after rechromatography. It would be possible to explain this finding if 50% of the preparation from dystrophic muscle was a protein contaminant not found in the normal preparation. However, only traces of impurity were indicated by starch-gel electrophoresis, density - gradient centrifugation (Fig. 2) and ultracentrifugal analysis.

Differences revealing species variation between normal mouse and rabbit kinases were found only by 'fingerprinting' (cf. Figs. 4a and 4c). Dance &

Watts (1962) showed that the number of peptides obtained after tryptic digestion was approximately half that expected from the known content of lysine plus arginine. Although variation in particular peptide spots in the 'fingerprint' of the mouse enzyme suggests a number of amino acid differences between the creatine kinases of the two species, the occurrence of a similar number of peptides in both makes unlikely the presence of any major contaminating protein in the mouse preparations. It also suggests that, like the rabbit enzyme, mouse creatine kinase has a dimeric structure.

The creatine kinase from dystrophic muscle is indistinguishable from the normal mouse enzyme and the rabbit enzyme by physical criteria. However, its specific activity is only half that of the normal mouse enzyme and investigation of the enzymic and chemical properties revealed a number of differences that suggest that one of the two essential catalytic thiol groups (Watts, Rabin & Crook, 1961, 1962) is missing. Thus the kinase from dystrophic muscle contains only one essential thiol group/mol. in the reaction of the enzyme with iodoacetamide (Fig. 7) or with 5,5'-dithiobis-(2-nitrobenzoic acid) (Table 4). The reaction of the total thiol groups of creatine kinase with 5,5'-dithiobis-(2-nitrobenzoic acid) in the presence of guanidine hydrochloride (Table 4) also shows the disappearance of only one thiol group/mol., indicating that the loss has not resulted from the formation of an internal disulphide bond. This suggests that the cysteine at the catalytic site has been replaced by another amino acid, which would be compatible with the hereditary nature of the disease. Since the electrophoretic mobility of the enzyme from dystrophic muscle on starch gel at pH 8.5 is unchanged this would have to be either aspartate or glutamate. Watts (1964) has shown that the essential thiol groups contribute negative charges to the enzyme at pH 8.5, since blocking with iodoacetamide decreases the electrophoretic mobility but iodoacetate does not. The additional peptide detected by 'fingerprinting' (Fig. 4) had the mobility in both dimensions of glutamic acid. However, as glutamic acid could not be released from the denatured enzyme by tryptic digestion unless the trypsin used was contaminated with chymotrypsin (Thomson *et al.* 1964), more evidence is required before this finding can be accepted.

The kinetic data are compatible with the finding of one defective catalytic site/enzyme molecule and suggest that, in addition, the change is great enough to modify slightly the properties of the remaining catalytic site. Although the enzymes from normal and dystrophic muscle have identical Michaelis constants for ATP and creatine at pH 9.0 (Table 2), the use of ADP as a non-competitive inhibitor for creatine yielded a small but significant difference

in the inhibition constant (Table 2). The difference in protection against iodoacetamide inhibition by substrate equilibrium mixtures (Fig. 6) is also in accord with these data, since ADP is again involved. This suggests that the modifying effect of the inactive site on the active catalytic site may be primarily associated with nucleotide binding. These data are also important because they reveal differences between the two enzymes that do not depend on the absolute enzyme concentration.

This could explain our finding that Laevadosin inhibited creatine kinases from both normal and dystrophic muscle in the forward direction but to different extents (Fig. 5). Laevadosin is generally considered to be of rather doubtful value in the treatment of human muscular dystrophy (Pearce *et al.* 1964). The concentration used in the present work to inhibit creatine kinase was very high. At physiological concentrations the effect was not detectable, so that the present data should not be considered to have clinical significance.

In summary, our studies suggest that the creatine kinase of dystrophic mouse muscle differs from that of normal muscle by having, on average, half of its catalytic sites rendered inactive as a result of a mutation of the essential thiol group, possibly, although by no means certainly, to glutamate. Watts *et al.* (1962) found a preparation of creatine kinase from rabbit muscle that, like the enzyme from the dystrophic mouse, contained only one active catalytic site/mol., but, in contrast, it contained two reactive thiol groups/mol. although with different reactivities. At the time it was not known whether this unusual rabbit enzyme represented a hereditary defect or an isolation artifact. In the light of the present work the former now seems more likely.

Studies on the ontogeny of chicken and rat creatine kinases (Eppenberger, Eppenberger, Richterich & Aepli, 1964) and hybridization experiments by Dawson, Eppenberger & Kaplan (1965) have shown that they consist of separable sub-units. One explanation of the present finding that creatine kinase from dystrophic muscle contains a mixture of active and inactive sub-units is that the two sub-units forming a creatine kinase molecule are not absolutely identical and are coded by separate sections of DNA. This conclusion is compatible with the findings of Olson & Kuby (1964) that the rabbit enzyme has only one C-terminus/mol. of mol.wt. 81 000 and of Noltmann, Mahowald & Kuby (1962) that odd numbers of several amino acids occur in its composition.

The question then arises as to whether the two types of sub-unit combine randomly when they are synthesized or whether each enzyme molecule is composed of one sub-unit coded by each gene. If the former were true the kinase from dystrophic muscle would contain a mixture of fully active, half-active

and inactive enzyme molecules. However, this possibility is not supported by the iodoacetamide inhibition findings (Fig. 6). To cause the observed difference in substrate protection the half-active molecules, which would contain 50% of the total active sub-units in the enzyme from dystrophic muscle, would have to be completely protected by the substrate equilibrium mixture as the rate constant under these conditions is almost exactly half that of the normal (Table 3). Such a large change in the protection by substrates seems improbable in the light of Table 2, which shows no significant difference in the Michaelis constants for ATP and creatine. The more plausible inference is that each enzyme molecule from dystrophic muscle is composed of one active and one inactive sub-unit and the change in substrate protection (Fig. 6) is the direct result of the altered properties of the inactive sub-unit influencing each active sub-unit.

An alternative to the suggestion that the inactive sub-unit could arise directly as a result of a mutation in the structural gene is that a lesion in the protein-synthesis mechanism that is only manifested in certain situations could result in the wrong amino acid being inserted into one sub-unit but not into the other. Smoller & Fineberg (1965) have produced evidence that the myosin from dystrophic mouse muscle has an abnormal amino acid composition. Differences in the primary structure of two separate proteins are only compatible with a simple Mendelian pattern of inheritance if the primary lesion occurs in the protein-synthesis mechanism.

It is pertinent to ask whether a lesion in creatine kinase alone is sufficient to account completely for muscular dystrophy in mice. A lowering of normal creatine kinase concentration to half might impair muscular activity but might not be expected to destroy it completely unless it was also involved in the contractile mechanism. Yagi & Noda (1960) have shown that creatine kinase transfers phosphate from phosphocreatine to ADP bound to myofibrils, and Yagi & Mase (1962) have produced evidence to suggest that creatine kinase is bound close to the adenosine-triphosphatase site of myosin A with a dissociation constant of $6 \times 10^{-8} \text{ M}$. This is compatible with the finding by Perry (1954) that ATP produced through the creatine-kinase reaction reacts much more efficiently than ATP itself on the shortening of myofibrils, and that by Davies (1965) that fluorodinitrobenzene-poisoned muscle after stimulation contained ATP that could only be utilized for muscle contraction if a 10 min. rest period was allowed for some diffusion process to occur. Carlson & Siger (1960) estimated that in a single isometric twitch of iodoacetate-nitrogen-poisoned muscle 2-3 mol. of phosphocreatine was broken down/myosin mol. Thus there is ample evidence to suggest that, although phospho-

creatine is not the primary source of energy for muscular contraction (Cain & Davies, 1960), creatine kinase participates intimately in the catalytic mechanism *in vivo*. It may be that both catalytic sites on the kinase are necessary for normal muscle function, so that the loss of one catalytic site/mol. could have a disadvantageous effect out of proportion to the decrease in enzyme activity. The resultant inactivity would then result in muscular atrophy and the biochemical events found in common with disuse atrophy (MacCamman, 1963).

It is tempting to extrapolate these findings to human muscular dystrophy. Work is in progress to determine whether or not a similar lesion can be detected in human creatine kinase.

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