Adenosine Triphosphate-Creatine Phosphotransferase from Ox Brain

2. PROPERTIES AND FUNCTION*

By T. WOOD

Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, London, S.E. 5

(Received 11 April 1963)

The properties and structure of adenosine triphosphate-creatine phosphotransferase (EC 2.7.3.2) from muscle have been extensively investigated in recent years, with a view to elucidating the mechanism of catalysis and the function of the enzyme *in vivo* (for a review, see Kuby & Noltmann, 1962, pp. 515–596). However, apart from the preliminary investigation by Narayanaswami (1952), little attention appears to have been paid to the enzyme from cerebral tissue.

The enzyme from brain is known (Narayanaswami, 1952) to have an absolute requirement for magnesium or other bivalent cations, and to catalyse the reversible reaction:

From the nomenclature of Kuby, Noda & Lardy (1954a), the reaction proceeding from left to right will be referred to as the 'forward reaction' and that proceeding from right to left will be called the 'reverse reaction'. The 'forward reaction' predominates at pH 9 and the 'reverse reaction' at pH 7.

Since the purification of the enzyme from ox brain (Wood, 1963) its properties have been investigated and compared with the properties of the phosphotransferase of muscle. The effects on the reaction catalysed by the cerebral enzyme of changes in temperature, pH, substrate concentration and metal activator concentration, and maximal rates of phosphocreatine breakdown and resynthesis, have been compared with the rates measured *in vivo*.

MATERIALS

Enzyme. The enzyme used was peak I of the preparation described by Wood (1963) and had specific activity 73 units/mg. One unit of enzyme is defined as that amount [per ml. of reaction mixture, containing initially ATP (1 mM), creatine (24 mM), magnesium acetate (6 mM) and freshly prepared sodium thioglycollate (1 mM), at 30° and pH 9.0 (96 mM-glycine-NaOH buffer)] that will catalyse the phosphotransferase reaction with an apparent second-order velocity constant, K' , of 1.0 (Noda, Kuby & Lardy, 1955; Wood, 1963).

Substrates. Crystalline phosphocreatine disodium hydrate AMP, ADP and ATP were Sigma Chemical Co. products (St Louis, Mo., U.S.A.). Creatine was reagent grade (Hopkin and Williams Ltd., Chadwell Heath, Essex) recrystallized from water. Magnesium acetate was Hopkin and Williams Ltd. A.R. grade.

Activators and inhibitors. Sodium thioglycollate was Hopkin and Williams Ltd. reagent grade, recrystallized from 60% alcohol after treatment with decolorizing charcoal. 2-Mercaptoethanol was obtained from L. Light and Co. Ltd. (Colnbrook, Bucks.). *p*-Chloromercuribenzoic acid was a Sigma Chemical Co. product.

Buffers. Tris was Sigma Chemical Co. 121 grade, and glycylglycine was from the same source. Histidine and glycine were A.R. grade (British Drug Houses Ltd., Poole, Dorset). Imidazole was obtained from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. *N*-Ethylmorpholine was obtained from L. Light and Co. Ltd. and was redistilled at atmospheric pressure.

METHODS

Determination of initial reaction velocities of the forward reaction. The rate of reaction was followed by measuring the phosphocreatine formed from creatine and ATP. The phosphocreatine was determined as inorganic phosphate after hydrolysis with perchloric acid-ammonium molybdate, as described by Kuby *et al.* (1954a). Incubation mixtures (10 ml.) were prepared in a 30 ml. B19 standard ground-glass-joint test tube. The enzyme was diluted with a 4 mM solution of the buffer used and, in some experiments, bovine serum albumin was added with the enzyme to a final concentration of 5 μ g./ml. to stabilize the enzyme at the high dilutions used.

The substrate mixture was incubated for 10–15 min. in a water bath maintained at constant temperature ($\pm 0.1^\circ$) by a Technetemp thermostatic control unit [Technetemp (Cambridge) Ltd., Duxford, Cambs.]. The reaction was begun by adding 1.00 ml. of the enzyme from a 1 ml. tuberculin syringe. A 'zero-time' sample was withdrawn within 10 sec. and added to 5 ml. of the acid-molybdate mixture. Further 1.00 ml. samples were withdrawn by syringe at 2, 4, 6 and 8 min. The time-courses of the reactions were plotted and usually were found to be linear up to 8 min. Initial velocities were obtained from the slope of the curve at the origin.

Determination of initial reaction velocities of the reverse reaction. The rate of reaction was followed by measuring the creatine formed from ADP and phosphocreatine. The enzyme was inhibited by *p*-chloromercuribenzoic acid as described by Rosenberg & Ennor (1955). Incubation

* Part 1: Wood (1963).

mixtures were prepared usually from a stock mixture of magnesium acetate and buffer (glycine-NaOH, glycylglycine-NaOH or *N*-ethylmorpholine acetic acid), and suitable volumes of freshly prepared sodium phosphocreatine in 4 mM-glycine buffer, pH 9, ADP and 20 mM-sodium thioglycollate, pH 7.0, to give a volume of 9.00 ml. The enzyme was diluted in a 4 mM solution of the buffer, albumin added and the reaction was started by adding 1.00 ml. of the enzyme, as described above. Samples (1.00 ml.) were withdrawn at 0, 2, 4, 6 and 8 min. and injected into 10 ml. volumetric flasks containing 0.5 ml. of 200 mM-EDTA (dipotassium salt), 0.8 ml. of 2.5 mM-*p*-chloromercuribenzoic acid and 0.2 ml. of 2*N*-NaOH. Precautions were taken to prevent transfer of inhibitors to the reaction mixture.

After mixing, either the reagents for the estimation of creatine were added immediately or the flask was kept in ice until it was convenient to do so. Keeping in ice for up to 4 hr. did not produce any further breakdown of phosphocreatine.

Determination of creatine. The creatine in the flasks was determined by the method of Eggleton, Elsdon & Gough (1943). Since from run to run there was some slight variation in the depth of colour with standard amounts of creatine, and because the buffers used in the enzyme reaction mixture tended to interfere with colour development, standard amounts of creatine were always run at the same time as the enzyme samples. For this purpose a standard solution of creatine together with 1 ml. of a 100 mM solution of the buffer were added to inhibitors in a 10 ml. volumetric flask and the colour was allowed to develop.

Measurement of the inhibition of development of creatine colour. Portions (1 ml.) of substances likely to inhibit colour development were added, at the appropriate dilutions, to 10 ml. flasks containing 0.3 μ mole of creatine. The colour was allowed to develop and compared with that from a similar flask containing creatine alone; there was presumed to be no interference when the difference in *E* was less than 2%.

Construction of the pH-stability curve. Buffer solutions (0.2M) were prepared to cover the following ranges: pH 4.0-6.0, acetic acid-NaOH; pH 6.0-8.0, KH_2PO_4 - Na_2HPO_4 ; pH 9.0-11.8, glycine-NaOH. A portion (0.2 ml.)

of enzyme solution (11 mg. of protein/ml., sp. activity 15 units/mg.) was mixed with an equal volume of the buffer and placed in ice for 2 hr. For the mixture about pH 13, an equal volume of 2*N*-NaOH was added. At the end of the incubation period a sample of the enzyme was withdrawn, diluted with 4 mM-glycine and assayed immediately at pH 9.0.

Variation of reaction velocities with varying substrate concentrations. (i) Forward reaction: initial reaction velocities were measured in reaction mixtures containing variable amounts of ATP and magnesium acetate such that the ratio magnesium/ATP was 1. Similarly, the ATP concentration was kept constant at 4 mM, the magnesium concentration at 6 mM, and reaction velocities were measured over a range of creatine concentrations.

(ii) Reverse reaction: initial velocities were determined at pH 7.9, the pH optimum for the brain enzyme. With ADP as the variable substrate, phosphocreatine was maintained at 12 mM and magnesium acetate at 6 mM. When phosphocreatine was varied, reaction mixtures contained 2 mM-ADP and 6 mM-magnesium acetate.

Measurement of pH. Measurements were made on a model 23A pH-meter manufactured by Electronic Instruments Ltd., Richmond, Surrey.

Ultracentrifuge measurements. These were performed in a Spinco model E analytical ultracentrifuge with a 12 mm. $2\frac{1}{2}^\circ$ double sector cell. One sector contained 4.65 mg. of the enzyme/ml. of 0.1M-glycine-NaOH, pH 9.0, and the other sector contained the same solution with the presence of freshly prepared 20 mM-sodium thioglycollate. A sedimentation-velocity run was carried out at 420 40 rev./min. and 2° , with schlieren optics.

RESULTS

Assay of creatine. A plot of *E* at 525 $m\mu$ against the concentration of creatine gave a straight line through the origin for amounts of creatine ranging from 0 to 0.5 μ mole of creatine/10 ml. volume of final coloured solution, determined in the presence of the enzyme-inhibitor mixture but in the absence of interfering substances. Substances without effect, and those found to interfere with the colour development, are listed in Table 1. The interference by 1 mM-sodium thioglycollate was completely eliminated after reaction with the *p*-chloromercuribenzoic acid in the inhibitor mixture, as described by Ennor & Stocken (1948).

Tyrosine and tryptophan content. The determination was made on two separate preparations of the enzyme by the spectrophotometric method of Beaven & Holliday (1952). The results, together with the corresponding data for the rabbit-muscle enzyme, are given in Table 2. Both sets of values are in close agreement, indicating that there are seven tryptophan and 21 tyrosine residues/molecule, and these proportions are very similar to the seven tryptophan and 19 tyrosine residues/molecule found by Noltmann, Mahowald & Kuby (1962) from an amino acid analysis of the enzyme from rabbit muscle.

Table 1. *Substances interfering with the diacetyl colour reaction for creatine*

The compounds were tested by adding a known amount to a 10 ml. volumetric flask containing 0.3 μ mole of creatine, before the development of colour. The concentrations quoted are those in 1 ml. of the sample added to the flask. Experimental details are given in the text.

Interfering compounds	Concn. (mM)	Non-interfering compounds	Concn. (mM)
Sodium thioglycollate	1	Imidazole	100
Glycylglycine	120	<i>N</i> -Ethylmorpholine	100
Histidine	100	1 ml. of substrate mixture for reverse reaction, without enzyme or thioglycollate	—
Tris-acetate	100	1.5 ml. of enzyme-inhibitor mixture (see text)	—
Glycine	100		

Table 2. Tyrosine and tryptophan contents of adenosine triphosphate-creatine phosphotransferase from ox brain and from rabbit muscle

	Tryptophan (residues/mole)	Tyrosine (residues/mole)	Moles of tyrosine
			Moles of tryptophan
Brain enzyme (spectrophotometric method)*			
Sample 1	7.3	20.6	2.82
Sample 2	7.2	20.9	2.80
Muscle enzyme (spectrophotometric method)†	10	17	1.7
Muscle enzyme (by amino acid analysis)‡	7	19	2.7

* Calculated as described by Beaven & Holliday (1952).

† Da Costa & Friedburg (1960).

‡ Noltmann, Mahowald & Kuby (1962).

Reaction with AMP as the phosphoryl acceptor.

An incubation mixture was prepared containing 12 mM-phosphocreatine, 8 mM-magnesium acetate, 100 mM-*N*-ethylmorpholine-acetic acid buffer, pH 7.0, and the ADP usually present was replaced by 1 mM-AMP. No formation of creatine could be detected after addition of enzyme and incubation at 30°.

Reaction rate and enzyme concentration. The rate of the forward reaction at pH 9.0 was determined over a 20-fold range of enzyme concentration, and found to vary linearly with the concentration.

Effect of pH on the stability of the enzyme. The enzyme was found to be completely stable for 2 hr. at 0° over the range pH 5.5–11.8. On either side of these limits the enzyme was rapidly inactivated (Fig. 1). The lower limit, pH 5.5, was the pH chosen for elution of the enzyme from a diethylaminoethyl-Sephadex column during its preparation (Wood, 1963), and in no instance was loss of activity observed under these conditions.

Variation of reaction velocities with pH. Initial velocities at fixed concentrations of substrates were found to vary with pH (Fig. 2) in an analogous fashion to that observed by Kuby, Noda & Lardy (1954b) under similar conditions. The optimum pH for the forward reaction was at 9.0, the same as for the muscle enzyme, and at this pH the observed velocity in glycine buffer was close to the maximal velocity calculated from a Lineweaver & Burk (1934) plot. The same difference between the velocities at pH 9.0 in glycine and in glycyglycine buffer, found by Kuby *et al.* (1954b), was also observed. In the reverse reaction, the optimum pH was at 7.9 compared with 7.0 for the muscle enzyme, and at the former pH the velocity was only about half the maximal velocity derived from a Lineweaver-Burk plot, indicating that at the concentrations of substrates used the enzyme was still not saturated. A comparison of the phosphocreatine concentration (12 mM) with the Michaelis constant for phosphocreatine (K_m 10 mM, Table 5) confirms this.

Position of equilibrium. This was calculated from the phosphocreatine formed in the presence of an

Fig. 1. Stability of the enzyme at 0° and various pH values. Samples of an enzyme preparation of specific activity 15 units/mg. in 0.1M-buffer of the required pH were kept for 2 hr. in melting ice before 8000-fold dilution in 4 mM-glycine, pH 9.0, and assay at pH 9.0. Buffers: x, acetic acid-NaOH; O, phosphate; ●, glycine-NaOH; ▲, N-NaOH.

Fig. 2. Effect of pH on initial velocities of the forward and reverse reactions. (A) Forward reaction: 24 mM-creatine, 1 mM-ATP, 1 mM-sodium thioglycollate, 6 mM-magnesium acetate, enzyme 0.38 μ g./ml., glycyglycine-NaOH or glycine-NaOH buffer (100 mM). Temperature 30°. The pH indicated is that of the reaction mixture measured at the end of the run. The maximum velocity of the forward reaction at pH 9.0 was 0.063 μ mole/min./ μ g. (B) Reverse reaction: 12 mM-phosphocreatine, 1 mM-ADP, 1 mM-sodium thioglycollate, 8 mM-magnesium acetate, enzyme 0.143 μ g./ml., 120 mM-glycyglycine-NaOH buffer. Temperature 30°. Maximum velocity of reverse reaction in glycyglycine buffer at pH 7.9 was 0.140 μ mole/min./ μ g.

excess of enzyme added to an incubating mixture containing initially creatine (24 mM), ATP (1 mM), magnesium acetate (6 mM) and glycylglycine-NaOH buffer (100 mM), pH 7.0. A value 1×10^{-3} was obtained for the apparent equilibrium constant at 30°, which may be compared with the value 9.5×10^{-4} obtained by Noda, Kuby & Lardy (1954) in glycylglycine-NaOH buffer (100 mM), pH 7.4 at 30°, in the presence of 6–10 mM-Mg²⁺ ions. At 37° and pH 7.0 an apparent equilibrium constant 7.5×10^{-4} was measured with the brain enzyme. At

a final pH 9.2, in glycine-NaOH buffer (100 mM), with initially creatine (24 mM), ATP (1 mM) and magnesium acetate (6 mM), an apparent equilibrium constant 0.17 was obtained at 30°, which may be compared with a value 0.21–0.36 determined by the above authors in glycine-NaOH (100 mM), pH 8.9 at 30°, at a concentration of 6 mM-Mg²⁺ ions.

Activation of the enzyme. Preliminary studies during the purification of the enzyme showed that it could be activated by thiol compounds at all stages of purification (Wood, 1963). A fresh potassium chloride extract of brain showed only a small (20–40 %) increase in activity when assayed with 1 mM-sodium thioglycollate added to the reaction mixture. On storage at 5°, the activity, assayed in the absence of thioglycollate, was found to decrease progressively until, after 9 days, one preparation showed no activity at all, although full activity was restored in the presence of 1 mM-thioglycollate (Table 3). The progressive decline in activity could be prevented by storing the frozen solution at –20°, but loss occurred if the solution was subsequently thawed and stored at 5°.

Preincubation of the partially inactivated enzyme with 5 mM-thioglycollate and subsequent assay after a 100-fold dilution, in a medium without added thioglycollate, gave an activity slightly higher than that of the same preparation, without preincubation, assayed in the presence of thioglycollate (Table 4). Preincubation at 30° alone was also found to activate the enzyme a little. Surprisingly enough, storage at –20° in the frozen state was found to result in an increase in the activity assayed in the presence of thioglycollate.

Table 3. *Effect of storage on the activity of adenosine triphosphate-creatine phosphotransferase in a potassium chloride extract of ox brain*

Ox brain was extracted with 10 mM-KCl + 1 mM-EDTA as described by Wood (1963). Assays were performed in the presence and in the absence of 1 mM-sodium thioglycollate. Values are uncorrected for the small amount of adenosine-triphosphatase activity present during the first few days.

Treatment of extract	Activity (units/ml.)	
	Without thioglycollate	With thioglycollate
Freshly prepared	7.1	11.7
1 day at 5°	2.9	7.9
4 days at 5°	1.0	8.8
5 days at 5°	1.3	7.5
9 days at 5°	0.0	3.2*
	0.0	9.8
7 days at –20°	11.0	11.0
7 days at –20° and then	5.6	7.9*
1 day at 5°	5.6	10.8

* Assayed in the presence of 0.1 mM-sodium thioglycollate.

Table 4. *Effects of various treatments on the activity of a number of fractions obtained during the purification of adenosine triphosphate-creatine phosphotransferase*

Units of enzyme activity and the preparation of the various fractions were as described by Wood (1963). All measurements were made on fractions obtained during subsequent stages of a single enzyme preparation, except the magnesium acetate extract marked by an asterisk, which was from a different preparation. 'Aged' thioglycollate had been stored at 5° for 2 months and no longer smelled of mercaptans.

Fraction	Activity (units/ml.)		Treatment
	Without thioglycollate	With thioglycollate	
Initial extract, freshly prepared	15.0	16.2	None
Initial extract, stored for 5 days at 5°, then 1 day at –20°	0.6	10.0	None
	2.4	—	Preincubated for 15 min. at 30°, alone
	11.5	12.4	Preincubated for 15 min. at 30°, in 5 mM-thioglycollate
Magnesium acetate extract	0.0	86.4	None
Magnesium acetate extract*	105	15	'Aged' thioglycollate used in assay
1.75–2.4M-Ammonium sulphate fraction freshly prepared	—	267	None
	—	312	Preincubated for 15 min. at 30° in 5 mM-thioglycollate
1.75–2.4M-Ammonium sulphate fraction, stored 8 days at –20°	—	420	None
Pure enzyme, freshly prepared	132	166	None

* See protocol above.

Activation by thioglycollate was also observed when the enzyme reaction was followed in the reverse direction (Fig. 3).

Inactivation by 'aged' thioglycollate. On a number of occasions the addition to the reaction medium of old solutions of thioglycollate (stored in the refrigerator at 5°) was found to give rise to inhibition rather than activation (Table 3). Thus the presence of a 2-month-old sample of thioglycollate at 0.1 mM yielded a progressively increasing inactivation of the enzyme during an incubation for 8 min., and 1 mM concentration produced almost complete inactivation after 6 min. (Fig. 3).

Effect of temperature on reaction rates. The initial velocities of the forward and reverse reactions were measured at 20°, 30° and 37°, at concentrations of reactants similar to those expected to occur *in vivo*. [Forward reaction: creatine (10 mM), ATP (2 mM), magnesium acetate (6 mM), sodium thioglycollate (1 mM), glycine-NaOH buffer, pH 9.0 (100 mM). Reverse reaction; phosphocreatine (3 mM), ADP (1 mM), magnesium acetate (6 mM), sodium thioglycollate (1 mM), *N*-ethylmorpholine-acetic acid buffer, pH 7.9 (100 mM).] At 37° the time-course of the reaction indicated that some inactivation of the

enzyme was taking place during the incubation. However, in later experiments at this temperature this was decreased to a low value by the inclusion in the reaction mixture of bovine serum albumin as a protective agent. Apparent activation energies were calculated from linear Arrhenius plots. For the forward reaction, a value 10.4 ± 1.0 kcal./mole was obtained and 11.0 ± 2.0 kcal./mole for the reverse reaction. These values may be compared with the corresponding 11.2 ± 2 and 13.6 ± 3 kcal./mole obtained by Kuby *et al.* (1954b) with the muscle enzyme under somewhat similar conditions. It should be pointed out that these apparent activation energies describe the effect of temperature on both K_m and V_{max} for the reaction.

Variation of reaction velocity with magnesium concentration. Initial velocities of the forward reaction at pH 9.0 were determined in a reaction mixture containing ATP (1 mM) and varying concentrations of magnesium acetate. The curve obtained (Fig. 4A) did not show the normal rectangular hyperbolic relation between cofactor and velocity but had the same shape as that

Fig. 3. Inhibition by 'aged' sodium thioglycollate. Incubation mixtures for the reverse reaction contained phosphocreatine (1.2 mM), ADP (1 mM), magnesium acetate (6 mM), *N*-ethylmorpholine-acetic acid buffer (100 mM), pH 7.9, enzyme, 2 μ g./ml., bovine serum albumin, 10 μ g./ml. and the following concentrations of sodium thioglycollate: ●, none; ○, 1 mM (from a freshly prepared stock solution); ▲, 1 mM (from a stock solution 2 months old); △, 0.1 mM (from a stock solution 2 months old). Temperature 30°.

Fig. 4. Effect of magnesium concentration on the initial velocities of the forward and reverse reactions. (A) Forward reaction: incubation mixtures contained ATP (1 mM), creatine (24 mM), sodium thioglycollate (1 mM), enzyme, 0.41 μ g./ml., glycine-NaOH buffer (100 mM), pH 9.0, and varying concentrations of magnesium acetate. Temperature 30°. (B) Reverse reaction: incubation mixtures contained ADP (1 mM), phosphocreatine (10 mM), sodium thioglycollate (1 mM), enzyme, 0.58 μ g./ml., *N*-ethylmorpholine-acetic acid buffer (100 mM), pH 7.9, and varying concentrations of magnesium acetate. Temperature 30°.

obtained with the muscle enzyme (Kuby *et al.* 1954*b*), except that the peak was located at ratio magnesium/ATP 0.5 instead of at 1.0, and the asymptotic value of the velocity was only some 60 % of the peak value. The position of this peak for the brain enzyme was confirmed in three experiments with two separate preparations of the enzyme. In the reverse reaction at pH 7.9 (Fig. 4*B*) the shape of the curve was very similar to that obtained with the muscle enzyme (Nihei, Noda & Morales, 1961), approaching a maximum at a point corresponding to a ratio magnesium/ADP somewhat greater than 1.

Variation of reaction velocities with substrate concentrations. Values for K_m and V_{max} were calculated from the slopes and intercepts of Lineweaver-Burk plots, which were linear (Figs. 5 and 6). Values of these quantities and the corresponding values for the muscle enzyme are listed in Table 5.

Study in the ultracentrifuge. In each sector the material sedimented as a single, symmetrical peak and the two peaks could be superimposed. The sedimentation coefficients corrected to 20° in water were 4.65s and 4.66s. No evidence was obtained for dissociation of the enzyme in the presence of thioglycollate.

Fig. 5. Lineweaver-Burk plots for the forward reaction. ○, [ATP] varied: incubation mixtures contained creatine (24 mm), sodium thioglycollate (1 mm), magnesium acetate (concentration equal to the ATP concentration), glycine-NaOH buffer (100 mm), pH 9.0, enzyme, 0.407 μg./ml. Temperature 30°. ●, [Creatine] varied: incubation mixtures contained ATP (4 mm), magnesium acetate (6 mm), sodium thioglycollate (1 mm), glycine-NaOH buffer (60 mm), pH 9.0, enzyme, 0.58 μg./ml. Temperature 30°.

Fig. 6. Lineweaver-Burk plots for the reverse reaction. ○, [ADP] varied (right-hand ordinate): incubation mixture contained phosphocreatine (12 mm), magnesium acetate (6 mm), sodium thioglycollate (1 mm), *N*-ethylmorpholine-acetic acid buffer (100 mm), pH 7.9, enzyme, 0.29 μg./ml. Temperature 30°. ●, [Phosphocreatine] varied (left-hand ordinate): incubation mixtures contained ADP (2 mm), magnesium acetate (6 mm), sodium thioglycollate (1 mm), *N*-ethylmorpholine-acetic acid buffer (100 mm), pH 7.9, enzyme, 0.29 μg./ml. Temperature 30°.

Table 5. *Michaelis constants and maximal velocities of adenosine triphosphate-creatine phosphotransferase from brain and from muscle*

Values for the Michaelis constant (K_m) and the maximal velocity were all determined from Lineweaver-Burk plots. Figures for the muscle enzyme are from Kuby *et al.* (1954*b*), and values for the brain enzyme were calculated from Figs. 5 and 6. All values refer to a temperature of 30°.

Variable substrate	Brain enzyme		Muscle enzyme	
	K_m (mole/l.)	V_{max} (μmole/ min./μg.)	K_m (mole/l.)	V_{max} (μmoles/ min./μg.)
Reverse reaction, pH 7.9 (pH 7.0 for muscle enzyme)				
Phosphocreatine	1×10^{-2}	0.20	5×10^{-3}	1.1
ADP	6.5×10^{-4}	0.27	8×10^{-4}	1.1*
Forward reaction, pH 9.0				
ATP and Mg ²⁺ ions	5.4×10^{-4}	0.059	5×10^{-4}	0.18†
Creatine	3.3×10^{-3}	0.064	1.6×10^{-2}	0.18

* Calculated assuming that V_{max} was the same as when phosphocreatine was varied.
† Calculated assuming that V_{max} was the same as when creatine was varied.

DISCUSSION

Narayanaswami (1952) reported that a brain creatine phosphokinase was able to transfer a phosphoryl group from phosphocreatine to both AMP and ADP, but suggested that AMP may act as an acceptor only via the mediation of myokinase (EC 2.7.4.3). In the present investigation with the pure enzyme no reaction was observed with AMP as an acceptor, confirming the observations of Oliver (1955) who obtained the same result with a crude preparation from brain.

The properties of the enzyme from ox brain, in general, were similar to those of the enzyme from rabbit muscle reported by Kuby and his fellow workers (Kuby *et al.* 1954*b*; Noda *et al.* 1954); however, a detailed comparison of the two enzymes revealed certain marked differences in stability and catalytic behaviour. The pH-rate curves (Fig. 2) for the forward and reverse reactions were similar in shape and appearance to those of the muscle enzyme, save that the brain enzyme in glycylglycine buffer had a pH optimum for the reverse reaction of 7.9, compared with a pH optimum of 7.0 for the muscle enzyme with magnesium as an activating ion (Kuby *et al.* 1954*b*) and 7.2 with calcium as the activating ion (Ennor & Rosenberg, 1954). Noda, Nihei & Morales (1960) and Nihei *et al.* (1961) have shown that the apparent K_m values for the muscle enzyme are not appreciably affected by pH over the range 6.4–8.9, whereas V_{max} changes with change in pH. It would therefore appear reasonable to regard the changes in rate obtained in the present experiments as due to changes in V_{max} over the range pH 7.0–9.0. The differences in pH optima between the brain and muscle enzymes therefore probably reflect different effects of changes in pH on the ionization of their enzyme-substrate complexes. Interactions with the buffer may also be involved, since Kuby *et al.* (1954*b*) have demonstrated with the muscle enzyme a pronounced shift of the pH optimum of the reverse reaction, from pH 7.0 in glycylglycine, β -glycerophosphate and tris-sulphate, to pH 6.0 in histidine-hydrochloride.

A plot of relative velocity against pH (Fig. 2) analogous to that of Nihei *et al.* (1961) yielded two sigmoid curves crossing near their points of inflexion at pH 8.3. As suggested by Nihei *et al.* (1961) this point could represent the pK of a dissociating group on the enzyme-substrate complex, possibly an amino group. The enzyme was found to be stable over 8 min. when the measurements were made at 30° in glycine-sodium hydroxide buffer in the range pH 9.0–9.8, and the fall in rate above pH 9.0 may be attributed to the ionization of a thiol group at the active site as suggested by Kuby & Noltmann (1962, p. 561).

The sedimentation coefficients (Wood, 1963) and the tyrosine and tryptophan content of the enzymes from muscle and from brain are almost identical. However, the differences in electrophoretic mobilities at pH 8.6 and pH 9.0 reported previously (Wood, 1963) indicate a difference in the number of charged groups per molecule, and any similarity in size and amino acid composition does not appear to extend to the tertiary structures since the brain enzyme is more liable to inactivation by heat and by organic solvents (Wood, 1963) and is less sensitive to the effects of pH than the muscle enzyme, although a direct comparison of the pH-stability curves of the two enzymes may be invalidated by the difference in temperatures at which measurements were performed.

The activity of the enzyme, determined in the absence of thioglycollate, was found to undergo quite rapid changes on storage, presumably due to aerial oxidation of essential thiol groups required for activity, since loss of activity could be reversed completely by incubation with thioglycollate and other thiols (Wood, 1963) or partially by incubation alone. Contradictory reports (Kuby *et al.* 1954*a*; Ennor & Rosenberg, 1954; Oliver, 1955; Cho, Haslett & Jenden, 1960; Mahowald, Noltmann & Kuby, 1962) have appeared on the activation of the muscle enzyme by thiols and the phenomenon does not seem to have been easy to observe. By contrast, complete inactivation and reactivation of concentrated preparations of the partially purified brain enzyme has been noted on several occasions. Other evidence for the lability of essential thiol groups is provided by the observation that 'aged' thioglycollate added to a reaction mixture progressively inactivated the enzyme.

Values of K_m and V_{max} .

Kuby *et al.* (1954*b*) stated that in their experiments creatine, at a constant concentration of 24 mM, was not held at 'enzyme saturation concentrations' when the ADP concentration was varied, and advanced this as an explanation for the lack of agreement between the maximal velocities obtained from the Lineweaver-Burk plots when creatine and ATP concentrations were each varied independently. The Lineweaver-Burk plots obtained with the brain enzyme for the forward and reverse reactions at 30° showed a rather closer agreement of the intercepts than did the corresponding plots for the muscle enzyme obtained by Kuby *et al.* (1954*b*), presumably, for the forward reaction, because the K_m of the brain enzyme for creatine is much smaller than that of the muscle enzyme and, at the concentration of creatine used, the enzyme was 'saturated'. Conversely, since the K_m of the brain enzyme for phosphorylcreatine is twice that of the muscle enzyme, the 12 mM concen-

tration used in the experiments where the ADP concentration was varied was less than the 'enzyme saturation concentration'. However, the maximal velocity measured in these experiments was somewhat greater than when phosphocreatine was the variable substrate.

In their comprehensive and detailed discussion of the kinetics of the ATP-creatine-phosphotransferase reaction, Kuby & Noltmann (1962) concluded that the Michaelis constant for each of the substrates bound to the enzyme approximates to the dissociation constant for the ternary enzyme-metal-substrate complex involving that substrate. To quote: 'These data support the view that a "quasi-equilibrium" mechanism exists for ATP-creatine transphosphorylase' (Kuby & Noltmann, 1962, p. 579). In the light of this conclusion, a comparison of K_m for creatine and ATP (the magnesium concentration varied simultaneously) for the brain enzyme, with those for the muscle enzyme, leads to the deduction that the affinity for ATP (or the active species of ATP under the identical conditions used) of the two enzymes is about the same, and that the brain enzyme has a greater affinity for creatine than has the muscle enzyme. A comparison of K_m for phosphocreatine shows that, conversely, the affinity of the brain enzyme for this substrate is less than that of the muscle enzyme.

The two values for V_{max} , calculated from Lineweaver-Burk plots of the forward reaction showed good agreement, as did those for the reverse reaction. However, the values obtained were only about one-third of the corresponding values of the maximal velocities of the muscle enzyme of a somewhat lower specific activity (54 units/mg.). The reason for this remains obscure but a possible explanation may lie in the differing effects of magnesium on the brain and muscle enzymes at the concentration used (6 mM).

Effect on the reaction of variation in magnesium concentration

The curves relating reaction velocities and magnesium concentration were of the same shape as those obtained with the muscle enzyme, at the same concentrations of ATP and ADP, by Kuby *et al.* (1954b). In the forward reaction, from the fact that over a range of ATP concentrations the peaks of the curves always lay at a point corresponding to a ratio total magnesium/total ATP 1, they concluded that a Mg-ATP complex was the 'true substrate' for the enzyme. Other workers have attributed the shape of the curve obtained in the forward reaction to the inhibitory effect of the SO_4^{2-} anion introduced with the added magnesium (Noda *et al.* 1960; Nihei *et al.* 1961). This explanation cannot be used to explain the similar shape of the curve for the brain enzyme, since magnesium

was added as the acetate, and the same authors (Noda *et al.* 1960) have shown that at pH 9.0 the inhibitory effect and degree of association of this anion with magnesium is very small. For the brain enzyme, the rate of the forward reaction fell markedly as the ratio total magnesium/total ATP increased above 1 and reached an asymptotic value 40% below the rate of the peak, compared with an asymptotic value only 20% below the peak height with the muscle enzyme.

The peak for the brain enzyme was located at a ratio total magnesium/total ATP 0.5. If we follow the argument of Kuby *et al.* (1954b) mentioned above, this would indicate the species $Mg[ATP]_2$ to be the 'true substrate' for the enzyme. If we reject this idea (see Kuby & Noltmann, 1962, p. 570, for a full discussion) then a ratio 0.5 probably indicates a higher affinity for magnesium of the brain enzyme than of the muscle enzyme. The decline of the rate at ratios greater than 1 may be explained, as suggested by Kuby & Noltmann (1962, p. 573), by an increased formation of the complex $Mg_2[ATP]$ with a consequent reduction of the concentration of the 'true substrate' $Mg[ATP]^{2-}$.

Calculated rates of the forward and reverse reactions under conditions assumed to exist in vivo

Ox brain was found to contain 24 ± 11 units of enzyme/g. of tissue (Wood, 1963) and guinea-pig brain amounts of the order of 60 units/g. From these figures, the specific activity of the enzyme (73 units/mg. of protein; Wood, 1963) and the data presented here it is possible to calculate the maximal rates of the enzymic reaction under conditions *in vivo*, provided that an estimate can be made of the pH and likely concentrations of substrates within the cell. For the latter purpose it will be assumed that a content of 1 μ mole/g. of tissue of a substrate will approximate to a concentration of 1 mM within the cell. Thus a value of 10 μ moles/g. was found by Thomas (1956) for the total creatine content of slices of guinea-pig cortex fixed immediately after removal from the animal, and the following values were found by Minard & Davis (1962) for the phosphorylated intermediates of normal rat brain: phosphocreatine, 3.5 μ moles/g.; ATP, 2 μ moles/g.; ADP, 0.2 μ mole/g. The free magnesium content of the tissue is unknown, but the total magnesium content of rat brain was determined by Ames & Nesbitt (1958), who found 6.8 μ moles/g. of tissue. The intracellular pH has been discussed by Caldwell (1956), who concluded that the normal intracellular pH of nerve cells and muscle fibres was 6.8-7.0. For the purposes of the calculations, an intracellular pH 7.0 was assumed for guinea-pig cerebral tissue, and the rate of the reverse reaction (phosphocreatine breakdown) at 37° and substrate concentrations of 3 mM-phospho-

creatine, 1 mM-ADP and 6 mM-magnesium was computed. Similarly the maximal rate of the forward reaction (phosphocreatine resynthesis) was calculated at pH 7.0 at 37° and concentrations of 10 mM-creatine, 2 mM-ATP and 6 mM-magnesium. Corrections were made for the change in rate with temperature from the data obtained in this investigation. The correction for the change in rate with pH assumes that there was no change in the shape of the pH-rate curve over the range 30–37°. The intermediate steps in the calculation and the final rates are given in Table 6.

The maximal calculated rate of phosphocreatine breakdown of 6900 μ moles/hr./g. of tissue may be compared with the observed maximal rate of 1400 μ moles/hr./g. of tissue in slices of guinea-pig cerebral cortex subjected to electrical stimulation (Heald, 1954), and the observed rate of resynthesis of 150 μ moles/hr./g. of tissue with the calculated maximal rate of 200 μ moles/hr./g. of tissue. It should be noted that in the calculation of maximal rates, no correction has been made for the inhibiting effect of the products of the reaction in either direction; however, initially these effects will be small and are neglected here. A small increase in pH, from 7.0 to 7.5, can almost double the rate of phosphocreatine resynthesis.

Equilibrium constant and amounts of phosphorylated intermediates in vivo

The apparent equilibrium constant determined during this work agreed with that determined by Noda *et al.* (1954) at 30°; in addition the value of the constant at pH 7.0 and 37° was obtained (conditions that were not used by these authors).

The only rapid reaction *in vivo* in which creatine and phosphocreatine are known to participate is that catalysed by ATP-creatine phosphotransferase.

Consequently these two compounds are not located in the middle of a chain of enzymic reactions but are situated at a 'dead end', serving to buffer the steady-state concentrations of ADP and ATP, substances which lie on numerous other reaction pathways (McIlwain, 1959). The respiratory rate of normal cat brain is of the order of 120 μ moles of oxygen/g./hr. (Geiger & Magnes, 1947); assuming a ratio P/O 3, that the guinea-pig brain has a similar respiratory rate to that of the cat and that the majority of energy-consuming reactions are mediated by ATP, this represents a turnover of 720 μ moles of ATP/g./hr. in the steady state for guinea-pig brain. It was calculated earlier that there is enough ATP-creatine phosphotransferase in guinea-pig brain to catalyse phosphocreatine breakdown at 6900 μ moles/g./hr. and resynthesis at 200 μ moles/g./hr. Therefore in the normal guinea-pig brain the phosphotransferase reaction would be expected to be at, or near, equilibrium and the concentrations of phosphocreatine and creatine should be related to the steady-state concentrations of ADP and ATP by the equilibrium constant of the phosphotransferase reaction.

In an attempt to verify this, Minard & Davis (1962) used a very efficient rapid freezing method to fix rat brain and calculated the function:

$$K'_{CK} = \frac{[ADP] \times [\text{phosphocreatine}]}{[ATP] \times [\text{creatine}]}$$

The substances in brackets represent the amounts present in μ moles/g. of fresh brain. Minard & Davis (1962) found that K'_{CK} for intact, unstressed animals was 0.052 ± 0.0018 , and that when animals were stressed K'_{CK} fell in proportion to the amount of AMP formed in the organ. However, since the equilibrium constant of the phosphotransferase reaction decreases as the pH falls (Noda *et al.* 1954),

Table 6. *Rates of phosphocreatine breakdown and resynthesis under the conditions postulated to exist in guinea-pig cerebral tissue in vivo*

Rates of the ATP-creatine-phosphotransferase reaction were calculated at 37° and pH 7.0, assuming an enzyme content of 60 units/g. of tissue (equivalent to 800 μ g. of pure enzyme, specific activity 75 units/mg. of protein, in 1 g. of the tissue). Correction factors for changes of rate with changes in pH and temperature were applied.

Substrates	pH	Temp.	Rate (μ mole/min./ μ g. of enzyme)
(a) Rate of phosphocreatine breakdown			
1 mM-ADP, 3 mM-phosphocreatine, 6 mM-Mg	7.9	37	0.200
1 mM-ADP, 3 mM-phosphocreatine, 6 mM-Mg	7.0	37	0.144
Maximal rate under conditions postulated <i>in vivo</i> 6900 μ moles/hr./g. of tissue			
(b) Rate of phosphocreatine resynthesis			
2 mM-ATP, 10 mM-creatine, 6 mM-Mg	9.0	37	0.055
2 mM-ATP, 10 mM-creatine, 6 mM-Mg	7.5	37	0.007
2 mM-ATP, 10 mM-creatine, 6 mM-Mg	7.0	37	0.0041
Maximal rate under conditions postulated <i>in vivo</i> 200 μ moles/hr./g. of tissue			

the fall in K'_{CK} with increasing AMP content may be explicable on the basis of an accompanying fall in pH. The value for K'_{CK} of unstressed animals was some 70 times that of the apparent equilibrium constant of the phosphotransferase reaction determined here. A number of explanations for this discrepancy may be advanced.

(a) The reaction was not at equilibrium. This seems most unlikely, since it involves more phosphocreatine being present in the steady state than at equilibrium.

(b) The intracellular pH of cerebral tissue is higher than 7.0. There is no evidence for this, and from the data of Noda *et al.* (1954), at 30° in the presence of 6 mM-magnesium, the intracellular pH would have to be in the region of 8.0 for the apparent equilibrium constant to lie in the range 0.04–0.05.

(c) There is considerable compartmentation of the intermediates, so the overall ratio ATP/ADP differs considerably from that governing the phosphotransferase reaction. Creatine and phosphocreatine are usually considered to be localized in the cytoplasm, but Seraydarian, Mommaerts & Wallner (1962) have shown 60% of the ADP in frog skeletal muscle to be localized in the myofibrils, where it is believed to be attached to actin.

(d) A separate system for the phosphorylation of creatine exists. A number of other enzymic reactions able to perform this function have been reported, but none has been substantiated (see Kuby & Noltmann, 1962, p. 522). However, a tight coupling of mitochondrial oxidative phosphorylation to phosphocreatine formation via a phosphotransferase which is either part of the mitochondria or bound to its surface could give rise to a ratio phosphocreatine/creatine bearing no relation to the ratio ADP/ATP in the bulk of the cell. Some evidence for such a possibility is provided by the discovery of appreciable ATP-creatine-phosphotransferase activity in a mitochondrial fraction from guinea-pig brain (T. Wood & P. D. Swanson, unpublished work).

At the present time, an explanation along the lines of (c) and (d) appears to be the most likely for cerebral tissue.

SUMMARY

1. The properties of adenosine triphosphate-creatine phosphotransferase prepared from ox brain were investigated and compared with those of the muscle enzyme.

2. Inhibition was produced by *p*-chloromercuribenzoate and 'aged' sodium thioglycollate, and fresh sodium thioglycollate could give rise to activation. On storage of partially pure preparations at 5°, the activity assayed in the absence of thioglycollate gradually declined, although the activity

assayed with thioglycollate present showed little change. It is suggested that thiol groups essential for activity are sensitive to aerial oxidation.

3. The enzyme was unable to catalyse a reaction with adenosine monophosphate as the phosphoryl-acceptor. It contained similar amounts of tyrosine and tryptophan to the muscle enzyme but had a considerably greater electrophoretic mobility at pH 8.0 and 8.6.

4. The optimum pH for the 'forward reaction' was 9.0 and for the 'reverse reaction' was 7.9. The enzyme was stable at 0° over the range pH 5.5–11.8. Reaction rates in both directions were measured at 20°, 30° and 37°, and the activation energies that were calculated were very close to those for the muscle enzyme.

5. Maximal velocities for the 'forward' and 'reverse reactions' and Michaelis constants for each of the four substrates creatine, phosphocreatine, adenosine diphosphate and adenosine triphosphate were determined. The effect of the magnesium concentration on the reaction was also investigated. The apparent equilibrium constant of the reaction at pH 7.0 was determined at 30° and at 37°.

6. From the data obtained, maximal rates of phosphocreatine breakdown and resynthesis in guinea-pig cerebral tissue under conditions postulated to exist *in vivo* were calculated and compared with the observed values. Observed concentrations of phosphorylated intermediates *in vivo* are discussed in the light of the measured equilibrium constant of the phosphotransferase reaction and the function of the enzyme.

I am indebted to Professor H. McIlwain for helpful discussion and to Dr D. B. Gammack, who carried out the run in the ultracentrifuge, which was provided by the Medical Research Council. Able technical assistance was rendered by Mr H. Russell. This investigation was supported in part by the Air Force Office of Scientific Research, OAR, through the European Office, Aerospace Research, United States Air Force.

REFERENCES

- Ames, A. & Nesbitt, F. B. (1958). *J. Neurochem.* **3**, 116.
- Beaven, G. H. & Holliday, E. R. (1952). *Advanc. Protein Chem.* **7**, 320.
- Caldwell, P. C. (1956). *Int. Rev. Cytol.* **5**, 229.
- Cho, A. K., Haslett, W. L. & Jenden, D. T. (1960). *Biochem. J.* **75**, 115.
- Da Costa, W. A. & Friedburg, F. (1960). *J. biol. Chem.* **235**, 3134.
- Eggleton, P., Elsdon, S. R. & Gough, N. (1943). *Biochem. J.* **37**, 526.
- Ennor, A. H. & Rosenberg, H. (1954). *Biochem. J.* **57**, 203.
- Ennor, A. H. & Stocken, L. A. (1948). *Biochem. J.* **42**, 557.
- Geiger, A. & Magnes, J. (1947). *Amer. J. Physiol.* **149**, 517.
- Heald, P. J. (1954). *Biochem. J.* **57**, 673.
- Kuby, S. A., Noda, L. & Lardy, H. A. (1954a). *J. biol. Chem.* **209**, 191.

- Kuby, S. A., Noda, L. & Lardy, H. A. (1954*b*). *J. biol. Chem.* **210**, 65.
- Kuby, S. A. & Noltmann, E. A. (1962). In *The Enzymes*, vol. 6. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- McIlwain, H. (1959). *Ciba Found. Symp.; Regulation of Cell Metabolism*, p. 127. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: J. and A. Churchill Ltd.
- Mahowald, T. A., Noltmann, E. A. & Kuby, S. A. (1962). *J. biol. Chem.* **237**, 1535.
- Minard, F. N. & Davis, R. V. (1962). *J. biol. Chem.* **237**, 1283.
- Narayanaswami, A. (1952). *Biochem. J.* **52**, 295.
- Nihei, T., Noda, L. & Morales, M. F. (1961). *J. biol. Chem.* **236**, 3203.
- Noda, L., Kuby, S. A. & Lardy, H. A. (1954). *J. biol. Chem.* **210**, 83.
- Noda, L., Kuby, S. A. & Lardy, H. (1955). In *Methods in Enzymology*, vol. 2, p. 605. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Noda, L., Nihei, T. & Morales, M. F. (1960). *J. biol. Chem.* **235**, 2830.
- Noltmann, E. A., Mahowald, T. A. & Kuby, S. A. (1962). *J. biol. Chem.* **237**, 1146.
- Oliver, I. T. (1955). *Biochem. J.* **61**, 116.
- Rosenberg, H. & Ennor, A. H. (1955). *Biochim. biophys. Acta*, **17**, 261.
- Seraydarian, K., Mommaerts, W. H. F. M. & Wallner, A. (1962). *Biochim. biophys. Acta*, **65**, 434.
- Thomas, J. (1956). *Biochem. J.* **64**, 335.
- Wood, T. (1963). *Biochem. J.* **87**, 453.

Biochem. J. (1963) **89**, 220

Studies on the Mechanism of Action of Adenosine 5'-Triphosphate-Creatine Phosphotransferase

INHIBITION BY MANGANESE IONS AND BY *p*-NITROPHENYL ACETATE

By D. C. WATTS

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

(Received 8 April 1963)

In the reaction catalysed by creatine kinase (adenosine 5'-triphosphate-creatine phosphotransferase, EC 2.7.3.2):

Mn^{2+} ions may replace Mg^{2+} ions as the activating metal ions (Kuby, Noda & Lardy, 1954*b*; Geiger, 1956; Griffiths, Morrison & Ennor, 1957; Cho, Haslett & Jenden, 1960). Ennor & Rosenberg (1954), and Cho *et al.* (1960) also observed that preincubation of creatine kinase with Mn^{2+} ions caused inactivation of the enzyme. During the present studies it was found that the presence of some substrates enhanced the inhibitory effect of added Mn^{2+} ions. This phenomenon has been investigated in greater detail. The reactive sulphydryl groups found to be essential for catalytic activity (Mahowald & Kuby, 1960; Watts, Rabin & Crook, 1961) appear to be the sites of inactivation. The results also provide evidence that these sulphydryl groups are situated close to the substrate-binding sites.

The inhibition of creatine kinase by *p*-nitrophenyl acetate was reported by Watts, Rabin & Crook (1962), and used to distinguish enzyme preparations with one and two catalytic sites/

molecule. Though loss of enzyme activity could be attributed to reaction of the essential sulphydryl group for the enzyme preparation with one catalytic site/molecule, for preparations containing two sites/molecule some reactive sulphydryl groups remained after complete loss of enzyme activity had occurred. The present paper concerns the further investigation of the nature of this loss of activity with the normal enzyme containing two catalytic sites/molecule. The results indicate the presence of an essential amino group, whose integrity is essential for the maintenance of enzyme conformation, which changes its *pK* during the transphosphorylation reaction.

MATERIALS AND METHODS

Materials

Creatine kinase. This was prepared by 'method B' of Kuby, Noda & Lardy (1954*a*). Except where the crystalline enzyme is specified, purification was carried out to the precrystallization stage. The specific activity is given for each sample in the Results section. For the preparations with high specific activity [above 54 'Kuby units', as defined by Kuby *et al.* (1954*a*)] the ethanol used in the purification procedure was redistilled from potassium hydroxide (Nihei, Noda & Morales, 1961), and all solutions