CYCLOCREATINE PHOSPHATE AS A SUBSTITUTE FOR CREATINE PHOSPHATE IN VERTEBRATE TISSUES. ENERGETIC CONSIDERATIONS

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SUMMARY

The measured equilibrium constant of the reaction, P-creatine^{2-} + cyclocreatine $\rightleftharpoons$ creatine + P-cyclocreatine^{2-}, catalyzed by creatine kinase in the presence of a trace of MgATP, is 26 at 37°. P-Cyclocreatine has a V_{max} 0.6% that of P-creatine and a K_m of 3.7 mM, vs. 2.8 mM for P-creatine, with chick breast muscle creatine kinase at pH 7.0. These results suggest that the high ratio of P-cyclocreatine/cyclocreatine (ca. 45) previously observed in breast muscle of cyclocreatine-fed chicks, compared to the much lower ratio of P-creatine/creatine (ca. 2) in the same tissue, primarily reflects free energy relationships between the synthetic and natural phosphagens.

Rowley et al. (1) have synthesized a number of potential analogs of creatine and shown that 1-carboxymethyl-2-iminoimidazolidine (cyclocreatine) is an excellent substrate for creatine kinase in vitro (Reaction 1). Griffiths and Walker (2) found that cyclocreatine-fed chicks accumulated up to 60 mM P-cyclocreatine in the sarcoplasmic water of breast muscle. These authors suggested (2) that the unexpectedly high ratio of P-cyclocreatine/cyclocreatine (ca. 45) observed in chick breast muscle, compared with the much lower ratio of P-creatine/creatine (ca. 2), primarily reflected near-equilibrium conditions for Reaction 1

$$\text{Cyclocreatine} + \text{MgATP}^{2-} \rightleftharpoons \text{MgADP}^{1-} + \text{P-cyclocreatine}^{2-} + \text{H}^{+} \quad (1)$$

$$\text{P-Creatine}^{2-} + \text{cyclocreatine} \underset{(\text{tr. MgATP})}{\rightleftharpoons} \text{creatine} + \text{P-cyclocreatine}^{2-} \quad (2)$$

in view of the high concentration of creatine kinase in skeletal muscle.

In this paper we report evidence in support of this suggestion. Our approach was to determine the apparent equilibrium constant of Reaction 2 in the presence of creatine kinase and a trace amount of MgATP.

MATERIALS AND METHODS

Cyclocreatine was synthesized as described elsewhere (2). Creatine kinase was purified from chicken breast muscle (3) to a S. A. of 250 μmol/

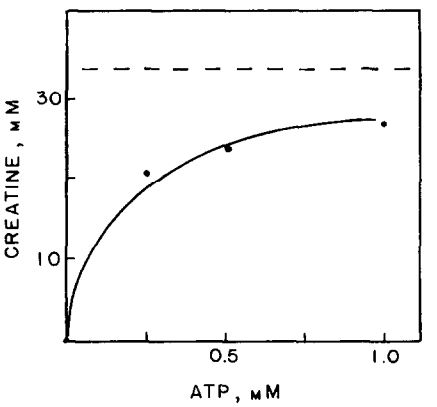

Fig. 1. Curve showing that attainment of the equilibrium of Reaction 2, catalyzed by creatine kinase, requires ATP. Initial conditions were identical to the first experiment of Table I. After incubation for 15 min, the creatine formed from P-creatine + cyclocreatine was determined colorimetrically. The equilibrium concentration would be 33.5 mM creatine.

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min/mg protein at 30° and pH 9.0, assayed by the pH-stat method. Only one band was observed on SDS gels. Enzyme was stored at 4° in 50 mM Tris.HCl, pH 8.0, and 14 mM mercaptoethanol. Colorimetric assays for the reactants of Reaction 2 were performed as described for the same four compounds in chick breast muscle (2). P-Cyclocreatine and P-creatine concentrations were obtained by substracting concentrations of cyclocreatine and creatine from the corresponding total values obtained after heating aliquots at 65° in 0.2 M perchloric acid for 15 min (P-creatine) and 45 min (P-cyclocreatine). K_{eq} of Reaction 2 was measured after incubation of reactants for up to 8 hours at 37° in 5 ml total volume containing: Mg acetate, 2 mM; ATP, 1 mM; creatine kinase, 3 mg; and 50 mM of either triethanolamine.HCl, pH 7.0, or Na glycine, pH 9.0. Kinetic constants for creatine kinase were determined by coupled enzyme assays (a) in the forward direction by measuring ADP formed by NADH disappearance (4), and (b) in the reverse direction by measuring ATP formed by NADPH appearance (5). The reverse direction was also assayed by colorimetric determination of cyclocreatine or creatine formed (2). Initially cyclocreatine was chemically phosphorylated in this laboratory (by G. R. Griffiths) with $POCl_3$ and NaOH and the product purified by column chromatography on Dowex-1(HCO_3^-). We found only the enzymically active isomer to be formed. However, the use of LiOH is preferable for large scale synthesis (G.L. Kenyon, personal communication). Cyclocreatine (45 g) was dissolved in 275 ml of 3 M LiOH and cooled to 0° with a dry ice-ethanol bath during the stepwise addition over 2 hours of a total of 120 ml of $POCl_3$ and 1000 ml of 3 M LiOH. The LiOH was added at intervals to keep the pH above 13. After 2.3 hours, the Li_3PO_4 was removed by filtration, and the supernatant solution was adjusted to pH 7.6 with 5 N HCl; 5 volumes of 95% ethanol were then added. The next day the fine precipitate was removed by filtration. The solution was cooled to -10° for 12 hours, then returned to room temperature to allow formation of crystals, which were filtered, washed with cold water and cold ethanol, and dried in a vacuum over P_2O_5. Yield: 25 g. The product was recrystallized from water-ethanol, washed, dried, and stored at -10°. The dilithium salt: (a) has an IR spectrum like that reported by Rowley et al. (1) for the enzymic product; (b) consists of only one active isomer as determined by coupled enzyme assay (5); (c) is hydrolyzed to cyclocreatine after 45 min at 65° in 0.2 M perchlo-

TABLE I

Determination of Equilibrium Constant of Reaction 2

Conditions	P-Creatine	Cyclocreatine	Creatine	P-Cyclocreatine	K_{eq}
Initial	40	40	0	0	
Final	6	7	31	32	23.6
Initial	0	0	40	40	
Final	7	6	30	36	25.7
Initial	35	35	35	35	
Final	11	13	59	60	24.8
Initial	50	50	20	0	
Final	11	10	54	42	20.6
Initial	5	5	25	25	
Final	5	5	27	27	29.2

Concentrations (mM) of reactants are given at 0 time and after incubation for 6 to 8 hours at 37°.

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ric acid (2); and (d) has pK_a's of approximately 3.1, 4.5, and 10.7. The active isomer is now known to be the 3-phosphono, rather than the 2-phosphono (1), derivative from X-ray crystallographic analysis (G. N. Phillips and F. A. Quiocho, in preparation)(cf. unpublished data cited in (6)).

RESULTS

Equilibration of the four reactants in Reaction 2 requires not only creatine kinase but also MgATP (or MgADP), as shown in Fig. 1. These results provide additional evidence against a double displacement mechanism for creatine kinase from chick breast muscle. An ATP (or ADP) concentration of 1 mM was selected for K_{eq} determinations in order to achieve equilibrium in a convenient length of time without markedly perturbing the equilibrium values obtained.

Table I summarizes the equilibrium data for Reaction 2 obtained from five separate experiments at pH 7.0, employing different initial concentrations of the four reactants. The average value for K_{eq} was 24.8. When ADP was substituted for ATP, the average K_{eq} was 28.4. At pH 9.0 the K_{eq} was 25.7. We observed that, like P-creatine, none of the pK_a's of P-cyclocreatine occur in the physiological range, so K_{eq} of Reaction 2 should be pH-independent

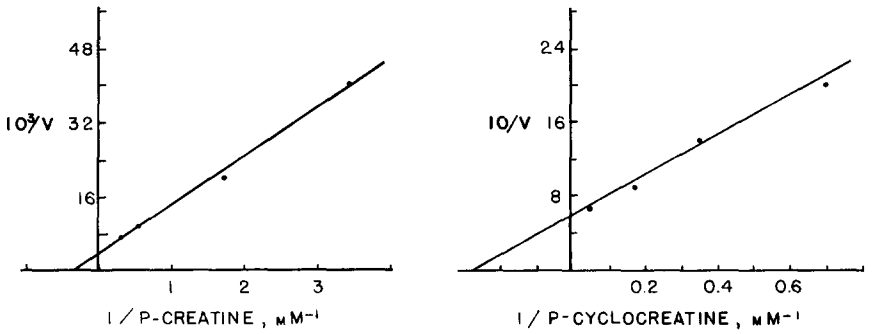

Fig. 2. Double-reciprocal kinetic plots for the reverse reaction catalyzed by chick breast muscle creatine kinase at pH 7.0 and 37°. Incubation mixtures contained 37 mM Mg acetate, 11 mM ADP, and 29 mM triethanolamine.HCl, pH 7.0. P-Cyclocreatine: K_m = 3.7 mM; V_{max} = 1.82 μmol/min/mg protein. P-Creatine: K_m = 2.8 mM; V_{max} = 313 μmol/min/mg protein.

over a broad range. The overall average of 16 determinations of K_{eq}, with ATP or ADP and at pH 7.0 or 9.0, was 25.8 ± 4.7 SD.

Kinetic data for the reverse of Reaction 1 catalyzed by chick breast muscle creatine kinase, assayed by the colorimetric method, indicated a K_m of 3.7 mM for P-cyclocreatine vs. 2.8 mM for P-creatine and a V_{max} for P-cyclocreatine 0.6% that for P-creatine at pH 7.0 and 37° (Fig. 2). Values obtained by coupled enzyme assay (5) at pH 7.0 and 37° were K_m's of 3.6 mM for P-cyclocreatine and 3.2 mM for P-creatine, and V_{max} for P-cyclocreatine 0.7% that for P-creatine. Kinetic data for the forward reaction, assayed by coupled enzyme assay at pH 7.0 and 37° (4), indicated a K_m of 55 mM for cyclocreatine vs. 20 mM for creatine and a V_{max} for cyclocreatine 45% that for creatine with chick breast muscle creatine kinase (cf. 7). Substitution of these kinetic parameters into the respective Haldane relationships for the reaction catalyzed by creatine kinase (8) when cyclocreatine or creatine are substrates has provided an independent check on the K_{eq} observed for Reaction 2. The ratio of K_{eq} for Reaction 1 obtained from the Haldane equation divided by K_{eq} for the corresponding reaction with creatine and P-creatine as reactants gives a value of 26, in agreement with the measured K_{eq} for Reac-

tion 2. In this calculation the assumptions were made that the K_m's for MgATP and MgADP are similar in both reactions.

DISCUSSION

This work was performed to evaluate P-cyclocreatine as a potential synthetic phosphagen from both the thermodynamic and kinetic standpoints. It is difficult to determine the equilibrium constant of Reaction 1, and the free energy of hydrolysis of P-cyclocreatine relative to ATP, at physiological pH because of the numerous other equilibria involved at pH's near the pK_a's of MgATP and MgADP (8,9). Additional complexities, such as determinations of intracellular pH and concentrations of free Mg^{2+} and unbound ADP in pertinent compartments, would be involved if equilibrium concentrations of these reactants were to be estimated in intact tissues, e.g. before and during muscular exercise (cf. 10,11). On the other hand, Reaction 2, which represents an equilibrium presumably attained in resting breast muscles of cyclocreatine-fed chicks (2), is relatively independent of both pH over the physiological range and dissociation constants involving chelated adenine nucleotides. The observed K_{eq} for Reaction 2 of 26 at 37° (Table I) indicates that this synthetic phosphagen pair has approximately 2 kcal/mol less free energy than the natural phosphagen pair, i.e. a free energy of hydrolysis only slightly higher than that of ATP. The high ratio of P-cyclocreatine/cyclocreatine in resting breast muscle of cyclocreatine-fed chicks (2) thus can be explained by the free energy relationships of Reaction 2, without invoking intracellular compartmentation or additional equilibria.

The physiological consequences of substitution of a major portion of the natural phosphagen in vertebrate tissues by this more stable (2) synthetic phosphagen of lower free energy should prove to be of considerable interest. For example, it might be predicted that, in animals previously fed cyclocreatine during rapid growth, resting fast-twitch muscle cells would have a larger total reservoir of high-energy phosphate, but the availability of that reservoir would be kinetically limited during a series of rapid contractions, when compared to similar cells of control animals.

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