Biochimica et Biophysica Acta, 397 (1975) 377—394
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BBA 67561

PARTIAL PURIFICATION AND PROPERTIES OF THIAMINE PYROPHOSPHOKINASE FROM PIG BRAIN

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(Received December 24th, 1974)

(Revised manuscript received April 1st, 1975)

Summary

Pig brain thiamine pyrophosphokinase (ATP: thiamine pyrophosphotransferase, EC 2.7.6.2) was purified 260-fold over extracts of brain acetone powder. A direct, radiometric assay was used to follow the purification. By isoelectric focusing, the purified enzyme appeared to have an isoionic point of approx. pH 4.2, but these preparations were still not homogeneous by disc-gel electrophoresis nor by analytical ultracentrifugation.

The purified enzyme has a broad pH optimum extending from pH 8.3 to 9.3 in 0.028 M phosphate/glycylglycine buffers. For optimal enzymatic activity, the ratio of magnesium to ATP must be fixed at 0.6, which suggests that for this ATP-pyrophosphoryl transfer reaction, the enzymatically preferred reactant may be $Mg(ATP)_2^{6-}$. A preliminary study of the kinetics of the reaction reveals that the enzyme may function via a partial "ping-pong" mechanism; on this basis, dissociation constants for ATP_t and for thiamine were evaluated. Pyrithiamine, butylthiamine, ethylthiamine, and oxythiamine appeared to be competitive inhibitors with respect to thiamine as the variable substrate, and their inhibitor dissociation constants were calculated. The relatively poor affinity of oxythiamine to the enzyme emphasizes the 4-amino group in the pyrimidine ring as one of the specificity requirements for thiamine pyrophosphokinase. Preliminary values for the apparent equilibrium coefficient of the thiamine pyrophosphokinase-catalyzed reaction, in terms of total species, has been approximated at several initial concentrations of reactants: e.g. $K'_{eq,app}$ =

Abbreviations: a subscript "t" is used to represent total concentrations; a subscript "eq", equilibrium concentrations; a subscript "f", for forward reaction; and a subscript "app" an apparent value.

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$\frac{[AMP_t]_{eq}[TDP_t]_{eq}}{[ATP_t]_{eq}[Th]_{eq}} = 0.9 \cdot 10^{-5} - 1.6 \cdot 10^{-5}$, at initial $[ATP_t]_{initial} = \frac{[Mg_t]_{initial}}{0.6} =$

$9.66 \cdot 10^{-3}$ M; and $[Th]_{initial} = 1 \cdot 10^{-6}$ and $2 \cdot 10^{-6}$ M, respectively, where TDP, Th, t and eq represent thiamine diphosphate, thiamine, total concentration and equilibrium concentration, respectively.

Introduction

In 1937, Lohman and Schuster [1] had found that thiamine diphosphate was an essential coenzyme for yeast pyruvate decarboxylase (EC 4.1.1.1) [3]. Since that time, thiamine diphosphate has also been shown to be an essential coenzyme for pyruvate dehydrogenase [2], α-ketoglutarate dehydrogenase (EC 1.2.4.2) [3], transketolase (EC 2.2.1.1) [4—6], phosphoketolase (EC 4.1.2.9) [7], and the oxidative decarboxylation of glyoxylic acid [8]. Therefore, if the vitamin-like action of thiamine is to be completely understood, the mechanism(s) of the pyrophosphorylation of thiamine to its active coenzyme form, thiamine diphosphate, must also be determined.

The enzyme responsible for the pyrophosphorylation of thiamine, thiamine pyrophosphokinase, ATP: thiamine pyrophosphotransferase (EC 2.7.6.2), was first purified from yeast by Kaziro and co-workers [9,10] who had shown that thiamine is pyrophosphorylated by a transpyrophosphorylation from ATP, according to the reaction:

$$\text{Thiamine} + ARP_\alpha P_\beta P_\gamma \rightleftharpoons ARP_\alpha + \text{Thiamine } P_\beta P_\gamma. \quad (1)$$

In addition, thiamine pyrophosphokinase has been partially purified from rat liver [11—15], rat brain [16], and pig heart [17,18]. A preliminary purification from rat brain was carried out in this laboratory [16] in an effort to gain a greater understanding of the differential effects of the two thiamine antagonists, pyrithiamine and oxythiamine on thiamine diphosphate-dependent enzymes in brain and other tissues. Thus, Johnson and Gubler [16] had found pyrithiamine and oxythiamine to be competitive inhibitors for the rat brain enzyme, with pyrithiamine the more potent (e.g. pyrithiamine $K_i = 1.3 \cdot 10^{-7}$ M and oxythiamine $K_i = 1.5 \cdot 10^{-4}$ M). Pig heart thiamine pyrophosphokinase has also been reported to be completely inhibited by pyrithiamine and oxythiamine [18], and pyrithiamine a competitive inhibitor of rat liver thiamine pyrophosphokinase [15].

Rindi et al. [19] found that oxythiamine diphosphate was present in the tissues of rats after the administration of oxythiamine and claimed that the oxythiamine was phosphorylated by thiamine pyrophosphokinase. Johnson and Gubler [16] had found that, in the presence of ATP and Mg^{2+}, the rat brain enzyme could synthesize a product from oxythiamine which would inhibit transketolase, but, if either ATP or Mg^{2+} were eliminated, the inhibitor

was not synthesized, an observation supporting the idea that oxythiamine can act as a substrate for thiamine pyrophosphokinase.

The objective of this present study was to elucidate some of the properties of brain thiamine pyrophosphokinase and those factors affecting the pyrophosphorylation of thiamine.

Materials and Methods

Pig brain was employed instead of rat brain [16] because of the larger amounts of material required for the purification.

The disodium salt of adenosine 5′-triphosphate (assay 99%), pyrithiamine hydrobromide, oxythiamine hydrochloride, thiamine hydrochloride and glycylglycine (free base) were obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals and solvents used were reagent grade. Thiamine ([2-^{14}C]thiazole) hydrochloride was a product from Amersham/Searle, Arlington Heights, Ill.; Amberlite CG50, 200—400 mesh, chromatographic grade cation-exchange resin, from Mallinckrodt Chemical Works, St. Louis, Mo.; Sephadex G-25 and Sephadex G-100 modified dextran gels from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.; and Ampholine carrier ampholytes, with pH ranges of 3—5, 3—6, and 3—10, were obtained from LKB Instruments, Inc., Rockville, Maryland.

The ion-exchange resin, Amberlite CG50, was prepared according to the procedure of Sharma and Quastel [20], converted to the H^+ form, washed with water until neutrality, and stored under water.

Alumin C_γ was prepared according to Willstätter [22] and allowed to age approx. 4 years.

Estimations of radioactivity were made by the addition of 1-ml aliquots of the column effluents to 10-ml aliquots of scintillation cocktail, prepared according to Bray [23].

Assay of thiamine pyrophosphokinase

Aliquots of the following solutions were added to 13 × 100 mm culture tubes: 0.10 ml of 0.5 M glycylglycine buffer, pH 7.8, at 37°C which is also 0.12 M in Mg^{2+}, 0.05 ml of 0.4 M ATP neutralized to pH 7.3, 0.10 ml of 0.002 M thiamine, which contained about 300 000 cpm of [^{14}C]thiamine, the appropriate volume of enzyme solution (added last) and water to give a final volume of 0.90 ml. After a reaction time of 1 h (usually at 37°C), 0.10 ml of 50% trichloroacetic acid was added to stop the reaction and to precipitate the protein, which was removed by centrifugation. 0.10-ml aliquots were chromatographed on 4 × 0.5 cm microcolumns of Amberlite CG-50, which were developed with 3 ml of water. An aliquot of the effluent was counted, and the thiamine diphosphate produced was calculated from its radioactivity (blank corrected), with the appropriate dilution factors and total volume of effluent collected.

Protein concentrations were determined by the method of Warburg and Christian [24]. One unit of enzyme activity was defined as equal to one nmol of substrate converted to product per hour, under the conditions of the assay. The concentration of substrate in nmol was computed from the measured cpm

and by the experimentally determined specific radioactivity of the thiamine solution; it was assumed that the specific activity of the thiamine diphosphate produced by the thiamine pyrophosphokinase reaction would be the same as the specific activity of the thiamine solution used.

Estimations of kinetic "constants" were facilitated by the use of a Hewlett-Packard Calculator-plotter (Model 9100B) which uses multiple linear regression for plotting the data.

Determination of equilibrium position

A preliminary estimation of the equilibrium position for the reaction catalyzed by thiamine pyrophosphokinase was made at 1 and 2 μM initial thiamine concentrations and at several relatively high initial ATP_t concentrations, (where $MgSO_4$ = 0.6 ATP_t) ranging from approx. $5 \cdot 10^{-3}$ M to approx. $3 \cdot 10^{-2}$ M, with about 30 units of enzyme and in the presence of $5.56 \cdot 10^{-2}$ M glycylglycine, pH 7.8, at 37° C. Aliquots were removed at 5 and 9 h to establish the equilibrium state and the concentrations of thiamine diphosphate as determined above. From the equilibrium concentrations of thiamine diphosphate (TDP), the stoichiometry of the reaction (Eqn 1), and the assumption that $[ATP_t]_{equilibrium} \cong [ATP_t]_{initial}$, since $[ATP_t]_{initial} \gg [Th]_{initial}$, where Th denotes thiamine, the apparent equilibrium coefficient was calculated from the relations:

$$K_{eq,app} = \frac{[AMP_t]_{eq}\,[TDP_t]_{eq}}{[ATP_t]_{eq}\,[Th]_{eq}} = \frac{[TDP_t]^2_{eq}}{[ATP_t]_{initial}([Th]_{initial} - [TDP_t]_{eq})} \qquad (2)$$

Purification of thiamine pyrophosphokinase

The pig brains were obtained gratuitously from Bartholomew Slaughter House, Springville, Utah. The brains were removed within 45 to 75 min after the death of the animal and were placed on ice for transport to the laboratory. After having been washed to remove blood clots and bone chips, 50-g portions of the brains were homogenized in 500 ml of acetone (pre-cooled to −20° C) for 15 s in a Waring Blender at high speed. The acetone was removed by suction filtration, and the filter cake was homogenized three more times, following the same procedure. (After the first homogenization, two filter cakes could be combined and carried through the rest of the procedure.) The acetone was removed from the final filter cake by powdering it in a hood at room temperature, and the acetone powder was stored in a desiccator over P_2O_5 at 0—5° C. In this form, it would be stored without loss of activity for at least 1 year.

The acetone powder was extracted by stirring for 3 h in 10 volumes (w/v) of 0.15 M NaCl. All operations were carried out at 0—5° C, unless otherwise indicated. With the use of a Potter-Elvehjem type homogenizer with a teflon pestle, aliquots of the acetone powder suspension were homogenized for 5 min, and the homogenate was centrifuged at 34 800 × *g* for 20 min. The supernatant fractions were saved and the residue rehomogenized in four volumes (four times original weight of acetone powder) of the salt solution, followed by centrifugation; the supernatant solutions were then combined.

10 ml of 1% protamine sulfate solution (pH approx. 7—8) per 100 ml of

extract were slowly added with constant stirring. The stirring was continued for 20 min after the last of the protamine sulfate had been added, and the precipitate was removed by centrifugation at 34 800 × *g* for 15 min.

The pH of the supernatant solution was then lowered to 5.8 by the slow addition of 0.5 M acetic acid with continuous stirring; the clear solution was distributed in 15 × 150 mm culture tubes and incubated in a 52° C water bath for 5 min, after which time it was cooled immediately in an ice bath. The precipitate formed was removed by centrifugation at 15 000 × *g* for 20 min. The supernatant solution was then fractionated with $(NH_4)_2SO_4$ by the slow addition of solid $(NH_4)_2SO_4$ to give 0.52 saturation (31.35 g/100 ml). After standing for 20 min, the solution was centrifuged as before and the precipitate was redissolved in a small amount of 0.5 M glycylglycine buffer, pH 7.8. In this state, the activity was quite stable, with almost no loss of activity, when stored for 2 weeks at 0—5° C.

$(NH_4)_2SO_4$ was removed from the resuspended precipitate by gel filtration through a 2.5 × 40 cm column of Sephadex G-25 (coarse) equilibrated with 0.005 M glycylglycine buffer, pH 7.3. The pH of the eluate, containing the majority of the protein (60—80 ml), was lowered to 4.3 by the slow addition of 0.5 M acetic acid with continuous stirring: 0.02 volume of 0.5 M acetate buffer, pH 4.3, was added and the solution allowed to stand overnight in the cold room. A small amount of precipitate was removed by centrifugation at 10 800 × *g* for 20 min, and the supernatant solution was diluted to give a protein concentration of less than 10 mg/ml. The thiamine pyrophosphokinase activity was adsorbed on alumina C_γ at a gel to protein ratio of 0.33. After the suspension had been stirred for 20 min, the gel was removed by centrifugation at 34 000 × *g* for 10 min, and the supernatant solution was treated with another equal portion of gel by the procedure given above. To avoid further dilution of the protein solution, the gel was collected by centrifugation at 34 000 × *g* for 10 min and the sedimented gels were combined and then resuspended in the eluent solution.

The thiamine pyrophosphokinase activity was eluted from the alumina C_γ gel by the following procedure: 10 ml of eluent (described below) per 100 mg of gel were added, the gel was suspended with stirring for 20 min and was collected by centrifugation at 34 800 × *g* for 10 min. The phosphate solutions employed as eluents and their order of use were as follows: 0.1 M solutions of NaH_2PO_4 with their pH values adjusted to 4.4, 5.1 and 6.1, respectively. The protein in each of the fractions was precipitated by the slow addition of $(NH_4)_2SO_4$ to give 0.85 saturation (0.608 g/ml); the precipitates were individually resuspended in small volumes of 0.25 M glycylglycine, pH 7.8, and then assayed for the enzyme activity. The most active fractions were pooled for use in the next step.

The combined active fractions from the alumina C_γ step were applied to a 2.5 × 40 cm column of Sephadex G-100 and the column was developed with 0.005 M glycylglycine, pH 7.4. The peak of activity was collected after about 50 ml had passed through the column.

Fractionation by isoelectric focusing followed. The solutions for filling the column were prepared as given by the manufacturer [21], with the anode at the bottom of the column. Phosphoric acid was used in preparing the solu-

tion at the anode and ethylenediamine for the solution at the cathode. The enzyme solution was mixed with the "light" solution to give the desired volume, and the column was loaded, using a pump and an LKB gradient mixer at a filling rate of 4 ml/min or less. The current was applied and the electrofocusing continued until the current, at constant voltage, decreased to a constant value in about 24—72 h, dependent on the pH range of the ampholine mixture (after initial experiments with wide-range ampholines, pH 3—10, only narrow-range, pH 3—6, ampholine mixtures were used). At the end of the run, the column was unleaded as directed [21] with the flow rate adjusted to less than 1 ml/min. Fractions were collected at 5-min intervals, and the pH of each was determined. Most of the thiamine pyrophosphokinase activity was eluted in fractions with pH values between 4.0 and 4.3, but fractions with pH values from 3.7 to 4.5 were all checked for activity.

Results

Table I shows that a 260-fold purification of pig brain thiamine pyrophosphokinase was accomplished with an overall recovery of 4.5%. This represents the highest degree of purification thus far obtained for a brain preparation of this enzyme. Tests for possible contamination by thiamine diphosphatase and ATPase or 5'-AMPase activities were negative under the standard conditions of assay.

The sensitivity of the thiamine pyrophosphokinase assays was dependent upon the specific radioactivity of the thiamine solution used, and, in the standard assay (1500 cpm/nmol), the lower limit of detection was about 0.1 nmol of thiamine diphosphate. Duplicate assays were accurate to within ±7%. Under standard assay conditions (0.0222 M ATP; $2.22 \cdot 10^{-4}$ M thiamine; 0.0123 M $MgSO_4$; 0.0556 M glycylglycine, pH 7.8, at 37°C), the amount of thiamine diphosphate produced during the assay was linear with time for a period up to about 2 h, or for approx. 10% of the total amount of thiamine (about 20 nmol) converted to thiamine diphosphate. The initial velocity of thiamine diphosphate synthesis was shown to be directly proportional to the concentration of

TABLE I

PURIFICATION OF PIG BRAIN PYROPHOSPHOKINASE

Acetone powder, 58.7 g.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Extract	560	8192	3121	0.38	100
Protamine sulfate	594	5061	2859	0.56	92
Heat treatment	574	2569	1837	0.72	59
$(NH_4)_2SO_4$	33	948.6	1495	1.54	47
Sephadex G-25	68	887.4	1289	1.45	41
Acid treatment	71	850.3	1139	1.34	37
Alumina G_γ gel	9.2	47.7	372	7.8	11.9
Sephadex G-100	50.5	25.1	247	9.8	7.9
Isoelectric focusing	4.7	1.4	141.4	98.9	4.5

enzyme added over the range of approx. 3—30 nmol thiamine diphosphate synthesized/h. Thus, the direct radiometric assay has proven to be both a highly sensitive and reliable index of the enzymatic activity. In the case of the determination of the K_m for thiamine (Fig. 4, below), because of the comparatively low concentrations of substrate explored, a solution of thiamine of approx. ten times the specific radioactivity used in the usual assay was employed (approx. 1700 cpm/nmol measured ≡ 14 Ci/μmol), which permitted a 2 pmol limit of detection of thiamine diphosphate.

pH optimum for pig thiamine pyrophosphokinase

The pH optimum for the purified enzyme was determined, using a mixed phosphate/glycylglycine buffer system, at a fixed concentration of 0.028 M for each buffer component. Instead of two separate buffers, a combination of buffers was used to insure adequate buffering capacity throughout the pH range and that any effects of the buffer species on the enzyme activity might be manifested equally throughout the pH range under investigation. Each pH value was measured directly after all the reaction mixture components were added. As can be seen from Fig. 1, there appears to be an apparent optimum pH for purified pig brain thiamine pyrophosphokinase, extending from about 8.3 to 9.3 under these conditions; however, data beyond pH 9.3 were difficult to gather in view of the known instability of the thiazolium ring, and it is not known if this represents a broad plateau, rather than a distinct optimum.

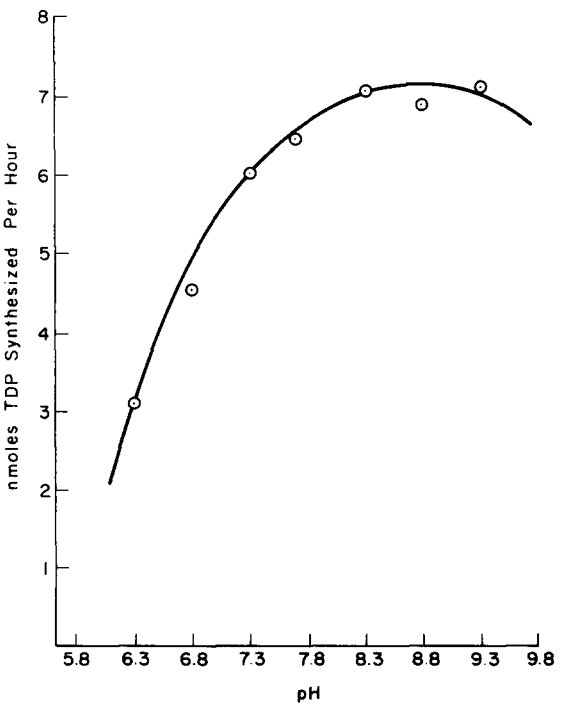

Fig. 1. pH optimum for pig brain thiamine pyrophosphokinase in glycylglycine/phosphate buffer, 0.0278 M, in each buffer component. The pH values were adjusted with HCl or NaOH, and the pH of each reaction mixture was determined experimentally, with the "standard" enzyme assay concentrations of substrates and Mg employed (see test). TDP, thiamine diphosphate.

Optimum $[Mg_t]/[ATP_t]$ ratio

As shown in Fig. 2, Mg is required for enzymatic activity, and at a fixed concentration of ATP_t with Mg_t varied (Fig. 2A), or at a fixed concentration of Mg_t with ATP varied (Fig. 2B), relatively sharp optimum ratios of Mg_t to ATP_t are observed, with a rapid decrease of activity on either side of this optimum. If

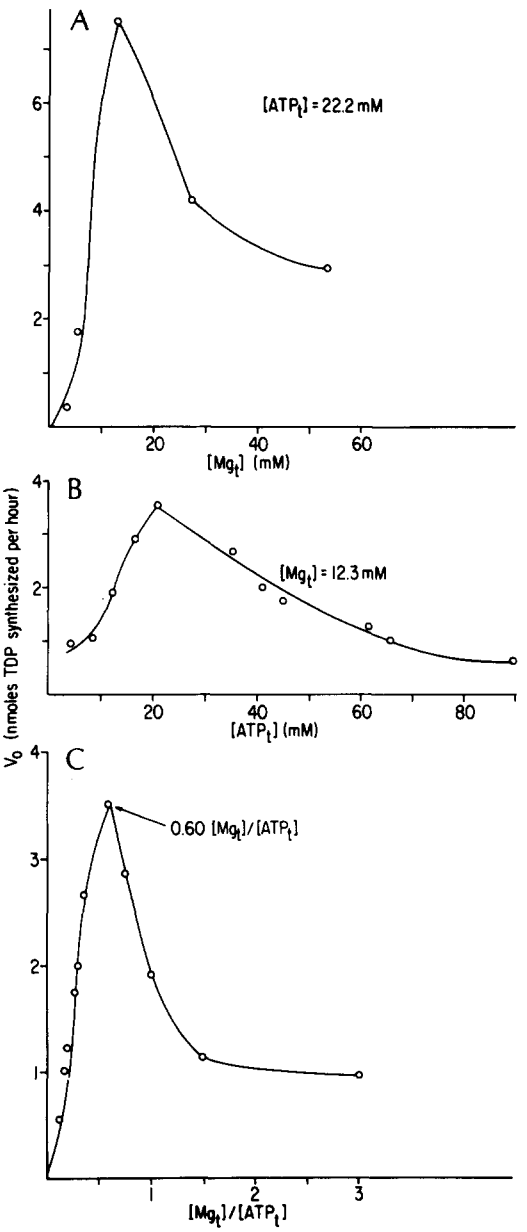

Fig. 2. Effect of the ratio of $[Mg_t]/[ATP_t]$ on thiamine pyrophosphokinase activity. The enzyme was assayed by the standard procedure (see text), except that the concentration of added Mg_t was varied in A at 0.0222 M fixed ATP_t concentration; in B, the concentration of ATP_t added was varied at a fixed Mg concentration of 0.0123 M; in C, the data of B are recalculated in terms of the ratio of $[Mg_t]/[ATP_t]$. TDP, thiamine diphosphate.

the data of Fig. 2B are recalculated in terms of the ratio of $[Mg_t]/[ATP_t]$, a value of 0.6 is observed for the optimum (Fig. 2C). Although a complete analysis of the concentrations of free Mg^{2+}, of the magnesium chelates of ATP, and of the ionizable uncomplexed species of ATP has not been attempted in the manner that was conducted for ATP-creatine transphosphorylase (see Kuby and Noltmann, [33]), it is tempting to suggest that for this transpyrophosphorylation reaction, the $Mg(ATP)_2^{6-}$ species might be the actual pyrophosphoryl donor.

Kinetic studies

Initially, it was assumed that a type of Michaelis-Menton kinetics might be followed, and, in Fig. 3, a double reciprocal plot is presented of the rate of thiamine diphosphate synthesis versus the initial ATP_t concentration, at a fixed $[Mg_t]/[ATP_t] = 0.6$, and at an initial thiamine concentration of $2.22 \cdot 10^{-4}$ M. Under these conditions, an apparent K_m for ATP was found to be 0.059 M, which is an unusually high value for most transfer reactions involving ATP. A preliminary determination of the apparent K_m for thiamine was also made from a double reciprocal plot of v_o versus the initial thiamine concentration, as shown in Fig. 4, where the K_m for thiamine was found to be only $4.1 \cdot 10^{-6}$ M, an extraordinarily low value for most non-nucleotide substrates involved in transfer reactions and almost a factor of 10^{-4} smaller than the value for ATP. The data presented in Fig. 5 demonstrate that, within experimental error, pyri-

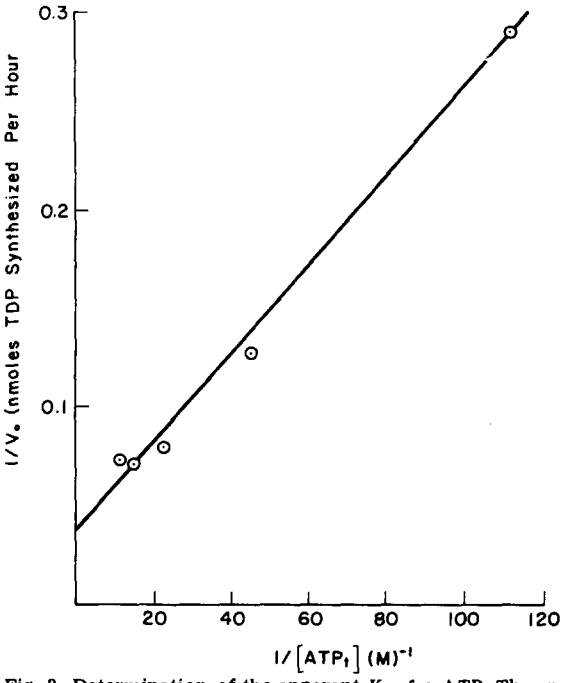

Fig. 3. Determination of the apparent K_m for ATP. The enzyme was assayed by the standard method (see text), except that the concentration of ATP was varied as shown in the ordinate, and the concentration of magnesium was simultaneously varied to give a constant $[Mg_t]/[ATP_t]$ ratio of 0.6 at a fixed initial concentration of thiamine of $2.22 \cdot 10^{-4}$ M. TDP, thiamine diphosphate.

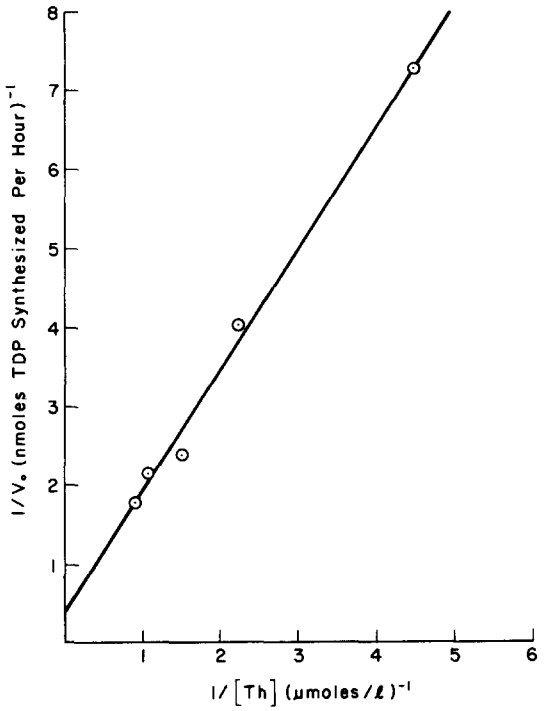

Fig. 4. Determination of the apparent K_m for thiamine at varying thiamine concentrations and a fixed initial concentration of $ATP_t = 2.22 \cdot 10^{-2}$ M; $[Mg_t]/[ATP_t] = 0.6$; a specific activity of $[^{14}C]$thiamine of approx. 14 Ci/μmol was employed for these studies. The concentrations of enzyme differed in Figs 3—5, and thus they are not comparable at given substrate concentrations. TDP, thiamine diphosphate; Th, thiamine.

thiamine, oxythiamine, butylthiamine and ethylthiamine all appear to be linear competitive inhibitors with respect to thiamine as the variable substrate, and with calculated apparent inhibition constants (K_i) of $1.4 \cdot 10^{-6}$, $6.8 \cdot 10^{-4}$, $2.1 \cdot 10^{-6}$, and $0.8 \cdot 10^{-6}$ M, respectively. One notes that of these four thiamine analogs, only oxythiamine appears to have a relatively poor affinity for the enzyme, pointing to the specificity requirement of the 4-NH_2 group of the pyrimidine ring to the enzyme. For these preliminary kinetic studies, different preparations of enzyme of differing specific activities were employed at different times in Figs 3—5; and, consequently, the enzymatic velocities are not comparable from experiment to experiment at given values of the substrates concentrations.

The kinetics were also studied in more detail, taking cognizance of the fact that in a bisubstrate reaction the kinetics of one substrate is often influenced by the concentration of the second. For this study, an enzyme preparation from the alumina C_γ gel step was used (see above). Since relatively high concentrations of ATP were to be employed, a large ATP stock solution was prepared, the concentration of which was determined enzymatically (using the hexokinase-glucose-6-phosphate dehydrogenase system) and found to be only 80% of the calculated value (0.32 vs 0.40 M calculated). Prior to this point, the calculated ATP concentrations had been employed. Fig. 6 presents the results

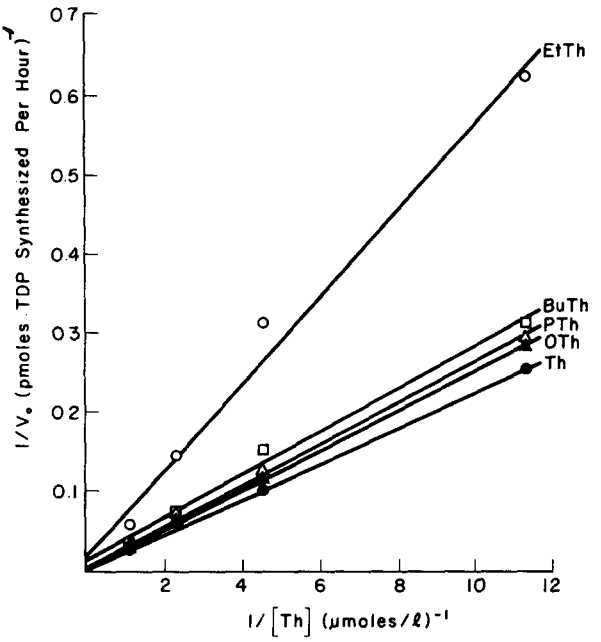

Fig. 5. Competitive inhibition with respect to thiamine of pig brain thiamine pyrophosphokinase by oxythiamine, pyrithiamine, butylthiamine, and ethylthiamine. The initial concentrations of the various inhibitors were as follows: ●, thiamine alone; ▲, oxythiamine, $8.89 \cdot 10^{-5}$ M; △, pyrithiamine, $2.22 \cdot 10^{-7}$ M; □, butylthiamine, $4.44 \cdot 10^{-7}$ M; and ○, ethylthiamine, $1.11 \cdot 10^{-6}$ M. A fixed initial concentration of $ATP_t = 2.22 \cdot 10^{-2}$ M with $[Mg_t]/[ATP_t] = 0.6$ was employed.

of a study in which the initial ATP concentration was varied at a fixed ratio of $0.6 = [Mg_t]/[ATP_t]$ and at five initial fixed thiamine concentrations. The converging lines of the family of double reciprocal plots is indicative of the formation of a ternary enzyme-thiamine-ATP complex (or possibly the E-Th-$Mg(ATP)_2^{6-}$ complex) with an apparent dissociation constant for ATP from an enzyme binary complex ($\overline{K}_A$), equal to 0.029 M, provided the m mechanism followed the rate expression:

$$v_{o,f} = \frac{V_f}{1 + \frac{K_A}{(A)} + \frac{K_B}{(B)} + \frac{\overline{K}_A K_B}{(A)(B)}} \tag{3}$$

Also, even though the $[Mg_t]/[ATP_t]$ ratio was maintained at a constant value of 0.6, at the highest concentrations of ATP employed (0.0322 M), inhibition of the reaction, especially at low thiamine concentrations, was very noticeable (see Fig. 6), and possibly was the result of inhibition of an ATP^{4-}-uncomplexed species (see e.g. Kuby and Noltmann [33] for comparable study on ATP-creatine transphosphorylase). The results of the above experiment, in which thiamine was plotted as the variable substrate with the initial ATP concentration held fixed at several values, are given in Fig. 7. Surprisingly, parallel lines are obtained for thiamine as the variable substrate and indicative of a reaction proceeding by a "ping-pong" mechanism [34] through a covalent intermediate of enzyme-substrate species. For this type of mechanism $\overline{K}_A K_B = 0$ in Eqn 3,

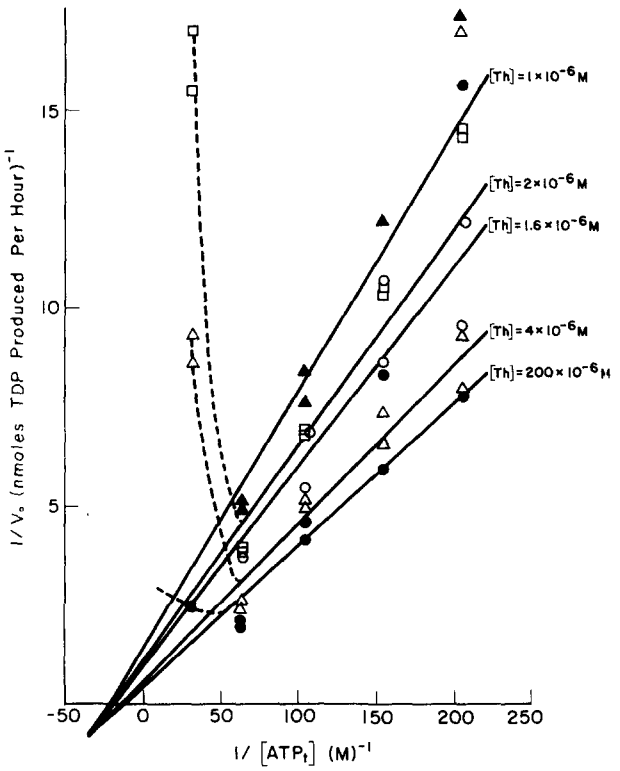

Fig. 6. A plot of the $1/v_o$ vs $1/ATP_t$ concentrations at various fixed initial concentrations of thiamine, and at a fixed ratio $[Mg_t]/[ATP_t] = 0.6$. Values represent duplicate determinations: —●——●—, $2.00 \cdot 10^{-4}$ M thiamine; △——△, $4.0 \cdot 10^{-6}$ M thiamine; □——□, $2.0 \cdot 10^{-6}$ M thiamine; ○——○, $1.6 \cdot 10^{-6}$ M thiamine; ▲——▲; $1 \cdot 10^{-6}$ M thiamine. Standard assay conditions and procedure otherwise employed (see Materials and Methods).

and a reciprocal velocity equation with thiamine (Th) (B) as the variable substrate may be written as:

$$\frac{1}{v_{o,f}} = \frac{K_B}{V(B)_f} + \frac{1}{V_f}\left[1 + \frac{K_A}{(A)}\right]; \tag{4}$$

where $K_B = K_{Th}$, (B) = [Th], $K_A = K_{ATP}$, and (A) = $[ATP]_t$. It can be seen (Eqn 4) that the slope of each parallel line is equal to K_B/V_f with the Y-intercept equal to $1/V_f\ (1 + K_A/(A))$. From a secondary plot of the Y-intercept versus 1/(A) (Fig. 7B), values for $1/V_f$ (from the intercept), and K_A/V_f (from the slope) are obtained, thus permitting an evaluation of K_A and V_f; K_B is obtained from the primary slopes, together with a value of V_f from the secondary plot. An average of $5.0 \cdot 10^{-6}$ M for $K_B = K_{Th}$ is obtained (see legend to Fig. 7A), and a value for $K_A = K_{ATP} = 5.5 \cdot 10^{-2}$ M, which is approx. twice the value of $\overline{K}_A$ assigned for a "sequential" mechanism (see above). Although the data have been statistically evaluated; until the mechanism may be more rigorously defined (see Discussion below) statistical uncertainties are of little meaning and hence are not presented here.

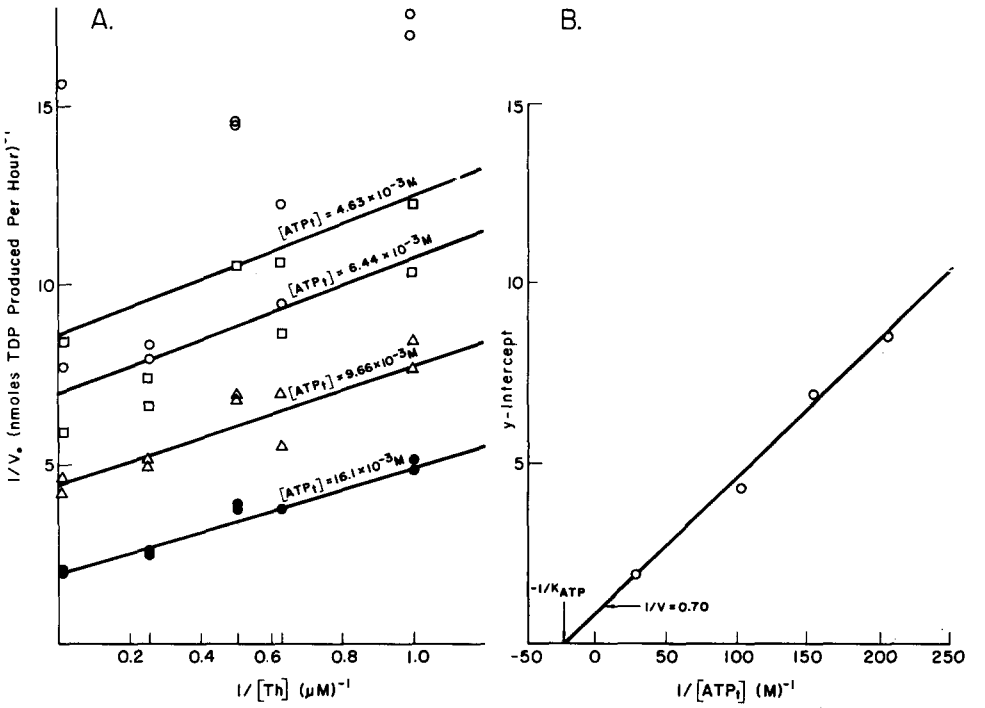

Fig. 7. (A) $1/v_o$ vs $1/[Th]_o$ at several fixed values of ATP_t, and $[Mg_t]/[ATP_t] = 0.6$; other conditions the same (see Materials and Methods). Th, denotes thiamine. ●———●, $16.1 \cdot 10^{-3}$ M ATP_t; △———△, $9.66 \cdot 10^{-3}$ M ATP_t; □———□, $6.44 \cdot 10^{-3}$ M ATP_t; ○———○, $4.63 \cdot 10^{-3}$ M ATP. Values of $K_B = K_{Th}$ calculated for each curve (see Text. Eqn 2), are as follows: $4.3 \cdot 10^{-6}$ M (●———●), $4.9 \cdot 10^{-6}$ M (△———△), $5.4 \cdot 10^{-6}$ M (□———□), $5.4 \cdot 10^{-6}$ M (○———○), yielding an average value of $5.0 \cdot 10^{-6}$ M. (B) Secondary plot of data given above, fitted to the expression (from Eqn 2, see text) for Y-intercepts: Y-intercept = $1/V_f + 1/V_f K_A(A)$; where K_A was found to be $5.5 \cdot 10^{-2}$ M.

Equilibrium studies

Table II summarizes the data obtained for the preliminary estimations of the apparent equilibrium coefficient (Eqn 2) estimated for the 12 sets of initial reactant concentrations. A comparatively wide variation in the calculated values for $K_{eq,app}$ is observed over the range of $(ATP_t)_{initial}$ concentrations selected, but with an apparent convergence to a value which lies between $0.9 \cdot 10^{-5}$ and $1.6 \cdot 10^{-5}$ at the lower range of ATP_t initial concentrations studied, i.e. at $5 \cdot 10^{-3}$–$6 \cdot 10^{-3}$ M.

No attempt, at present, has been made to evaluate a thermodynamic equilibrium constant in terms of the metal complexed nucleotides (cf. Kuby and Noltmann [33], calculations for the rabbit muscle ATP-creatine transphosphorylase-catalyzed reactions) until (a) values for the chelation constants of magnesium and thiamine diphosphate may be estimated, and (b) an evaluation is made of those metal chelates of ATP which actually participate as reactants; although at true thermodynamic equilibrium, one may define an equilibrium constant in terms of selected chelate species of the nucleotides.

It is clear, however, that the equilibrium position for this reaction is very far towards the side of the reactants, and that the transpyrophosphorylation reaction is actually thermodynamically unfavorable.

TABLE II

DETERMINATION OF THE EQUILIBRIUM POSITION FOR THE THIAMINE PYROPHOSPHATE-CATALYZED REACTION, AT pH 7.8, 37°C IN $5.56 \cdot 10^{-2}$ M GLYCYLGLYCINE

Th, thiamine.

$[ATP]^*_{initial}$ (mM)	$[Th]_{initial} = 2 \times 10^{-6}$ M		$[Th]_{initial} = 1 \times 10^{-6}$ M
	$K_{eq, app}$ at 5 h ($K \times 10^5$)	$K_{eq, app}$ at 9 h ($K \times 10^5$)	$K_{eq, app}$ at 5 h ($K \times 10^5$)
32.2	0.252	0.252	0.258
16.1	3.9	3.4	1.5
9.66	2.5	1.6	0.9
6.44	3.3	1.3	0.9
4.83	4.1	1.6	1.1

* $[Mg_t]/[ATP_t] = 0.6$.

A note on the purity of the enzyme

By sedimentation velocity, the enzyme preparation from the electrofocusing step displayed two distinct components and one fraction from the alumina C_γ gel step of high specific activity (83 units/mg) by polyacrylamide disc-gel electrophoresis, revealed five distinct protein bands, after staining with Coomassie blue. A preliminary attempt was made to estimate the subunit molecular weights in the final preparation, using a Tanford column of agarose swelled in 6 M guanidinium chloride as described by Palmieri et al. [35], but, because of the inhomogeneity of the enzyme preparation, this determination and other estimations of its molecular properties were unsuccessful.

Discussion

The direct, radiometric assay used in this study was simpler and far more sensitive that the coupled enzyme systems employed by earlier workers (see e.g. refs 16 and 25), and it alleviated the necessity for the several enzymes used in these coupled enzyme assays. Furthermore, for the kinetic studies, all extraneous reaction components required for the coupled enzyme assays, which might prove inhibitory, could be eliminated.

The 260-fold purification of pig brain thiamine pyrophosphokinase compares favorably with other purifications of the mammalian enzyme reported (e.g. refs 14,16,18). The most highly purified preparations of pig brain enzyme were quite unstable, especially in solutions of low ionic strength.

The optimum $[Mg_t]/[ATP_t]$ ratio of 0.6 observed in this study is close to that reported by Deus et al. [26] for purified rat liver thiamine pyrophosphokinase in contrast to the results of Mano and Tanaka [15]. The present observation suggests but does not prove the possibility that $Mg(ATP)_2^{6-}$ may be the enzymatically active reactant in the pyrophosphoryl transfer reaction. Since the chelation constant for $Mg(ATP)_2^{6-}$ is only approx. 400 at 37°C compared to approx. 90 000 for $MgATP^{2-}$ [33], the latter would be the major chelate species in solution. Thus, the relatively large measured value for K_{ATP} calculated from the total ATP_t concentration could be accounted for on this basis,

since the actual "substrate" concentration and K_{ATP} would be far smaller than the values assigned from the total concentrations, unless corrected for the relatively huge concentrations of $MgATP^{2-}$ and of ATP^{4-}, in solution, compared to $Mg(ATP)_2^{6-}$. The postulate of $Mg(ATP)_2^{6-}$ as the actual substrate could explain another apparent dilemma, viz., that the in vivo concentration of ATP in brain is reported to be only approx. 3 mM [27], a concentration which would drastically reduce the v_o of thiamine pyrophosphokinase if the measured constants were the actual constant in situ.

To date, there have been very few studies on the kinetics of the reaction catalyzed by thiamine pyrophosphokinase. This has probably been due, at least in part, to the lack of an enzyme preparation of sufficient purity. With the exception of Thome-Beau et al. [25], others have been content to report apparent K_m values for thiamine and ATP without taking into account the fact that the concentration of one substrate may effect the value of the K_m of the second substrate. As shown in Figs 3—5, it is possible to obtain apparent K_m values for ATP and thiamine, as well as apparent inhibition constants by thiamine analogs. But, as can be seen from Figs 6 and 7, the kinetics of this reaction are, in actual fact, rather complex. The data shown in Figs 6 and 7 indicate that the reaction may proceed by apparently two contradictory mechanisms, either through the formation of a ternary complex, or possibly through a covalent intermediate. The mechanism could be resolved by the detection of a pyrophosphoryl enzyme as well as by a detailed examination of the reverse reaction, together with a study of the nature of the inhibition by the products. Hence these kinetic data should be considered as preliminary only.

It should be noted, however, that a combination of experimental error and the magnitude of the $\overline{K}_A \cdot K_B$ term in the denominator of the rate equation (cf. Eqns 3 and 4) may present a limit to the detection of a point of convergence in the double reciprocal plots. Thus, if $K_B \ll \overline{K}_A$, and even if the products were not zero, a case of parallel plots may actually appear to result for a given reactant, but within experimental error, therefore, a sequential mechanism may in essence predominate. (One notes that K_B may by 10^{-4} smaller than $\overline{K}_A$).

The inhibitor constants given in Fig. 5 probably give an indication of the ability of the thiamine antagonists to compete with the vitamin as acceptors of the pyrophosphoryl group from a derivatized enzyme, which provides an explanation for the competitive type of inhibition to be found in each case. The fact that the most potent inhibitors all contain an amino group at position four on the pyrimidine ring supports the theory of Cerecedo [28] that the amino group at position four on the pyrimidine ring is necessary for effective binding of the substrate to the enzyme; moreover, this idea is reinforced by the relatively poor affinity of oxythiamine to the enzyme.

The fact that the kinetics, (with respect to thiamine as the variable substrate), are a function of the ATP_t concentration suggests that the values for the inhibitor constants may also, to some degree, depend on the ATP_t concentration. Thus, it may not be valid to make a comparison of the inhibition constants found in this study with those given in previous studies. It is interesting that the thiamine analogs which have been reported to cause neurological

symptoms, i.e. butylthiamine and pyrithiamine, both have K_i values of the same order of magnitude as the K_m for thiamine, but pyrithiamine is a much more effective antimetabolite. Therefore, the effectiveness of pyrithiamine as an antithiamine agent cannot be wholly ascribed to its effect on thiamine pyrophosphokinase. This conclusion is strengthened by the fact that ethylthiamine, which has been reported to have thiamine-type activity in vivo [29] and in vitro [15], was also found to have a K_i of the same order of magnitude as pyrithiamine. On the other hand, oxythiamine, which is a relatively poor antithiamine agent (compared to pyrithiamine), has a K_i 400 times greater than pyrithiamine. Therefore, there may be some correlation between the effectiveness as an antithiamine agent and the ability to compete with thiamine as an acceptor for the pyrophosphoryl group.

In this report, no direct proof was obtained as to whether these inhibitors were actually pyrophosphorylated by ATP in the presence of pig brain thiamine pyrophosphokinase. However, some investigators have reported evidence which suggests that the pyrophosphate esters of some inhibitors are actually enzymatically synthesized [16,19,30]. The fact that Koedam and Steyn-Parve [30] showed that pyrithiamine pyrophosphate was produced in the presence of purified rat liver enzyme lends credence to the idea that purified pig brain enzyme may also catalyze the formation of pyrithiamine pyrophosphate.

This study lends support to the concept that, in vivo, those antagonists with high affinity for thiamine pyrophosphokinase (pyrithiamine, butylthiamine, and ethylthiamine) may exert their inhibitory effects, in part, by competing with thiamine for the enzyme, thus preventing the pyrophosphorylation of thiamine. On the other hand, as discussed by Gubler [31], oxythiamine probably exerts its main inhibitory effect in vivo after it is pyrophosphorylated, by competing with thiamine diphosphate for binding to the various thiamine diphosphate-requiring enzymes. Yet it is still possible that the main point of inhibition of all the thiamine antagonists discussed is at the pyrophosphorylation step, with oxythiamine further inhibiting by binding to thiamine diphosphate-requiring enzymes. If one considers that a 5 to 1 ratio of pyrithiamine to thiamine is sufficient to bring about the symptoms of thiamine deficiency, whereas a 200 to 1 ratio of oxythiamine to thiamine is required to do the same [31,32], it would appear that it is important, for in vivo studies, to insure that a large excess of antagonist is present to inhibit the pyrophosphorylation of thiamine. But, whatever the case may be, it can be stated with certainty that the actions of the antagonists cannot be completely explained on the basis of their effects on brain thiamine pyrophosphokinase alone.

Lastly, the preliminary estimations of the apparent equilibrium coefficient of the reaction is deserving of mention. Thus, if 10^{-5} may be taken as a measure of the apparent equilibrium constant at pH 7.8, and 37°C, a value for $\Delta F'^{\circ}$ may be calculated to be approx. +7 kcal/mol, a very unfavorable reaction, which would be made even more unfavorable thermodynamically, if one considered the reaction between the uncomplexed species and the fact that one H^+ is required to maintain electrical neutrality for the reaction: $Th^+ + ATP^{4-} \rightleftharpoons AMP^{2-} + TDP^{3-} + H^+$ where Th and TDP denote thiamine and thiamine diphosphate, respectively. Thus, a minimum value of $10^{-5} \cdot 10^{-7.8} = 10^{-12.8}$ must be assigned for the equilibrium constant of this reaction, or a $\Delta F^{\circ} = +18$ kcal/mol.

One notes that, under comparable conditions, a pH-independent K_{eq} for the uncomplexed nucleotide species leads to a ΔF^{o} of 11.8 kcal/mol at 38°C for the ATP-creatine transphosphorylase reaction [33], which involves a phosphoryl group transfer in contrast to the pyrophosphoryl group transfer for the thiamine pyrophosphate-catalyzed reaction. It is likely that the larger free energy barrier in the pyrophosphoryl group transfer lies in the difference between the standard free energies of the following reactions: (1) the hydrolysis between the α and β phosphoryl groups of ATP to yield pyrophosphate and AMP, and (b) the hydrolysis of thiamine diphosphate at the 5-(β-hydroxyethyl) group of the methylthiazole ring to yield thiamine and pyrophosphate; neither of these two reactions have been quantitatively evaluated thermodynamically and it would be of great interest to further pursue the energetics of these interesting pyrophosphoryl group reactions.

Acknowledgments

Supported in part by grants (e.g. no. AM-02448) from the National Institutes of Health, and one from the National Science Foundation. In part, this work was taken from the Ph.D. thesis of J.W. Peterson.

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