Communication

Transition State Analogs for Thiamin Pyrophosphate-dependent Enzymes

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SUMMARY

Many of the transition states that are formed from thiamin pyrophosphate in enzymic reactions are expected to have structures in which the thiazolium ring of thiamin pyrophosphate has lost most of its positive charge. We have synthesized thiamin thiazolone pyrophosphate from the unphosphorylated compound. The sulphur-containing ring of thiamin thiazolone pyrophosphate is uncharged, and thus the compound resembles these transition states. In agreement with the prediction from the transition state theory of reaction rates, thiamin thiazolone pyrophosphate binds to *Escherichia coli* pyruvate dehydrogenase complex (EC 1.2.7.1) much more strongly than thiamin pyrophosphate itself. An upper limit for the value of the dissociation constant, calculated from the extent of inactivation of the enzyme by a low concentration of thiamin thiazolone pyrophosphate, is 5 × 10⁻⁵ M at 3° in 0.5 mM MgCl₂/10 mM potassium phosphate, pH 6.6. The dissociation constant for thiamin pyrophosphate under similar conditions is about 10⁻⁴ M. The kinetics of inactivation of pyruvate dehydrogenase complex by thiamin thiazolone pyrophosphate are first order with respect to both enzyme and thiamin thiazolone pyrophosphate; the value of the second order rate constant is 5.7 × 10⁵ M⁻¹ min⁻¹ at 3° in 0.5 mM MgCl₂/10 mM potassium phosphate, pH 6.6. An analysis of the decrease in the rates of inactivation caused by thiamin pyrophosphate indicates that thiamin thiazolone pyrophosphate binds to the coenzyme sites. We have also synthesized thiamin thiothiazolone pyrophosphate and obtained very similar results with this compound.

For most thiamin-PP⁺-dependent enzymic reactions, the major covalent changes that occur during catalysis are well established (1). A common feature of these mechanisms is that the high energy intermediates, unlike thiamin-PP itself, have structures in which the sulfur-containing ring is uncharged. For example, the initial steps in the thiamin-PP-dependent decarboxylation of pyruvate involve two metastable intermediates—the ylide, which is formed by abstraction of a proton from carbon 2 of the thiazolium nucleus, and the enamine, which is the immediate product of the decarboxylation of the pyruvate adduct.

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Abbreviations used are: thiamin-PP, thiamin pyrophosphate; TTP and TTPP, thiamin thiazolone phosphate and pyrophosphate; TTP and TTPPP, thiamin thiazozone phosphate and pyrophosphate (3-[(4-amino-2-methyl-5-pyrimidinyl)ethy]3-(3-hydroxyethyl)4-methyl 2(3H) thiazolone phosphate and pyrophosphate); TTPP and TTPPP, thiamin thiothiazolone phosphate and pyrophosphate (Chemical Abstracts name as for thiazolone, except 2(3H)thiazolonedione).

In this report we describe the synthesis of these compounds and provide evidence that, in accord with the prediction for transition state analogs (3, 4), they bind to the thiamin-PP sites of *Escherichia coli* pyruvate dehydrogenase complex much more strongly than does thiamin-PP itself.

MATERIALS AND METHODS

Thiamin disulfide was purchased from ICN Pharmaceuticals. *Escherichia coli* alkaline phosphatase and thiamin-PP chloride were products of the Sigma Chemical Co. The thiamin-PP was purified by chromatography on Bio-Rad AG1 (formate) resin (5), followed by passage through Bio-Rad AG-50W (ammonium) at pH 7 and lyophilization. Thiamin thiothiazolone was a gift from Dr. Frank Jordan, Rutgers University. It was recrystallized from chloroform-ethanol to give material of melting point 238-240° (literature value, 240-242° (6)).

* E. coli (Crookes strain) pyruvate dehydrogenase complex (33 mg/ml in 50 mM potassium phosphate, pH 7) was the generous gift of Dr. Lester Reed, University of Texas. According to Dr. Reed, the purity of the enzyme was 90 to 95% from analysis by sedimentation velocity ultracentrifugation and the specific activity was 24 units (micromoles of NADH formed per min) per mg. We found the activity of the enzyme to be 15 units per mg. This difference may be due to some loss of activity in transit or to slight differences in the assay procedure.

The enzyme was assayed by following the reduction of NAD⁺ by 340 nm with a Gilford recording spectrophotometer. The assay mixture contained 0.1 mM CoA, 1 mM cysteine, 2 mM NAD⁺, 1 mM sodium pyruvate, 0.1 mM thiamin-PP, 1 mM MgCl₂, and 100 mM potassium phosphate, pH 7.0 (7). Reaction was initiated by the addition of 2 μg or less of the enzyme to the mixture (final volume, 1.0 ml), which was equilibrated at a temperature such that the temperature after the addition of the enzyme was 30°. The rates were linear for at least 30 s. The enzyme showed no activity in the absence of added thiamin-PP. This fact suggests that it contains little or no bound coenzyme.
Synthesis of TTPP and TTTPP—Thiamin thiazolone, melting point 234-236° (literature value, 237°), was prepared from thiamin disulfide, according to the method of Sykes and Todd (6). The thiazolone (0.35 g) was phosphorylated with pyrophosphoryl chloride by the procedure that Hecht and Hawrelak (9) have described for the phosphorylation of 2',6'-O-methyladenosine. TTPP was isolated from the aqueous extract of the reaction mixture at pH 7 by adsorption on Bio-Rad AG1 resin in the formate form. The resin was washed with water and the compound, which bears no net charge at pH 2.5 due to protonation of the pyrimidine ring (pK about 5 (10)), was then released from the resin by adjusting the pH of the suspension to 2.5 with formic acid. The acidic extract was concentrated by rotary evaporation and then lyophilized. This yielded 1.1 g of TTPP that was, on the basis of its ultraviolet spectrum and total phosphorus content, about 70% pure by weight. The crude monophosphate (500 mg) was phosphorylated with mono[(3-n-butylammonium) phosphate and 1',1'-carbonyldimidazole in dimethyl formamide. This reaction was carried out by the procedure that Kozarich et al. (11) employed to prepare 2',5'-di-O-(1-methoxyethyl)adenosine 3'-diphosphate from the monophosphate, with the exception that TTPP was treated with 2 mol of tri-n-butylamine. The residue that remained upon evaporation of the dimethyl formamide was dissolved in water; and the aqueous solution, after adjustment to pH 6 with HCl, was applied to a column (2.4 x 9 cm) of Bio-Rad AG50W (ammonium) resin. TTPP was eluted between 0.6 and 1.6 liters of a gradient created through the introduction of 1 M ammonium acetate, pH 6, into 1 liter of water. Water and ammonium acetate were removed by rotary evaporation and then lyophilized. An aqueous solution of this crude product was adjusted to pH 8 with ammonia and passed through a short column of Bio-Rad AG50W (ammonium) resin. After lyophilization of the eluate, the residue was dissolved in several ml of water; the pH was adjusted to 8 with ammonia; and a mixture of acetone/ethanol (9/1, v/v) was added at 60° with vigorous mixing until a slight precipitate remained. The TTPP that separated upon cooling the mixture to 4° was dried under vacuum over P2O5. The yield was 300 mg. This procedure of passage through the ammonium form of Bio-Rad AG50W and precipitation from acetone/ethanol was also used to purify TTPP.

The preparations of TTP and TTTPP showed single ultraviolet-absorbing components of Rs 0.67 and 0.50, respectively, upon thin layer chromatography (Eastman cellulose plates with fluorescent indicator; ethanolic butanol/15 M sodium citrate, pH 4 (10/1/6 by volume) as the solvent). They exhibited single ultraviolet-absorbing components upon paper electrophoresis at pH 4 and 7 with mobilities similar to those of AMP and ADP, respectively. Analysis of the phosphoryl component (12) gave values of 8.0 and 8.3% for TTP and 13.3 and 13.6% for TTTP; the theoretical values for the monophosphate, with the exception that TTP was treated with 2 mol of tri-n-butylamine. The residue that remained upon evaporation of the dimethyl formamide was dissolved in water; and the aqueous solution, after adjustment to pH 6 with HCl, was applied to a column (2.4 x 9 cm) of Bio-Rad AG50W (ammonium) resin. TTPP was eluted between 0.6 and 1.6 liters of a gradient created through the introduction of 1 M ammonium acetate, pH 6, into 1 liter of water. Water and ammonium acetate were removed by rotary evaporation and then lyophilized. An aqueous solution of this crude product was adjusted to pH 8 with ammonia and passed through a short column of Bio-Rad AG50W (ammonium) resin. After lyophilization of the eluate, the residue was dissolved in several ml of water; the pH was adjusted to 8 with ammonia; and a mixture of acetone/ethanol (9/1, v/v) was added at 60° with vigorous mixing until a slight precipitate remained. The TTPP that separated upon cooling the mixture to 4° was dried under vacuum over P2O5. The yield was 300 mg. This procedure of passage through the ammonium form of Bio-Rad AG50W and precipitation from acetone/ethanol was also used to purify TTPP.

**RESULTS**

Specific Inactivation of Pyruvate Dehydrogenase Complex by TTPP—When pyruvate dehydrogenase complex was incubated with TTPP and subsequently assayed for activity, we found that the enzyme was inactivated (Table I). Related compounds caused little or no inactivation (Table I). It was possible to titrate the enzyme with TTPP (Fig. 1). There appeared to be one site per about 180,000 daltons. Since the *Escherichia coli* pyruvate dehydrogenase complex has a molecular weight of about 4.6 x 10° (14) and binds about 24 molecules of thiamin-PP (7), this value is in approximate agreement with that expected if the preparation of enzyme is substantially pure and if TTPP binds to the thiamin-PP sites. However, because of the less than maximal specific activity of our enzyme preparation, further investigation will be required to establish this stoichiometry. Here, our intention is to illustrate the potential usefulness of TTPP as an active site titrant.

In order to estimate an upper limit for the dissociation constant of TTPP bound to the enzyme, we treated a very low concentration of enzyme (3.3 mg/ml) with 10° M each compound at 7° in 0.5 mM MgCl2/10 mM potassium phosphate, pH 6.6, for 1 hour. Then the residual activity was determined by assay of an aliquot. The percent activity is the ratio of the activity after 1 hour to the initial activity in the absence of the compound.

| Compound added | % activity |
|----------------|------------|
| None           | 100        |
| Thiamin-PP     | 95         |
| Thiamin thiazolone pyrophosphate (TITPP) | <5 |
| Thiamin thiazolone phosphate (TTPP) | 97 |
| Thiamin thiazolone | 92 |
| Thiamin thiothiazolone phosphate (TTTPP) | <5 |
| Thiamin thiothiazolone phosphate (TTPP) | 87 |
| Thiamin thiothiazolone | 102 |
| ATP            | 90         |
| ADP            | 100        |

a Concentration was 10−4 M.
b Concentration was 5 x 10−5 M.

**FIG. 1.** Titration of the pyruvate dehydrogenase complex with TTPP. Enzyme at a concentration of 50 μg/ml was incubated with the stated concentrations of TTPP in 0.5 mM MgCl2/10 mM potassium phosphate, pH 6.6, at room temperature. After 20 min (O) and 1 hour (C), 10-μl aliquots were assayed for activity; ΔA/min is the initial rate of change in absorbance at 340 nm.
binding sites) with a slight excess of TTPP \((2.5 \times 10^{-4} \text{ M})\) at \(3^\circ\) in 0.5 mM MgCl\(_2/10\) mM potassium phosphate, pH 6.6. Aliquots were removed periodically and assayed for enzymic activity. After 8 hours, the activity was less than 5% of that of enzyme that was incubated in the absence of inhibitor. The enzyme alone decreased in activity by only 10% over this period. Thus, the concentration of the active sites associated with TTPP \((E)\) reached a value that was at least 19 times larger than that of uncomplexed sites \((E)\). Since the dissociation constant \((K_i)\) is equal to \([E] [I]/[EI]\) where \([I]\) is the concentration of unbound inhibitor, we calculate an upper limit for \(K_i\) of less than \((1/19) (2.5 \times 10^{-8} - (18/20) (1.7 \times 10^{-8}))\) or \(5 \times 10^{-10} \text{ M}\).

Kinetics of Inactivation of Pyruvate Dehydrogenase Complex by TTPP—The rate of loss of activity in the presence of TTPP was followed through the assay of samples from the reaction mixture at time intervals. With an excess of TTPP, the decrease in activity was first order (Fig. 2, closed circles). The values of the pseudo-first order rate constants for the inactivation of 8 \(\mu\)g/ml of pyruvate dehydrogenase by 1.25, 2.5, and \(5.0 \times 10^{-4} \text{ M} \) TTPP, at \(3^\circ\) in 0.5 mM MgCl\(_2/10\) mM potassium phosphate, pH 6.6, were found to be 0.006, 0.15, and 0.29 min\(^{-1}\), respectively. Thus, the rate constant is directly proportional to the concentration of TTPP, and the reaction is second order. The average value of the second order rate constant is \(5.7 \times 10^{-8} \text{ M}^{-1} \text{ min}^{-1}\).

Thiamin-PP decreased the rate of inactivation of pyruvate dehydrogenase complex by TTPP (Fig. 2). The simplest scheme for the interpretation of this effect is one in which the inhibitor \((I)\) can combine with each pyruvate dehydrogenase subunit \((E)\) (14, 15) of the pyruvate dehydrogenase complex only when thiamin-PP \((S)\) is not bound to the subunit, presumably because of competition for the same site:

\[
ES \xrightarrow{k_+} E + I \xrightarrow{k_-} EI
\]

Here \(K_i\) is the equilibrium dissociation constant for thiamin-PP and \(k_+\) is the second order rate constant for inactivation. If it is assumed that the rate of combination of enzyme with thiamin-PP is much larger than that of enzyme with TTPP so that the equilibrium between \(E\) and \(ES\) is maintained during the inactivation, the observed first order rate constant for formation of \(EI\) is given by

\[
k_{\text{obs}} = \frac{k_+ [I] K_S}{K_i + [S]} \quad \text{or} \quad \frac{1}{k_{\text{obs}}} = \frac{[S]}{k_+ [I] K_S} + \frac{1}{k_i [I]}
\]

The inserted plot in Fig. 1 shows that this equation describes the data. The value for \(K_i\), given by the ratio of the intercept to the slope, is \(9 \times 10^{-8} \text{ M}\).

Reversibility of Reaction of Pyruvate Dehydrogenase Complex with TTPP—Enzyme at a concentration of 15 \(\mu\)g per ml that had been inactivated by treatment with \(1.25 \times 10^{-7} \text{ M}\) TTPP was dialyzed against 1000 volumes of 0.5 mM MgCl\(_2/10\) mM potassium phosphate, pH 6.6, at \(3^\circ\) for 27 hours, and then further dialyzed for 24 hours against a second 1000 volumes of buffer. During this period, the activity of the enzyme rose from less than 0.25 to about 0.75 unit per mg. Identical treatment of enzyme that had not been inactivated resulted in a fall in activity from 15 to 12 units/mg. Thus only about 5% of the initial activity was recovered during dialysis. This minimal reactivation is not unexpected in view of the values of the rate constant for inactivation and the upper limit for the dissociation constant given above. From these, we can calculate an upper limit for the first order rate constant for dissociation of bound TTPP, the value of which corresponds to a half-time of 40 hours.

The nature of the interaction between TTPP and the enzyme appears to be noncovalent, since TTPP was released under denaturing conditions. Three milligrams of pyruvate dehydrogenase complex (16 nmol of TTPP binding sites) in 1 ml of 0.5 mM MgCl\(_2/10\) mM potassium phosphate was treated with 10 nmol of TTPP. This solution was dialyzed against water in order to remove the buffer and any unreacted TTPP. The dialysate was shaken with 0.10 ml of chloroform/ethanol (1/1), and the mixture placed in a boiling water bath for 5 min. After removal of the precipitated protein by centrifugation, the supernatant was concentrated under nitrogen and then subjected to thin layer chromatography in the system described under "Materials and Methods." An ultraviolet-absorbing compound with the mobility of TTPP and the intensity expected for about 10 nmol was detected.2 Enzyme that had not been treated with TTPP yielded no compound in this region of the chromatogram.

TTTPP—Experiments identical with those described above, with the exception of the titration of the binding sites and the recovery of inhibitor after denaturation, were also performed with TTPP. The results, including the values of the upper limit for the dissociation constant and of the second order rate constant for inactivation, were substantially the same as those obtained with TTPP (data not presented, except for that in Table I).

DISCUSSION

The effect of thiamin-PP upon the kinetics of inactivation of pyruvate dehydrogenase complex by TTPP indicate that TTPP and thiamin-PP compete for the same binding sites. Moe and

2 We have also carried out an experiment of the type described here with 5 mg of Escherichia coli pyruvate dehydrogenase complex kindly supplied by Dr. Richard Perham, Cambridge University; the same result was obtained.
Hammes (7) have found that the complex contains about 24 independent binding sites for thiamin-PP, with a single intrinsic dissociation constant. The value of this dissociation constant at 3° in 0.5 mM MgCl₂/10 mM potassium phosphate, pH 6.2, is $12 \times 10^{-6}$ M. Since our conditions differ from these only in the pH (6.6 rather than 6.2), it seems likely that the value of the dissociation constant for thiamin-PP under our conditions should not differ by more than a factor of 2 or 3; and the value of $9 \times 10^{-6}$ M that we obtain from the kinetic analysis is thus a reasonable one. A comparison of this value with the upper estimate of the dissociation constants for TTPP and TTPP shows that these inhibitors bind at least 20,000 times more tightly than the coenzyme. This finding is evidence that they are transition state analogs (3, 4) and furthermore, that the mechanisms that provided the basis for their design are correct. The remarkable difference between the affinity of the protein for the coenzyme and its affinity for the transition state analogs may be due to the positioning of the sulfur-containing ring of these compounds either in a nonpolar region of the apoenzyme or in proximity to a positively charged group of the apoenzyme. Transfer of the positively charged thiazolium nucleus from water to either a hydrophobic or a positively charged environment should be energetically much less favorable than transfer of the uncharged thiazolone nucleus. Spectroscopic studies (16, 17) have shown that the thiamin binding site of the related enzyme, pyruvate decarboxylase, is hydrophobic. On the other hand, the relative affinities for various coenzyme analogs have led to the suggestion that there is a positively charged group at the thiamin site of the enzyme transketolase (18).

Because of the common features of the mechanisms of thiamin-PP-dependent enzymic reactions (1), TTPP and TTPPP should prove to be potent inhibitors of other enzymes that function with this cofactor. The compounds should be useful for the titration of thiamin-PP binding sites and for metabolic studies in which the inhibition of a thiamin-PP-dependent enzyme is desired.

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