Reactivity of Pyridoxamine-Pyruvate Transaminase with Sulfhydryl Reagents*

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Wherever the point has been examined (e.g. References 1-6), pyridoxal phosphate enzymes have been found to require free sulfhydryl groups for activity. The functional role played by these groups, however, has not been clear. In glutamate-oxaloacetate transaminase, which has been studied most extensively, no spectrophotometric or enzymatic evidence for their participation in enzyme binding was at first obtained (4, 5). Recent preliminary results, however, indicated that certain free sulfhydryl groups are required for the binding of pyridoxamine phosphate by the apotransaminase (6).

To further clarify the role of sulfhydryl groups in enzymatic transamination, we have studied the simplified case provided by

Pyridoxamine + pyruvate \rightleftharpoons pyridoxal + L-alanine \hspace{1cm}(1)

pyridoxamine-pyruvate transaminase (7-9). Reaction 1, catalyzed by this enzyme, resembles closely a half-reaction of the more complicated over-all reaction catalyzed by the glutamate-oxaloacetate transaminase. Unlike the latter enzyme, pyridoxamine-pyruvate transaminase contains no prosthetic group and is unusually low in half-cystine residues (8). Finally, the binding of pyridoxal by pyridoxamine-pyruvate transaminase (8) resembles in part that of pyridoxal phosphate in glutamate-oxaloacetate transaminase (10, 11) in that an azomethine linkage to an ε-amino group of a lysine residue occurs in each case.

MATERIALS AND METHODS

Sulfhydryl reagents and most other chemicals were obtained from commercial sources. Pyridoxal-L-alanine was prepared by Dr. M. Ikawa in this laboratory by borohydride reduction of pyridoxal in the presence of excess L-alanine. The product was isolated by chromatography over Dowex 50, and was analytically pure. 2-Methyl-3-amino-4,5-bis(hydroxymethyl)-pyridine was a gift from Merck Sharp and Dohme and was supplied through the courtesy of Dr. Karl Folkers. Yeast alcohol dehydrogenase was from the Worthington Biochemical Corporation. Crystalline pyridoxamine-pyruvate transaminase was prepared as described elsewhere (8). Its activity was determined at pH 8.5 by following pyridoxal formation (Reaction 1) either spectrophotometrically at 400 m\textmu or by the phenylhydrazine procedure (7, 8). Its specific activity represents the micromoles of pyridoxal formed per min per mg of enzyme at the specified temperature. Other experimental procedures are given with the tables and figures.

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Fig. 1. Spectrophotometric titration of pyridoxamine-pyruvate transaminase with p-chloromercuribenzoate (pMB). Transaminase (0.6 mg, 5 mmole) was titrated at 25° in either 1.0 ml of 0.1 M Tris-chloride, pH 8.5 (Curve 1), or 1.0 ml of 8 M urea in 0.1 M potassium phosphate, pH 6.9 (Curve 2). Values represent absorbance differences between cuvettes with and without enzyme, corrected for dilution by added reagent, after reaction was complete (1 hour for Curve 1 or 20 min for Curve 2).

Fig. 2. A, comparative rates of binding of p-chloromercuribenzoate (Curve 1, right ordinate) and loss in enzymatic activity (Curve 2, left ordinate) of pyridoxamine-pyruvate transaminase. The reaction mixture contained, per ml, 50 μmoles of potassium phosphate (pH 7.0); 0.6 mg (5 mmoles) of pyridoxamine-pyruvate transaminase, and 50 mmoles of p-chloromercuribenzoate. Incubation temperature was 23°. Small aliquots were removed from the incubation mixture at various times for measurement of their enzymatic activity. B, comparative rates of binding of p-chloromercuribenzoate (Curve 1, left ordinate) and loss of bound pyridoxal (Curve 2, right ordinate) by the pyridoxal-transaminase complex. Conditions were the same as in A except that pyridoxal-transaminase complex was used in place of the free enzyme.

Effect of Substrates and Substrate Analogues on Inhibition by p-Chloromercuribenzoate

Since the pyridoxal-transaminase complex reacts with p-chloromercuribenzoate somewhat more slowly than does the free enzyme (cf. Curve 2, Fig. 2B), that disappearance of the 410 mμ peak actually reflects a decreased affinity of pyridoxal for the transaminase, and not just a change in its mode of binding, was shown as follows. Pyridoxamine-pyruvate transaminase (6 mg, 50 mmol) was allowed to interact with 100 mmol of pyridoxal, then treated with 150 mmol of p-chloromercuribenzoate at pH 7.0 for 1 hour at 23°. Protein was separated from low molecular weight compounds by passing the mixture over a column (1 X 10 cm) of Sephadex G-25 equilibrated with 0.01 M potassium phosphate, pH 7.0, and washing with this buffer.

Changes in absorbance at 412 mμ were noted at the times indicated.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Changes in absorbance at 412 mμ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Native enzyme</td>
</tr>
<tr>
<td></td>
<td>0.230</td>
</tr>
<tr>
<td></td>
<td>Denatured enzyme</td>
</tr>
<tr>
<td></td>
<td>0.255</td>
</tr>
<tr>
<td>5</td>
<td>0.00</td>
</tr>
<tr>
<td>10</td>
<td>0.00</td>
</tr>
<tr>
<td>15</td>
<td>0.300</td>
</tr>
<tr>
<td>30</td>
<td>0.304</td>
</tr>
</tbody>
</table>

Table I

Reaction of native and denatured pyridoxamine-pyruvate transaminase with 5,5'-dithio-bis(2-nitrobenzoic acid)

Two samples of pyridoxamine-pyruvate transaminase (0.6 mg, 5 mmol), each in 1.0 ml of 0.1 M Tris-HCl, pH 8.0, were incubated with 0.1 ml of 5,5'-dithio-bis(2-nitrobenzoic acid) at 23° in the presence and absence of 0.5% sodium dodecyl sulfate. Changes in absorbance at 412 mμ were noted at the times indicated.

The change in absorbance at 250 mμ at the point where transaminase activity is largely lost (Fig. 2A) corresponds to reaction of about two -SH groups per molecule of enzyme. Indeed, when reaction with limiting amounts of p-chloromercuribenzoate is allowed to go to completion, a linear decrease in activity of pyridoxamine-pyruvate transaminase is found until 2 eq of this mercural have been added, at which point almost complete loss of activity has occurred (Fig. 3). Thus, two -SH groups are (a) essential for activity of this enzyme, (b) in some way related to substrate binding, and (c) more sensitive to reaction with p-chloromercuribenzoate than the remaining four -SH groups. The fact that the decrease in activity (Fig. 3) is linear indicates that these two -SH groups are equally reactive with p-chloromercuribenzoate. This equivalent reactivity is emphasized by data of Fig. 4, which show that the rate of change in absorbance at 250 mμ following addition of 1 eq of p-chloromercuribenzoate is essentially identical with that following addition of a second equivalent, and very different from that observed on addition of the third equivalent.

Inhibition by p-chloromercuribenzoate was fully reversed by dialysis against buffered L-cysteine solution at pH 7.0, but not by dialysis against buffer alone (Table II).
zyme (Fig. 2, A and B, Curve 1) and since —SH groups are required for substrate binding (Fig. 2B), the possibility that inactivation of the enzyme by p-chloromercuribenzoate might be prevented by excess substrate was tested. At sufficiently high concentrations both pyridoxal and pyridoxamine prevented such inhibition (Fig. 5). Pyridoxine, pyridoxylalanine, and 2-methyl-3-amino-4,5-bis(hydroxymethyl)pyridine, all of which inhibit transaminase action competitively, also protected against inactivation by the mercurial. The effectiveness of these protective agents parallels closely their affinities for the enzyme as indicated by their $K_m$ or $K_i$ values (8). Especially noteworthy is the effectiveness of pyridoxyl-L-alanine, which is closely related in structure to an assumed enzyme-bound intermediate in the transamination reaction and has a very high affinity for the transaminase (8). These protective effects apparently result from physical or chemical interaction of the substrate (or analogue) with the active site of the enzyme in such a way that the essential —SH groups become inaccessible to the mercurial. No reversal of inhibition is observed if substrates

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**TABLE II**

<table>
<thead>
<tr>
<th>Percentage of initial activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equivalents of p-chloromercuribenzoate added</td>
</tr>
<tr>
<td>--------------------------------------</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
</tr>
<tr>
<td>2.0</td>
</tr>
<tr>
<td>3.0</td>
</tr>
</tbody>
</table>

* The specific activity of untreated enzyme was unchanged after dialysis against either cysteine or potassium phosphate.

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Pyridoxyl-L-Alanine

Pyridoxine

Pyridoxamine

3-Amino Analogue

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**Fig. 5.** Protective effect of substrates and substrate analogues against inhibition of pyridoxamine-pyruvate transaminase by p-chloromercuribenzoate. Pyridoxamine-pyruvate transaminase (0.048 mg, 0.4 mmole) was incubated with a 5-fold molar excess of p-chloromercuribenzoate in 0.2 ml of 0.1 M potassium phosphate buffer, pH 7.0, for 30 min at 23°C in the presence of various amounts of the indicated substrates or substrate analogues. The enzymatic activity was then determined in the standard assay. Pyridoxylalanine, pyridoxine, and its 3-amino analogue (2-methyl-3-amino-4,5-bis(hydroxymethyl)pyridine) are inhibitory substrate analogues (8). The extent of inhibition (never over 31%) due to their presence in the assay mixture was estimated separately, and the values plotted have been corrected accordingly.

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**Fig. 4.** Comparative rates of reaction of pyridoxamine-pyruvate transaminase with the first, second, and third equivalent of p-chloromercuribenzoate. To 0.6 mg (5.4 mmoles) of pyridoxamine-pyruvate transaminase in 1.0 ml of 0.1 M potassium phosphate buffer, pH 7.0, 5 mmoles of p-chloromercuribenzoate were added at 0, 20, and 40 min, as indicated by the arrows. Reaction was allowed to proceed at 23°C.

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**Fig. 3.** Stoichiometry between loss in enzymatic activity of pyridoxamine-pyruvate transaminase and added p-chloromercuribenzoate (pMB). Pyridoxamine-pyruvate transaminase (0.32 mg, 2.7 mmoles) was incubated in 0.5 ml of 0.1 M potassium phosphate, pH 7.0, with p-chloromercuribenzoate as indicated. After 15 min at 22°C, aliquots were removed, and their enzymatic activity was determined.
or substrate analogues are added to the incubation mixture after reaction with p-chloromercuribenzoate is complete. The co-substrates of the transaminase reaction, L-alanine and pyruvate, exhibit no protective effect in the absence of pyridoxal or pyridoxamine.

**Reaction of p-Chloromercuribenzoate with Pyridoxamine-Pyruvate Transaminase in Presence of Excess Pyridoxine**

The preceding results indicate that compounds that bind to the combining sites for pyridoxal (or pyridoxamine) protect the essential -SH groups of those sites from substitution by p-chloromercuribenzoate, but do not reveal the fate of the four -SH groups which seem to lie outside these sites. To clarify this problem, the transaminase was incubated with a 10-fold molar excess of p-chloromercuribenzoate in the presence of excess pyridoxine, then isolated from the reaction mixture by passage through a Sephadex column. The absorption of the protein fraction at 250 mp indicated that 4 moles of the sulfhydryl reagent had reacted per mole of enzyme (Table III). The activity of the transaminase treated in this way was about 40% of the untreated control. Enzyme treated in a similar fashion in the absence of pyridoxine reacted with the expected 6 moles of p-chloromercuribenzoate and showed no activity. Thus, pyridoxine protects from substitution only the two -SH groups which seem to lie outside these sites. To substitute the remaining four -SH groups reduces but does not eliminate this activity.

The catalytic properties of the modified enzyme carrying four p-chloromercuribenzoate residues have not been investigated extensively. Preliminary results indicate that its $K_a$ values for pyridoxamine, pyridoxal, pyruvate, and L-alanine are not greatly affected by the reaction under standard assay conditions.

**Table III**  
*Effect of pyridoxine on extent of reaction and activity of pyridoxamine-pyruvate transaminase following incubation with excess p-chloromercuribenzoate*

Reaction mixtures contained in 0.7 ml of 0.1 M Tris-HCl (pH 8.5), 1.2 mg (0.01 mmole) of pyridoxamine-pyruvate transaminase, and either 0.1 mmole of p-chloromercuribenzoate or 50 mmole of pyridoxine or both. After 16 hours at 23°C, protein was separated from other components of the reaction mixture by passage over a column (0.8 X 10 cm) of Sephadex G-25 equilibrated with 0.05 M Tris-HCl, pH 8.5. Activity and absorbance of the eluted protein were determined in the usual fashion.

<table>
<thead>
<tr>
<th>Alkylation agent</th>
<th>Pyridoxal formed with addition of various equivalents of p-chloromercuribenzoate (umole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.51 0.27 0.06 0.02</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>0.49 0.27 0.05 0.01</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>0.52 0.25 0.06 0.00</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>0.50 0.25 0.06 0.00</td>
</tr>
</tbody>
</table>

* Pyridoxal formed per 1.2 mg of pyridoxamine-pyruvate transaminase in 5 min at 23°C under standard assay conditions.

**Table IV**  
*Effect of alkylating agents on activity of pyridoxamine-pyruvate transaminase and its sensitivity to p-chloromercuribenzoate*

Reaction mixtures containing, in 0.5 ml, 50 mmole of potassium phosphate (pH 7.0), 0.06 mg (0.5 mmole) of transaminase, and 0.5 mmole of the indicated alkylating agent were incubated at 23°C. After 5 hours, 0.5, 1.0, or 2.0 mmole of p-chloromercuribenzoate were added. The enzymatic activity was determined 30 min later.

<table>
<thead>
<tr>
<th>Enzyme used</th>
<th>Specific radioactivity (cpm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxamine-pyruvate transaminase</td>
<td>9 0</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>375 2</td>
</tr>
</tbody>
</table>

**Effect of Alkylating Reagents on Activity of Transaminase**

The activity of pyridoxamine-pyruvate transaminase was not decreased by incubation with a 1000-fold molar excess of iodoacetate, iodoacetamide, or N-ethylmaleimide (Table IV). The sensitivity to p-chloromercuribenzoate after this treatment was unchanged, showing unequivocally that the two functionally essential -SH groups sensitive to this mercurial had not reacted with the alkylating reagents. Furthermore, incubation with excess radioactive iodoacetate resulted in no fixation of label to the transaminase, although yeast alcohol dehydrogenase was fixed with 0.1 umole of 1.2 mg of pyridoxamine-pyruvate transaminase in 5 min at 23°C under standard assay conditions.
These results show that two —SH groups of equivalent and high reactivity toward p-chloromercuribenzoate are required for catalytic activity of pyridoxamine-pyruvate transaminase, and that the loss in catalytic activity that occurs during reaction with the mercurial proceeds in parallel with a decrease, partial or complete, in ability of the enzyme to bind pyridoxal. Not only pyridoxal or pyridoxamine in excess, but also their inhibitory analogues, protect these two —SH groups from reaction. Since 2 moles of substrate or substrate analogue are normally bound per mole of enzyme (8, 9), it appears that one —SH group is required for each active site, and that these active sites show little or no interaction. The observations are consistent with any of several nonexclusive roles for these —SH groups, e.g. (a) direct participation in binding of the pyridine substrates or substrate analogues; (b) an essential catalytic role of an —SH group so placed that once it reacts with mercurial the active site, for steric or other reasons, no longer combines with substrate; or (c) a conformational role in which maintenance of a functional active site requires that the free —SH group be present even though this group is not directly concerned with either binding or catalysis. Both b and c require that the —SH group become inaccessible for reaction with p-chloromercuribenzoate when an excess of substrate (or substrate analogue) is present, i.e. when the binding site is fully occupied. It is clear that the four sluggishly reactive —SH groups lie outside the active sites of the enzyme, are not essential for catalytic activity, and are not prevented from reaction with p-chloromercuribenzoate by enzyme-substrate interaction. None of the six —SH groups in the native enzyme is accessible to attack by other sulfhydryl reagents tested, but they become so upon denaturation. It may be that the spatial similarity of pyridoxal to the p-chloromercuribenzoate residue contributes to the enhanced sensitivity of the two —SH groups at or near the active site.

These results resemble in some respects those obtained with glyceraldehyde 3-phosphate dehydrogenase. Interaction of this enzyme with a variety of —SH reagents (including iodoacetate) results in loss of activity and of bound DPN, and prior reaction with substrate protects the enzyme from inactivation with iodoacetate (17, 18). A catalytic role of the —SH group has been inferred from this and other evidence (19).

Although results with the pyridoxamine-pyruvate transaminase differ in many respects from those reported for the extramitochondrial glutamate-oxaloacetate transaminase (3–6), there are also many points of similarity. The latter enzyme has a similar molecular weight (approximately 116,000) and binds 2 moles of pyridoxal-P (or pyridoxamine-P) per mole of protein, apparently at independent sites on separate peptide chains (4).

The unmodified enzyme contains 12 to 14 —SH groups per molecule (4, 20), 6 of which (3 per pyridoxal-P) are especially sensitive to p-chloromercuribenzoate (4, 21). When the enzyme is purified by a procedure which involves heating with maleate, certain nonessential —SH groups are alkylated (20); in the modified enzyme thus obtained, two of the first three —SH groups titratable with p-chloromercuribenzoate are required for activity (5). Although reaction with excess p-chloromercuribenzoate does not remove the bound pyridoxal-P (4, 5), similar experiments with the pyridoxamine-P form of the enzyme show that this form of the coenzyme is released by treatment with p-chloromercuribenzoate (6). A relationship between —SH groups and binding of the substituted pyridines that serve as coenzymes or substrates thus appears established for both enzymes. Because the bacterial pyridoxamine-pyruvate transaminase contains so few —SH groups with such clearly separable properties, it would appear to offer especially favorable experimental material for the further analysis of this relationship.

REFERENCES


Reaction of Pyridoxamine-Pyruvate Transaminase with Sulfhydryl Reagents
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