Oxidation of the Carbanion Intermediate of Transaldolase by Hexacyanoferrate(III)*

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The transaldolase-dihydroxyacetone carbanion intermediate formed in the reaction of transaldolase with its donor substrates fructose-6-P or sedoheptulose-7-P is susceptible to oxidation by hexacyanoferrate(III). The dihydroxyacetone moiety is oxidized to the corresponding 2-ketoaldehyde, i.e. hydroxy- pyruvaldehyde (CH₂OH·CO·CHO). This oxidation product is, in contrast to dihydroxyacetone, readily released from the enzyme. In the presence of hexacyanoferrate(III) transaldolase thus functions as an efficient catalyst of the oxidative cleavage of its donor substrates fructose-6-P or sedoheptulose-7-P into hydroxy- pyruvaldehyde and glyceraldehyde-3-P or erythrose-4-P, respectively. Two moles of hexacyanoferrate(III) are released per mole of oxidatively cleaved donor substrate. The molecular activity for oxidative cleavage of fructose-6-P at a hexacyanoferrate(III) concentration of 0.5 mM is 0.65% of that for the normal transfer reaction with erythrose-4-P as the acceptor substrate. The present data emphasize the applicability of certain oxidants as trapping agents for enzymatic carbanion intermediates as proposed previously (Healy, M. J., and Christen, P. (1973) Biochemistry 12, 35-41).

Transaldolase, an enzyme of the pentose phosphate pathway, transfers the 3-carbon fragment dihydroxyacetone from a phosphorylated ketose to an acceptor aldehyde, e.g. sedoheptulose-7-P + glyceraldehyde-3-P = erythrose-4-P + fructose-6-P. The carbonyl group of the donor substrate forms a Schiff base intermediate with the ε-amino group of an active site lysyl residue. Cleavage of the C3–C4 bond releases a phosphorylated aldehyde and results in formation of a Schiff base carbanion of the dihydroxyacetone moiety. Protonation of this intermediate apparently does not occur and the Schiff base intermediate is not hydrolyzed unless C3 of the dihydroxyacetone moiety forms a new bond with an acceptor aldehyde (1).

Oxidation-reduction indicators such as hexacyanoferrate(III) have been reported previously to oxidize the carbanion intermediates of several enzymes and, hence, have been proposed as mechanistic probes for the detection of these intermediates (2). Detailed studies including product analysis on the oxidative trapping of enzyme-substrate intermediates have been carried out with the Schiff base forming class I fructose-1,6-P₂ aldolase from rabbit muscle (2-5) and the class II metalloaldolase from yeast (6). Similar trapping reactions have been found to occur with aspartate aminotransferase (2, 7), pyruvate decarboxylase (2, 8, 9), and 6-phosphogluconate dehydrogenase (2). Recently, such trapping reactions have gained additional interest since they are accompanied by an active site-directed chemical modification of the enzyme (10).

In the present work hexacyanoferrate(III) has been applied as a probe to the mechanism of action of transaldolase. The kinetic features of the oxidative trapping reaction have been determined and its products have been identified.¹

**EXPERIMENTAL PROCEDURE**

**Transaldolase**—Transaldolase (EC 2.2.1.2) from yeast (∼15 units/μg) was obtained as a suspension in ammonium sulfate from Boehringer. Before use it was dialyzed against 10 mM EDTA/100 mM triethanolamine-Cl (pH 7.6). Enzymatic activity was determined photometrically using fructose-6-P and erythrose-4-P as substrates and triose-P isomerase and glycerol-3-P dehydrogenase as auxiliary enzymes (12). The maximum concentration of transaldolase in the assay mixture was 0.03 units/ml. Protein concentration was determined photometrically with A₀.₅₀.₆₅ = 0.76 cm⁻¹ for a 1 mg/ml solution (13). Prior to reduction of the transaldolase-dihydroxyacetone complex with sodium borohydride transaldolase (3.3 units) was incubated with 4 mM fructose-6-P in 300 μl 0.1 M glycylglycine/10 mM EDTA/0.24 mM NADH (pH 8.4) containing 1 μg of triose-P isomerase and 10 μg of glycerol-3-P dehydrogenase for 15 min at room temperature (14). The reaction mixture was acidified to pH 6.0 with 5 n acetic acid, cooled to 0˚, and sodium borohydride (fresh 2 M solution in H₂O) was added in 10-μl portions at 3-min intervals. The pH was maintained between 6.0 to 6.5 by addition of 1 n acetic acid; 30 min after the last addition of borohydride the reaction mixture was dialyzed twice for 1 hour at 4˚ versus 200 volumes of 0.1 M triethanolamine-Cl/10 mM EDTA (pH 7.6). In a control experiment when fructose-6-P was omitted from the reaction mixture, 81% of the original transaldolase activity was still present.

**Other Materials**—L-Glycerol-3-P dehydrogenase (EC 1.1.1.8), triose-P isomerase (EC 5.3.1.1), glyceraldehyde-1 from yeast (S-lactoylglutathione methylglyoxal-lyase (EC 4.4.1.5)), fructose-1,6-P₂ aldolase (EC 4.1.2.13) from rabbit muscle, fructose-6-P diisomerase salt, fructose-1,6-P₂, tetraclobexylammonium salt, fructose-1,6-P₂, diisomerase salt, and NADH were obtained from Boehringer. Alka-

¹ A preliminary account of this work has been presented at the 10th Congress of the European Biochemical Societies, Paris, 1975 (11).

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line phosphate was prepared from Escherichia coli (15). Potassium hexacyanoferrate(III), tetrathionate, peroxidase, 2,6-dichlorophenolindophenol, EDTA, sodium borohydride, 2,4-dinitrophenylhydrazine, and reduced gluathione were from Fluka. Sedoheptulose-7-P barium salt was from Sigma. AG 1-X4 and AG 5OW-X4 ion exchange resins (Boehringer) and n-erythrose-4-P diethyl acetal monobarium salt (Boehringer) were treated with AG 5OW-X4 (H+ form) and hydrolyzed at pH 2 at 40°C for 48 hours or at 100°C for 5 min, respectively. The dicyclohexylammonium salt of the dimethyl ketol of dihydroxyacetone-P was chemically synthesized and converted to dihydroxycetone-P (16). Hydroyxypyruvaldehyde was synthesized enzymatically by aldolase-catalyzed oxidation of dihydroxyacetone-P with hexacyanoferrate(III) (2). Dephosphorylation with alkaline phosphatase (0.1 mg/ml at pH 7.6, 25°C, 30 min) yielded hydroyxypyruvaldehyde which was isolated from the reaction mixture by passing it over a AG 1-X4 (Cl- form) column (see below).

Product Analyses—Reduction of hexacyanoferrate(III) (cz = 1000 m−1 cm−1 (2)) was followed photometrically in a Unicam SP-1800 recording spectrophotometer. Glyceraldehyde-3-P was determined with triose-P isomerase and glyceraldehyde-3-P dehydrogenase (17). Erythrose-4-P was determined with transaldolase, triose-P isomerase, glyceraldehyde-3-P dehydrogenase, and fructose-6-P as donor substrate (18). The nonphosphorylated oxidation product was isolated from the reaction mixture (see Table 1) by passing a 3 ml sample through an AG 1-X4 (Cl- form) column (0.5 x 7.0 cm) equilibrated and eluted with distilled water. Ketoadhyde was determined in the eluate fractions with glyoxalase I and reduced glutathione as co-substrate (19). Thin layer chromatography the oxidation product was prepared in 50 mM imidazole-Cl buffer instead of triethanolamine-Cl because the latter interfered with the detection of ketoadhydes on the thin layer plates. The product of alkaline dismutation of the 2-ketoadhyde (0.1 n NaOH, 30°C, 30 min (19) followed by neutralization with cation exchange resin AG 5OW-X4 (H+ form)) was analyzed on silica gel plates with 1-propanol/H2O/ethylacetate (7/2/1, v/v) (20). Ketoadhydes were visualized with 2,4-dinitrophenylhydrazine spray (21). For analysis by thin layer chromatography the oxidation product was prepared in 50 mM imidazole-Cl buffer instead of triethanolamine-Cl because the latter interfered with the detection of ketoadhydes on the thin layer plates. The product of alkaline dismutation of the 2-ketoadhyde (0.1 n NaOH, 30°C, 30 min (19) followed by neutralization with cation exchange resin AG 5OW-X4 (H+ form)) was analyzed on silica gel plates with 1-propanol/H2O/ethylacetate (7/2/1, v/v) (20). Glyceric acid was detected with a 2,6-dichlorophenolindophenol spray (21).

RESULTS

Transaldolase when added in catalytic amounts to a solution of hexacyanoferrate(III) and the donor substrates fructose-6-P or sedoheptulose-7-P initiates a time-dependent reduction of hexacyanoferrate(III) (Fig. 1). The reaction occurs only in the presence of both the enzyme and the donor substrate and not in the presence of the acceptor substrates erythrose-4-P and glycer-

<table>
<thead>
<tr>
<th>Product</th>
<th>Fructose-6-P</th>
<th>Sedoheptulose-7-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyceraldehyde-3-P</td>
<td>1.29</td>
<td>0.59</td>
</tr>
<tr>
<td>Erythrose-4-P</td>
<td>0.60</td>
<td>0.31</td>
</tr>
<tr>
<td>Hydroyxypivaldehyde</td>
<td>0</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Table I: Quantitative determination of reaction products

Reaction conditions were as in Fig. 1; the volume of the reaction mixture was 3 ml. In order to compensate for the progressive inactivation of the enzyme (see "Discussion") the reaction mixture was supplemented with transaldolase (0.3 units/ml) after 30 and 60 min. After a reaction time of 60 min the reaction mixture was analyzed for the products as indicated under "Experimental Procedure". In control experiments when fructose-6-P or sedoheptulose-7-P were incubated with either transaldolase or hexacyanoferrate(III) neither glyceraldehyde-3-P, erythrose-4-P, nor hydroyxypivaldehyde were produced in detectable quantities.

FIG. 1. Transaldolase-catalyzed reduction of hexacyanoferrate(III) to hexacyanoferrate(II) in the presence of fructose-6-P (F6P) or sedoheptulose-7-P (S7P). The sample cuvettes contained 0.5 mM hexacyanoferrate(III) and 3 mM fructose-6-P or 3 mM sedoheptulose-7-P or no substrate (NONE) in 10 mM EDTA/100 mM triethanolamine-Cl (pH 7.6, 25°C). The reference cuvette contained 0.5 mM hexacyanoferrate(III) in the same buffer. At zero time transaldolase (TALD) was added to the sample cuvette (1 unit/ml of reaction mixture). The formation of hexacyanoferrate(II) was calculated from the decrease in absorbance at 420 nm (see "Experimental Procedure"). The initial rate of hexacyanoferrate(III) reduction corresponds to 0.65% of the rate of the normal transaldolase reaction with erythrose-4-P as the acceptor substrate at the same concentration of enzyme.
hyde-3-P is produced. With either substrate, fructose-6-P or sedoheptulose-7-P, an identical second product, produced in enzyme alone or of substrate alone (see Fig. 1).

FIG. 2. Rate of transaldolase-catalyzed hexacyanoferrate(III) reduction as a function of fructose-6-P (F6P) concentration. The enzyme concentration was 0.5 units/ml; other conditions, except for substrate concentration, were as in Fig. 1. The rates given here represent the increase in hexacyanoferrate(II) concentration per min, averaged over the time period from 1 to 5 min reaction time and are corrected for the rate of hexacyanoferrate(II) production occurring in the presence of enzyme alone or of substrate alone (see Fig. 1).

Oxidation of Transaldolase Intermediate

In this reaction hexacyanoferrate(III) may be replaced by other oxidants such as 2,6-dichlorophenolindophenol. The kinetic features of the reaction of 2,6-dichlorophenolindophenol with the transaldolase-substrate intermediate are quite similar to those of the reaction with hexacyanoferrate(III). In both cases the oxidation of the enzyme-substrate intermediate is accompanied by a relatively slow time-dependent decrease in enzymatic activity of transaldolase (see Ref. 10 and “Discussion”). Under the present conditions about 90% of the original transaldolase activity is lost within 30 min. The decreasing slope of the reaction progress curve (Fig. 1) reflects this time-dependent decrease in enzymatic activity and is not due to depletion of hexacyanoferrate(III) or substrate. However, with other oxidants, e.g. with porphyrindin or tetratromethane, the concomitant inactivation of transaldolase occurs much faster allowing the enzyme to perform only a few catalytic cycles.

DISCUSSION

The oxidation of a transaldolase-substrate intermediate by hexacyanoferrate(III) is quite analogous to the oxidation of carbanion intermediates of other enzymes reported previously (2–9). The reaction complies with the following criteria that have been defined (2) in order to verify the participation of an enzyme-substrate intermediate: (a) the reaction occurs only in the ternary system, i.e. in the presence of hexacyanoferrate(III) and enzyme plus substrate, but neither with hexacyanoferrate(III) and enzyme along nor with hexacyanoferrate(III) and substrate (or product) alone; (b) the reaction requires enzymatically active transaldolase; (c) the rate of reaction is proportional to the concentration of active enzyme and obeys saturation kinetics with respect to the concentration of the substrate; (d) the amount of substrate oxidized is stoichiometric to the amount of hexacyanoferrate(III) reduced.

A reaction pathway compatible with the reaction mechanism of transaldolase (24) and with the present kinetic data and product analysis is summarized in Scheme 1. In presence of an oxidant such as hexacyanoferrate(III) an additional pathway for the enzyme-bound dihydroxyacetone carbanion is opened, viz. oxidation to hydroxypyruvaldehyde. In contrast to dihydroxyacetone this oxidation product is readily released from the enzyme, possibly because of the absence of the carbanion-enamine resonance. Transaldolase thus acts as a catalyst for the continuous cleavage and oxidation of the donor substrate. In the presence of an acceptor substrate the transfer reaction competes with the oxidation of the donor substrate. In absence of an acceptor the oxidative cleavage of donor substrates becomes the sole reaction. Under the conditions used in Fig. 1 the molecular activity of transaldolase for oxidative cleavage is 0.65% of the activity for the normal transaldolase reaction with erythrose-4-P as acceptor substrate.

The detection of an oxidation-susceptible transaldolase-substrate intermediate expresses the applicability of oxidants as probes to detect carbanion intermediates of enzymatic reactions and to render them accessible to kinetic and structural analysis (2). In particular, the present data suggest that the reaction pathways of transaldolase and of class I fructose-1,6-P aldolase are very similar. Apparently, the analogy not only encompasses formation of a covalent Schiff base intermediate (24) but also the transitory chemical properties of the substrate moiety in the course of catalysis. Not all enzymes with carbanion intermediates will necessarily be susceptible to oxidative trapping reactions. Life-time of the intermediate, degree of electron delocalization, steric factors, and the rate of the concomitant inactivation of the enzyme will determine the yield and, hence, the detectability of the trapping reaction.

Progressive inactivation of the enzyme during the oxidation of the carbanion intermediate has recently been found to occur with several other enzymes too. The inactivation is due to an irreversible intramolecular reaction of groups at or near the active site with enzyme-bound intermediates or products formed during the oxidation of the substrate intermediate (10). The failure of tetratromethane (25) and porphyrindin to maintain continuous oxidation of the transaldolase intermediate in a manner similar to hexacyanoferrate(III) or 2,6-dichlorophenolindophenol might be due to the exceedingly rapid inactivation of the enzyme by this mechanism.

1 The concentration of fructose-6-P resulting in half-maximal rate of hexacyanoferrate(III) reduction, K_m = 2.4 x 10^{-4} M, is 1 order of magnitude lower than the value of K_m = 3.2 x 10^{-3} M for the normal transaldolase reaction (24). The difference between these two values suggests that the numerical value of K_m does not represent that of K', the dissociation constant of the enzyme-substrate complex. The value of K_m might rather correspond with the value of K' since the oxidation reaction consumes the enzyme-substrate complex at a rate 2 orders of magnitude slower than that of the normal transfer reaction.
The oxidation of the aldolase and transaldolase substrate intermediates might also be of physiological import. The reactions are unspecific with respect to the oxidant and might occur with intracellular oxidants \( \text{H}_2\text{O}_2 \) has been found to oxidize the aldolase-substrate intermediate although at a very slow rate (26). With both aldolase and transaldolase the oxidation product is a 2-ketoaldehyde (hydroxypyruvaldehyde phosphate or hydroxypyruvaldehyde, respectively). 2-Ketoaldehydes produced by oxidation of substrate intermediates of aldolase, transaldolase, and possibly other related enzymes might thus represent natural substrates for the ubiquitous and very active glyoxalase system (cf. Ref. 27).

REFERENCES

Oxidation of the carbanion intermediate of transaldolase by hexacyanoferrate (III).
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