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Crosslinked Crystalline Horse Liver Alcohol Dehydrogenase as a Redox Catalyst: Activity and Stability toward Organic Solvent

KANG MIN LEE, MOHAMED BLAGHEN, JEAN-PIERRE SAMAMA, AND JEAN-FRANÇOIS BIELLMANN

Laboratoire de Chimie Organique Biologique, Associé au CNRS, Institut de Chimie, Université Louis Pasteur, 1 Rue Blaise Pascal, 67008 Strasbourg, France

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The holoenzyme crystals of horse liver alcohol dehydrogenase–NADH–DMSO complex were crosslinked with glutaraldehyde. A coupled activity test with ethanol and cinnamaldehyde as substrates was performed on the crosslinked enzyme crystals. The enzymatic activity was preserved and the coenzyme was found to be firmly bound to the enzyme crystals. These crystals can be used as redox catalysts with no addition of coenzyme. The crosslinked crystals were more stable toward dimethoxyethane than the enzyme in solution. Zinc ion salts reinforced this stability. Thirty percent of the initial activity was found in a medium containing 84% (v/v) organic solvents.

INTRODUCTION

A major problem in the use of $NAD(P)^+$-dependent dehydrogenases as catalysts in organic synthesis is recycling of the expensive cofactor. A variety of solutions to this problem have been presented. For example, pyridinium and flavine derivatives have been used to reoxidize the reduced coenzyme (*1, 2*). Covalent binding of a coenzyme analog to the enzyme, coimmobilization of a polymerizable coenzyme analog and the dehydrogenase, entrapment in a polyacrylamide gel (*3, 4*) and site-directed immobilization of dehydrogenases with a bis-NAD^+ analog on agarose beads (*5–7*) prevent diffusion of the coenzyme from the enzyme.

We present here a new solution to this problem. The representativity of the triclinic crystalline ternary complex of horse liver alcohol dehydrogenase as a catalytically active form of the enzyme was demonstrated by microspectrophotometric measurements on the crystalline enzyme–NADH–DMSO[1] complex (*8*). The spectral evolution of these crystals showed that bound coenzyme was reversibly oxidized by the appropriate substrates. Besides the enzymatic activity in the crystalline state, these experiments showed that the coenzyme was firmly bound to the enzyme. The strong binding of the coenzyme to the enzyme in the crystal-

[1] Abbreviations used: DMSO, dimethylsulfoxide; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}ethanesulfonic acid; MPD, 2-methyl 2,4-pentanediol.

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line state makes possible the use of this coenzyme–enzyme complex as a redox catalyst.

METHODS

1. General. 1,4-Dihydroxynicotinamide adenine dinucleotide (NADH) was from Boehringer at 100% purity. Glutaraldehyde was purchased from Roth (Karlsruhe) as a 25% aqueous solution. 2-Methyl 2,4-pentanediol was from Eastman–Kodak and was twice distilled. *E*-Cinnamyl alcohol (EGA Chimie) was sublimed prior to use and *E*-cinnamaldehyde (Aldrich) was distilled. Dimethoxyethane (monoglyme) (Aldrich) was of 99% purity.

All other reagents were of the highest quality available and were used without purification. Water was doubly distilled in a quartz apparatus.

Enzyme manipulations were carried out at 4°C and, after crystal insolubilization, at 20°C.

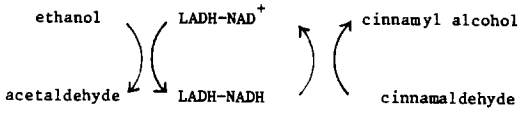
The membranes were Amicon ultrafiltration membranes XM-300 or glass microfiber paper Whatman GF/D when specified.

2. Crystallization. Alcohol dehydrogenase was purchased as a crystalline suspension from Boehringer. Crystals were centrifuged and dissolved in 50 mM TES buffer, pH 7.0, made 0.2 M in sodium chloride. After centrifugation, the enzyme solution was extensively dialyzed against 50 mM TES buffer, pH 7.0, at 4°C. The enzyme concentration was determined spectrophotometrically at 280 nm using a 0.455 optical density for a 1 mg/ml enzyme solution (*9, 10*). For the crystallization experiment (*10, 11*), the enzyme concentration was 10–12 mg/ml. Dialysis bags containing 0.7 to 1 ml of the enzyme solution were closed at each end and placed in flasks containing 5 mg NADH and 0.5 ml DMSO in 10 ml 50 mM TES buffer, pH 7.0. After equilibration (12 h), distilled 2-methyl 2,4-pentanediol (MPD) (250 μl) was added. This was repeated twice after 24 h. MPD (100 μl) was added daily until crystals appeared (concentration of MPD 7–10% (v/v)). After 1 week, MPD (200 μl) was added every 2 days until its concentration was 25%. The medium was then left for 1 week before the crosslinking.

3. Crosslinking of enzyme crystals. A 25% glutaraldehyde solution (125 μl) was added to the outer solution of the crystallization experiment. After 1 h, the bags containing the enzyme were placed in 50 mM TES buffer, pH 7.5 (10 ml). The crosslinked enzyme crystals can be stored at 4°C for several months.

4. Enzyme assay. Assays were conducted in a standard Amicon ultrafiltration cell 8410 (10 ml). This cell was equipped with Amicon ultrafiltration membrane XM-300 except for assays with dimethoxyethane at concentrations above 32% and for protein quantity determination in which glass microfiber paper membranes Whatman GF/D were used. After the crosslinking step, an aliquot of the suspension of crystals was transferred to the Amicon cell. The supernatant was removed by air pressure and replaced with 50 mM TES buffer, pH 7.5 (5 ml). This washing procedure was repeated five times at 20°C. The last filtrate showed no absorption at 250–280 nm.

The enzymatic activity was shown to be due to crosslinked crystals in the



SCHEME 1

following ways. Crosslinked crystals were left for 24 h at 8°C in the presence of 50 mM TES buffer, pH 7.5 (0.7 ml). The supernatant was removed through the membrane by air pressure. The coenzyme (NAD^+ or NADH) and the enzyme present in the filtrate were detected by the absorption at 260 and 340 nm and by adding NAD^+ solution (final concentration 1 mM) and ethanol (0.1 M) and following the absorption increase at 340 nm. Some samples were dialyzed before enzyme activity determination.

The activity of crosslinked alcohol dehydrogenase crystals was determined through a coupled assay in which the reduction of cinnamaldehyde to cinnamyl alcohol was monitored (Scheme 1).

In a preliminary study, the ethanol concentration was varied from 50 mM to 6 M and that of cinnamaldehyde from 10^{-5} to 10^{-2} M. The highest activity was found with 50 mM TES buffer, pH 7.5, 1 mM cinnamaldehyde, 4 M ethanol in a total volume of 3 ml. We chose these conditions for carrying out our standard assay. After 10 min, the solution was expelled from the Amicon cell by air pressure and extracted twice with cyclohexane (5 ml × 2). The organic phase was concentrated by partial vacuum evaporation to 0.5 ml and aliquots were analyzed by high-performance liquid chromatography on a Varian Model 5000 apparatus equipped with a C-18 reversed phase column (Waters). The products were eluted with an acetonitrile–water 1 : 1 mixture (v/v) at 0.5 ml/min and detected by uv absorption at 260 nm. The activity was defined as the ratio of the peak areas corresponding to cinnamyl alcohol and to cinnamaldehyde. The column was washed with pure acetonitrile and equilibrated with the eluant before each experiment.

After they were washed with 50 mM TES buffer, pH 7.5, the crystals on the membrane were ready for a new experiment; they could be stored wet at 4°C.

The activity of the enzyme in 50 mM TES buffer, pH 7.5, 4 M EtOH, 1 mM NADH, 1 mM cinnamaldehyde was determined by the decrease in the absorption at 282 nm after appropriate dilution.

5. Determination of enzyme concentration. After determination of their activity, the crosslinked enzyme crystals on the glass microfiber paper membrane were transferred to a glass tube and incubated with 0.1 M sodium hydroxide solution (3 ml) for 6 h at 60°C. Another membrane with soluble alcohol dehydrogenase (0.3 mg) and NADH (5×10^{-8} mol) was incubated in the same manner along with a membrane without any additive. The amino acid content of the three solutions was determined according to Lowry (*12, 13*).

6. Effect of dimethoxyethane addition on the activity of crosslinked enzyme crystals. The crosslinked crystals were washed with 50 mM TES buffer, pH 7.5. Assays were then conducted in presence of increasing volumes of dimethoxyethane stepwise from 0 to 48% expressed in v/v total. Between assays the crystals were washed in buffer (2 × 3 ml).

These results were compared to those obtained for enzyme in a solution of 50 mM TES buffer, pH 7.5, in the presence of 10^{-3} M NAD^+, 4 M ethanol, and dimethoxyethane (0 to 16% v/v total). In this case, the activity was determined spectrophotometrically through the appearance of reduced coenzyme (340 nm).

7. Effect of zinc ion addition. The effect of zinc ion, added as zinc chloride or zinc sulfate (1 mM) to the assay medium of crosslinked and solution enzyme(s), was determined in the presence of increasing amounts of dimethoxyethane.

RESULTS

Crystallization and Insolubilization

Alcohol dehydrogenase ternary complexes with NADH and the inhibitor DMSO were prepared in a manner similar to that described for the preparation of crystals used in the X-ray investigations of this complex (*10, 11*). The rate of 2-methyl 2,4-pentanediol addition was slightly modified in order to have small crystals where the diffusion parameter as minimized. The insolubilization was tried at 0.5, 1, and 2% glutaraldehyde (v/v) for 1 hour at 4°C. The best compromise between stability of the crosslinked crystals and enzyme activity was achieved at 1% glutaraldehyde. Microscopic examination of the crosslinked crystals revealed the expected parallelipipedon shape and optical activity under polarized light. Whereas native crystals decomposed at room temperature or in the absence of 2-methyl 2,4-pentanediol, crosslinked crystals did not dissolve in buffer at 20°C and retained their activity after several months at 4°C.

Crystal Activity

Analysis of the filtrate after 24 h incubation of crosslinked crystals of alcohol dehydrogenase–NADH–DMSO complexes in buffer showed that they did not release enzyme or coenzyme in detectable amounts.

In order to test the activity of the crosslinked crystals, we used the coupled assay depicted in Scheme [1]. The experiments were conducted on about a quarter of the enzyme crystals formed in one bag containing 7 mg of alcohol dehydrogenase. With this amount of enzymes, a yield of above 50% cinnamyl alcohol was produced within 10 min.

The protein content of the crosslinked crystals on the membrane was determined using amino acid determination according to Lowry after alkaline treatment of the membrane. The glass microfiber paper did not release any material reacting in the Lowry determination. The quantity of crosslinked enzyme on the membrane was about 1 mg and its activity expressed in millimoles of cinnamyl alcohol produced in 1 min per milligram of enzyme, under our assay conditions was 0.17 mmol/min · mg. The enzyme in buffer at the same concentrations of ethanol and of cinnamaldehyde in the presence of 1 mM dinucleotide had an activity of 0.65 mmol/min · mg.

We found that the crosslinked crystals had the same activity after extensive washing with buffer (10 × 10 ml).

TABLE I

ACTIVITY OF THE ENZYME IN SOLUTION AND OF THE INSOLUBILIZED ENZYME CRYSTALS

Dimethoxyethane content (%)[a]	Activity (%)[b]			
	In solution	Insolubilized crystals[c]	In solution in the presence of Zn^{2+}[d]	Insolubilized crystals treated with Zn^{2+}[c–e]
0	100 (0.65 mmol/min · mg)	100 (0.17 mmol/min · mg)	100	100
8	54	80 (85)[f]	58	100
16	34	75 (77)	35	100
24		55 (69)		95
32		26 (31)		86
40		24 (27)		76
48		16 (22)		50

[a] v/v total.

[b] Percentage of activity in absence of dimethoxyethane—for conditions see text.

[c] In our assays above 50% of the cinnamyl alcohol produced was the initial activity.

[d] Zinc ion as zinc chloride or zinc sulfate (1 mM).

[e] The crystals were treated once with zinc ion as zinc chloride or zinc sulfate (1 mM), washed, and subjected to assay or the assays were done in the presence of zinc ions.

[f] Data obtained with a different preparation of insolubilized crystals.

Effect of Dimethoxyethane on the Activity

We studied the effect of the addition of dimethoxyethane to the assay medium on the stability and activity of the alcohol dehydrogenase–NADH complex in crosslinked crystals. Measurements were made after 10 min reaction (Table 1). As a comparison, the effect of dimethoxyethane addition on the enzyme in solution (10^{-3} M NAD^+, 4 M ethanol) was studied in order to have a similar content of organic solvent in both cases, the important point being the relative decrease in activity (Table 1). Alcohol dehydrogenase in solution was more sensitive than the crystalline form to dimethoxyethane. At 16% dimethoxyethane, more than 66% of the activity was lost in solution while 75% of the activity remained for the crosslinked crystals. The effect of dimethoxyethane seemed to depend on the sample of crystals and on the number of dimethoxyethane treatments as shown by the results in Table 1 for two different batches of insolubilized crystals. The initial activity could not be regained after treatment with dimethoxyethane at concentrations above 16%.

Effect of Zinc Salts on the Activity

Zinc chloride and zinc sulfate addition had a remarkable effect on the enzyme activity of crosslinked crystals in the presence of dimethoxyethane. In the presence of zinc ion (1 m*M*) the activity remained close to 100% at 24% dimethoxyethane (Table 1). One treatment with zinc ion solution for 3 min was sufficient to

give this stabilizing effect and the stability was retained even after extensive washing of the crystals with buffer (10 × 10 ml).

The activity of zinc ion treated crystals did not depend on the presence of zinc ion in the activity test. With concentrations of dimethoxyethane up to 24%, the initial activity could be regained by washing the crystals (2 × 3 ml) before the assay. Above that concentration, the activity loss seemed irreversible. Thirty percent of the activity was found with 60% dimethoxyethane; taking into account the ethanol content, the organic solvents actually amounted to 84% (v/v). With soluble enzyme, zinc ion did not have a stabilizing effect and at higher concentrations zinc chloride and zinc sulfate were competitive inhibitors with respect to ethanol ($K_i = 4 \times 10^{-2}$ *M*).

DISCUSSION

The rationale of our study originated from the knowledge acquired on liver alcohol dehydrogenase from kinetic and structural investigations. Solution studies had shown that the reaction sequence followed an ordered bi-bi mechanism in which the nicotinamide coenzyme was bound first and was released last during each turnover (*14*). X-Ray structure determinations demonstrated that the protein had a different conformation in the apoenzyme (*15*) and in enzyme–coenzyme complexes (*11*). Binding of the cofactor induced a large conformational change in the protein whose function was to provide the proper set of interactions between coenzyme and protein residues and to increase the hydrophobicity of the active center (*16*).

In a number of dehydrogenases, the holoenzyme (coenzyme–enzyme) crystallized with cell symmetry and/or dimensions different from those of the apoenzyme. This change reflected in general an extended conformational change in coenzyme binding (*16, 17*). Cytoplasmic malate dehydrogenase seemed to undergo conformational changes upon coenzyme binding, but the coenzyme may be exchanged in the holoenzyme. Insolubilized crystals of malate dehydrogenase released the coenzyme and then were used in the same way as the soluble enzyme (*18*). 6-Phosphogluconate dehydrogenase bound the coenzyme with no major conformational change, inducing a modification of the crystallographic system (*19*).

Enzymatic catalysis in the crystalline state has been demonstrated in other systems such as cytoplasmic dehydrogenase (*18*), D-glyceraldehyde-3-phosphate dehydrogenase (*20*), chymotrypsin (*21*), carboxypeptidase A (*22, 23*), carboxypeptidase B (*24*), and ribonuclease S (*25*). Experiments on crystals (*8*) have reported that alcohol dehydrogenase was active in the crystalline complexes and that the coenzyme was firmly bound to the enzyme.

However, all crystalline complexes of alcohol dehydrogenase dissolved when the precipitating agent 2-methyl 2,4-pentanediol was removed. To overcome this problem, we stabilized the crystal architecture by crosslinking the enzyme–coenzyme complexes with glutaraldehyde. This selective reagent for free amino groups has been widely used on protein crystals (*26*), both for structural analysis pur-

poses (*27–29*) and for kinetic studies (*29, 30*), and on protein solutions (*31*). It is a mixture of glutaraldehyde and oligomeric aldol condensation products (*32–34*).

It was necessary to study the enzymatic activity through the coupled assay depicted in Scheme 1; when formed, enzyme–NAD^+ complex was converted to enzyme–NADH complex by ethanol whose concentration was found to be optimum at 4 M, representing an organic solvent concentration of 24% in the assay medium. The optimum concentration of cinnamaldehyde was found to be 10^{-3} M. The use of an XM-300 ultrafiltration and glass microfiber membranes was most convenient for repeated work with a constant amount of microcrystals and semicontinuous analysis of the reaction products.

The activity of the crosslinked crystals was 0.17 mmol/min · mg of protein. It is likely that in the system we used, contact between the crosslinked crystals and the solution was not achieved properly; the crosslinked crystals should prove to be more efficient in a flow system. The enzyme in solution had an activity of 0.65 mmol/min · mg of protein. The crystals were probably so small that the diffusion was not rate determining.

The use of water soluble enzymes for synthesis was limited by the sensitivity of these enzymes to organic solvents. The covalent binding of the protein on insoluble matrices (*35*) stabilized the enzymes toward organic solvent, and small amounts of water were enough to protect the protein against organic solvent denaturation (*36–38*). Other approaches consisted of the use of reversed micelles and of microemulsion as a microheterogeneous medium for enzymatic reactions (*39–41*).

The effect of organic solvent on the activity of alcohol dehydrogenase in solution has been studied (*42*) and it was found as reported here that 5% of the activity remained when 20% (v/v) dimethoxyethane was present in the assay medium.

Crosslinked crystals of alcohol dehydrogenase–coenzyme were more resistant to added dimethoxyethane than the enyzme in solution (Table 1). At 24% dimethoxyethane, that is, 50% total organic solvent concentration, 55 to 69% of the activity was preserved. Dimethoxyethane had the same effect on the enzyme in solution whether zinc ions were present or not. In contrast, zinc ions maintained the activity in the crystals at a constant value up to 24% (v/v) dimethoxyethane. In the absence of dimethoxyethane, the enzyme activity in solution and in the crosslinked crystals was not changed in the presence of 10^{-3} M zinc chloride or sulfate.

One zinc ion, located at the active site of alcohol dehydrogenase, is essential for activity (*43*). If the activity loss with dimethoxyethane addition was related to a modification of active site zinc content of the protein, added zinc salts would also stabilize the enyzme in solution, which is not seen.

The stabilization by zinc salts was persistent and was distinct from the inhibition of the enzyme reaction. Once the crosslinked crystals had been soaked in zinc containing solutions, the activity was constant upon dimethoxyethane addition even when the assays were conducted without zinc salts. It therefore seemed that the stabilization in the crystalline state resulted from an unknown intermolecular effect on the structure. Stabilization of protein crystal structure has been described in the case of thioredoxin where copper ions were required for the

crystallization (*44, 45*). The copper ion was found to be located in the intermolecular contact zone (*46*).

This zinc ion effect has a definite advantage for the use of these crosslinked enzyme–coenzyme crystals as redox catalysts. Indeed, their increased stability toward organic solvents is a solution to the problem of the poor solubility in water of many organic substrates. The lack of specificity of the enzyme allows an easy recycling of the bound cofactor so that we now have a stereospecific Wagner–Meerwein–Pondorf reagent for working under mild conditions. The easy preparation and handling of the crosslinked crystals should encourage the synthetic chemist to consider this system as a valuable tool.

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