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Electron transfer from NADH bound to horse liver alcohol dehydrogenase (NAD^+ dependent dehydrogenase): Visualisation of the activity in the enzyme crystals and adsorption of formazan derivatives by these crystals

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Abstract

The crystals of holoenzyme from native and cross-linked alcohol dehydrogenase exhibit electron transfer from NADH to phenazinium methosulfate (PMS), and then to the tetrazolium salt sodium 3,3′-{1-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzenesulfonate (XXT). The slow dissociation of the cofactor and/or the conformational change associated can now be bypassed. The reduction product, formazan, did not diffuse out of the crystals in buffer and the crystals turned colored. In the presence of dimethyl sulfoxide or dimethoxyethane, the formazan diffused out to the solution. The reaction rates were found to be, respectively, 18% and 15% of the redox reaction rate of ethanol with cinnamaldehyde, close to the activity determined for the enzyme in solution in the presence of dimethoxyethane. The use of system PMS-tetrazolium salt is a useful tool to

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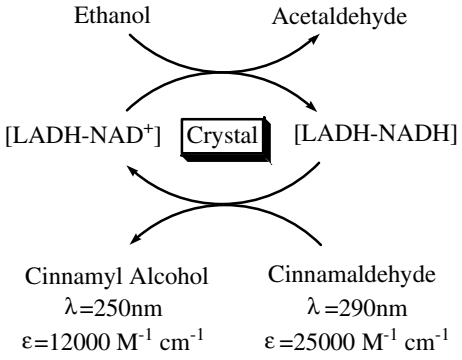
visualize the activity of dehydrogenases and other electron transferring systems in the crystalline state. The adsorption of formazan by the alcohol dehydrogenase crystals occurs in solution.

Keywords: Alcohol dehydrogenase; Electron transfer; Tetrazolium salt; Formazan; Adsorption; Crystal; Cross-linking

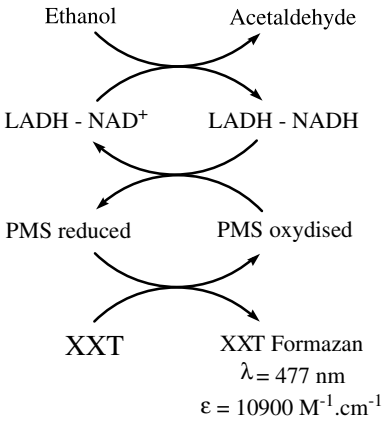
1. Introduction

The slow dissociation of the cofactor from the holoenzyme and/or the associated conformational change limits the studies of the mechanism of number of enzymes. Is there a way to eliminate this slow step? Alcohol dehydrogenase of horse liver is a good example for such studies. Indeed the cofactor dissociation rate contributes to a great extent to the overall rate [1]. The suppression of these steps would amount to reduce the enzyme-bound NAD^+ or to oxidize the enzyme-bound NADH. Studies of linking covalently the coenzyme to the enzyme showed that the coenzyme is not locked in the active site [2]. So we need a method to lock the coenzyme in the active site to study its reactivity with electron (or hydride) transferring agent. In earlier studies we and later others have shown that the NAD^+/NADH is locked to the active site in the horse liver alcohol dehydrogenase holoenzyme crystals [3–6]. The activity test had to rely on the redox properties of the holoenzyme, reducing cinnamaldehyde to cinnamyl alcohol while ethanol is oxidized to acetaldehyde (Scheme 1) [4]. Dismutation of cinnamaldehyde to cinnamic acid and cinnamyl alcohol could occur, but according to the data published for benzyl alcohol should be a minor reaction next to the reactions of Scheme 1, but this point has not been examined here, since the relative rates are of importance for the work presented here [7,8]. The cofactor is firmly bound to the enzyme in this crystalline form and can not be washed out [4]. In contrast the cofactor will bind and dissociate in the apoenzyme crystals [5]. So we have the conditions to study the oxidation of NADH while bound to the enzyme.

We then turned our attention to a cofactor regeneration which we had used previously for the enzyme in solution: the reduction of a tetrazolium salt by NADH mediated by a



Scheme 1. Coupled activity test using ethanol–cinnamaldehyde with LADH crystal.



Scheme 2. Activity test for LADH using PMS as electron transferring system and tetrazolium salt XXT as final electron acceptor reduced to XXT formazan.

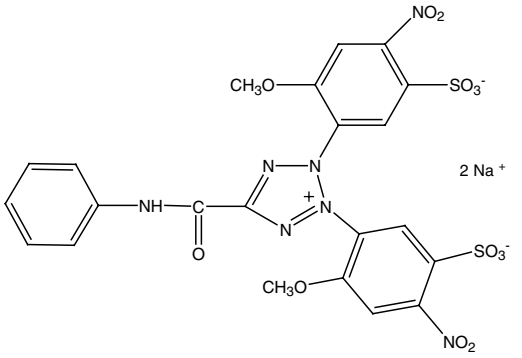
phenazinium salt to NAD^+ and the highly colored formazan, the chromophore to follow the reaction (Scheme 2). The turn over of this system is quite high [9]. We report here the results of the study of this system on crystalline horse liver alcohol dehydrogenase.

2. Materials and methods

2.1. Materials

NAD^+, NADH, $NADP^+$, and NADPH were products from Boehringer. Acetonitrile, *E*-cinnamaldehyde, *E*-cinnamyl alcohol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), dimethoxyethane (DME), 25% glutaraldehyde solution in water, 2-methyl-2,4-pentanediol, phenazinium methosulfate (PMS) were products from Aldrich. Disodium 3,3′-{1-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzenesulfonate (XXT), and the corresponding formazan were prepared in our laboratory by Nguyen Trong as published [10].

Formula XXT



XXT (disodium 3,3`-{1-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzenesulfonate).

2.2. Horse liver alcohol dehydrogenase

The suspension of horse liver alcohol dehydrogenase from Boehringer (100 mg) was centrifuged at 20,000 t/min for 1 h at 5 °C. The supernatant was disregarded. The precipitate was dissolved in 50 mM Tes buffer, 0.2 M NaCl, pH 7 (3 mL). This solution was centrifuged at 20,000 t/min for 2 h:30 at 5 °C to remove the denatured enzyme. The supernatant was dialysed overnight to a 50 mM Tes buffer, pH 7 (1 L). The solution was centrifuged at 20,000 t/min for 1 h at 5 °C. The concentration of the enzyme was determined by the absorption at 280 nm ($A = 0.455\ \mathrm{mg^{-1}\ cm^{-1}\ mL}$). The activity test (1 mL) was performed at 25 °C in 0.1 M glycine–NaOH buffer, pH 9 (1 mL). The solution is 2.1 mM NAD^+, 0.6 mM ethanol, and the enzyme solution (about 22 μg) was added. The absorption of NADH at 340 nm ($\varepsilon = 6220\ \mathrm{M^{-1}\ cm^{-1}}$) was monitored.

2.3. Determination of the maximum velocity in the XXT coupled test

The 50 mM Tes buffer, pH 7.5, was (17.2–258) mM ethanol, (0.03–0.5) mM NAD^+, (0.02–0.2) mM PMS, (0.06–0.8) mM XXT (1 mL). LADH (0.3 μg) in same buffer (total volume 1 mL) was added and the reaction was monitored at 477 nm ($\varepsilon = 10{,}900\ \mathrm{M^{-1}\ cm^{-1}}$) for 1 mn.

2.4. Crystallization and cross-linking of horse liver alcohol dehydrogenase

The holoenzyme crystals were obtained in 50 mM Tes buffer with methyl-2-pentane-diol-2,4 as described. The crystals were cross-linked with glutaraldehyde (1%) as described [4]. The cross-linked crystals can be stored at 4 °C for several months without any activity loss [4].

2.5. Experiments with enzyme crystal

2.5.1. Native LADH

One crystal of LADH was placed in the precipitating medium (10 μL) for LADH in the well solution on a microscope plate. Then a 18.7 M ethanol, 8.8 mM PMS, and 8.8 mM XXT solution (1 μL), was injected into the droplet. The color change was monitored visually under the microscope.

2.5.2. Cross-linked crystals of LADH

The cross-linked crystals were washed with bidistilled water by sonication in order to remove a brown polymer at their surface. They were collected by centrifugation. One crystal in 50 mM Tes buffer, pH 7.5, 0.8 mM PMS, 0.8 mM XXT, 1.7 M ethanol (10 μL) was placed under the microscope. The color change was monitored under the microscope at 4 °C in the dark.

2.5.3. Release of XXT formazan from cross-linked crystals in the presence of organic solvents

The cross-linked crystals were incubated at 37 °C for 3 h:30 in a solution 81 μM XXT formazan. The red crystals were transferred to 50 mM Tes buffer, pH 7.5 (1 mL) containing ethanol or DMSO or DME at determined concentration. The XXT formazan released from the crystals was determined by absorption at 477 nm.

2.5.4. Activity determination of LADH cross-linked crystals

All the following incubations were carried out with gentle stirring.

2.5.4.1. With cinnamaldehyde. The cross-linked crystals were suspended at 37 °C in a 50 mM Tes buffer, pH 7.5 (final volume 2 mL) to which a 0.16 M solution of cinnamaldehyde in acetonitrile (12 μL) and ethanol (466 μL) were added. Aliquots (10 μL) were taken every 5 min and diluted in 50 mM Tes buffer, pH 7.5 (990 μL). The concentration of cinnamyl alcohol was monitored by its adsorption at 250 nm ($\varepsilon = 12{,}000\ M^{-1}\ cm^{-1}$).

2.5.4.2. Coupled test with XXT in the presence of ethanol. The crystals 50 mM Tes buffer, pH 7.5, 36% ethanol (V/V), 64 μM XXT, 10 μM PMS (1 mL) were incubated at 37 °C with gentle stirring. After centrifugation, the absorption of the formazan at 477 nm in the supernatant was determined.

2.5.4.3. In the presence of DMSO or of DME. Crystals in 50 mM Tes buffer, pH 7.5, 50% DMSO or 50% DME (V/V), 64 μM XXT, 10 μM PMS, and 100 μM ethanol (1 mL) were incubated at 37 °C. After centrifugation, the absorption of the formazan was determined.

2.5.4.4. In the presence of pyrazole. Crystals in 50 mM Tes buffer, pH 7.5, 50% DME (V/V), 64 μM XXT, 10 μM PMS, 100 μM ethanol, and 100 μM pyrazole (1 mL) were incubated at 37 °C. After centrifugation the absorption of the formazan was determined.

3. Results

We prepared the triclinic horse liver alcohol dehydrogenase crystals according to established procedures and their cross-linking was done with 1% final concentration of glutaraldehyde [4]. Their size was in the 0.2–0.4 mm range. At first we used the assay with cinnamyl alcohol–cinnamaldehyde [4]. The test amounts to determine the increase of the cinnamyl alcohol concentration, the reductant being ethanol. Since it is not easy to determine the amount of enzyme, the activity measured with cinnamyl alcohol determines the enzyme crystal. All the following experiments were done in the presence of phenazinium methosulfate. The initial experiments were carried out with tetrazolium salt MTT. The crystals of the holoenzyme native and cross-linked turned blue in this test but the solution remained colorless. The tetrazolium salt is reduced to the formazan, a blue dye. So NADH is oxidized while bound to the enzyme. But the fact that the formazan remains in the crystals makes any rate determination difficult and is by itself puzzling. However it is known that MTT formazan has a low solubility and this is the reason of its use in biology [11]. The fact that the MTT formazan did not diffuse out of the crystal, could be due to its precipitation inside the crystals or to the formation of a bond with the holoenzyme.

So we turned to the sodium 3,3′-[1-[(phenylamino)carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid (XXT). XXT had been prepared for its solubility and that of its formazan in water for a number of biological tests [10]. Since the reaction of the reduced phenazine species with the tetrazolium salt likely occurs in solution, it is not surprising the XXT can replace MTT as final electron acceptor. So we did further studies with XXT and report the results here.

For the enzyme in solution, the saturating concentrations in reagents are 86 mM ethanol, 0.1 mM in PMS, 0.3 mM NAD^+, 0.1 mM XXT with LADH in solution. Under these

conditions the reaction rate is 2.3 μmol min^{-1} mg^{-1}. The cross-linked crystals of enzyme were then treated with XXT. At first the solution was slightly yellow and the crystal was transparent. The crystal turned red with time at first at the outside and the color invaded all crystal. But the reaction medium remained again colorless and on longer reaction time the crystals turned dark. After 12 h the crystals were taken out of the reaction medium and were placed in water and the water remained colorless. The spectroscopy of the solution confirmed that the formazan did not diffuse out.

We studied also native crystal with the same system. The crystal was then placed in the precipitating medium and a solution containing ethanol, PMS and XXT in the same medium was injected. And again the same events were observed: coloration of the crystal and no coloration of the medium. Thus the precipitation of the MTT formazan in the crystals is not the explanation of the coloration of the crystals. Since the XXT formazan is soluble, we diffused this formazan in the crystal and determined the conditions where it will diffuse out. The colored crystals were transferred to a solution containing ethanol or DMSO or DME. In increasing the amount of solvent, the crystals discharged their color at about 35% ethanol or 50% DMSO or 50% DME (V/V). Now we can try to determine the reaction rate in the presence of these solvents.

Cross-linked crystals whose activity in the cinnamaldehyde test had been determined, were incubated in the presence of 35% ethanol, PMS and XXT and the appearance of formazan in solution was monitored at 477 nm. No linear relation with time was found and after washing with buffer containing 35% ethanol the crystals remained colored and were inactive in the cinnamaldehyde test. In presence of 50% DMSO, for one crystal whose activity in the cinnamaldehyde test was 0.72 nanomol min^{-1}, the appearance of the formazan was monitored at 477 nm. The absorption increase was linear for 40 min and the rate was 0.135 nanomol min^{-1} (18% of the rate of the cinnamaldehyde test) (Fig. 1). In the presence of 50% of DME, the linear increase of the absorption extended to 30 min and the rate was 1.2 nanomol min^{-1} (15%) compared to 8 nanomol min^{-1} in the cinnamaldehyde test (Fig. 2). If the reaction in 50% DME was carried out in the presence of 100 μM pyrazole an inhibitor (K_i 20 μM) [12], the reaction rate was 6% of the rate

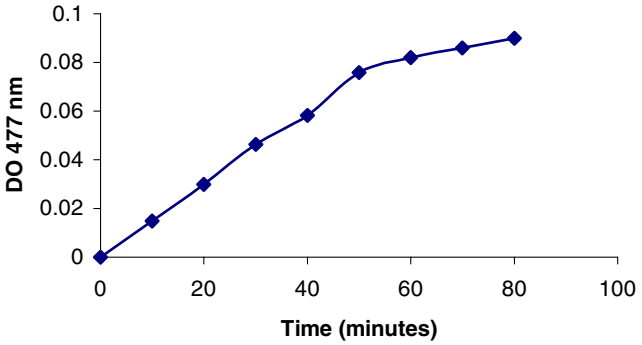


Fig. 1. Coupled test in the presence of DMSO (50%), absorption increase at 477 nm increase in function of time with one crystal holoenzyme: 50 mM Tes buffer (pH 7.5) 100 μM ethanol, 10 μM PMS, 64 μM XXT in the presence of 50% of DMSO. This crystal had an activity of 0.75 nmol min^{-1} in cinnamaldehyde test and in the presence of 50% DMSO the activity was 0.135 nmol min^{-1}.

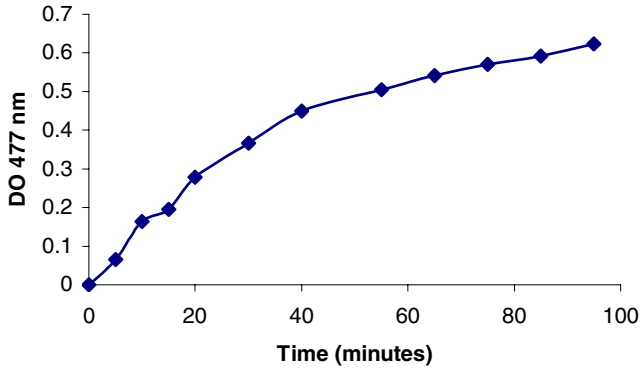


Fig. 2. Coupled test in the presence of DME (50%), absorption increase at 477 nm increase in function of time with one crystal holoenzyme: 50 mM Tes buffer (pH 7.5), 100 μM ethanol, 10 μM PMS, 64 μM XXT in the presence of 50% of DME. This crystal had an activity of 8 nmol min^{-1} in the cinnamaldehyde test and in the presence of 50% DME the activity was 1.2 nmol min^{-1}.

in the cinnamaldehyde test. The crystals left about 2 h in these DMSO or DME solution were inactive in cinnamaldehyde test.

4. Discussion

In the assay with phenazinium methosulfate–tetrazolium salt on alcohol dehydrogenase crystals, we did found that these crystals were active in this assay. Since NADH does not dissociate from holoenzyme crystals [4], its oxidation to NAD^+ must occur on the enzyme. So further studies on the holoenzyme in solution can be undertaken to show that the reaction rate can be greater with the electron transferring agents [13]. But the formazan, the final reduction product, stays inside the native and cross-linked enzyme crystals. This does not allow the monitoring of the reaction. So we then turned to other electron accepting systems like potassium ferricyanide, paraquat, methylene blue, quinoxaline, methylene blue and dichloroindophenol and found that the reaction rate was small or nil (results not shown). So we explored the reasons why the formazan did not diffuse out of the crystals. How about the dimensions of the channels present in the LADH crystals? According to the model available from data bank, the channels in alcohol dehydrogenase holoenzyme (triclinic crystals) are quite wide, around 35 Å and flat molecules like the tetrazolium salts and the formazans should diffuse in and out without any constraints. Indeed the dimensions of the substituted tetrazolium ring in 2,3,5-triphenyltetrazolium dichloro(1,3,5,-triphenylfromazanato)cobaltate (II) has the dimensions in the three axes of about 11.8, 11.8, and 8.8 Å [14]. For the formazan the dimensions are depending on the isomer [15] around 15 and 12 Å [16]. The space limitations for the diffusion seems not to be the explanation.

The formazan formed from MTT is quite insoluble in buffer. So the precipitation of the formazan crystals inside the enzyme lattice could the reason why the formazan did not diffuse out. Another possibility is that a covalent bond was formed with the holoenzyme.

The next step was to prepare a tetrazolium salt whose reduced species the corresponding formazan is soluble in the buffer. A sulfonated tetrazolium salt XXT (disodium

3,3′-{1-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzenesulfonate) was prepared [10]. This reagent in the presence of NAD^+–PMS and of ethanol was first tried with the enzyme in buffer and then with the enzyme crystals. Again the formazan did not diffuse out of the crystals. Since this formazan is of high solubility, its precipitation inside the crystal was less likely. So the hypothesis of the binding of the formazan to the crystalline enzyme had to be envisioned.

So we studied the solvent effect on binding of XXT formazan to the enzyme cross-linked crystal in buffer. The crystal turned red during the incubation with the formazan. This crystal transferred to fresh buffer did not discharge the color, but in the presence of increasing amount of solvents the crystals discharged their color at about 35% of ethanol or 50% DMSO or 50% DME. Now we could study the reaction in the presence of these solvents. The formazan should diffuse out of the crystals into the solution and it should be possible to monitor the reaction in respect of time. In presence of DMSO or DME the activity was 18% and 15% of that in the cinnamaldehyde test. The crystals were inactive after this solvent treatment. If the reaction was carried out in presence of pyrazole, an inhibitor [12], the activity was reduced, in agreement that the reaction occurs at the active site.

The solvents disrupt the reversible binding of formazan to the enzyme and its diffusion out of the crystal is now possible. Indeed later experiments carried out on aldose reductase with electrospray mass spectrometry showed that the XXT formazan binds to aldose reductase [17]. So the fact that the formazans do not diffuse out of the crystals could be due to their binding to the enzyme and this would explain that only the crystals were colored and no chromophore appeared in the solution. This binding must occur outside of the active site, because the reaction does not show any product inhibition. The adsorption of fluorescein and xanthene dyes by lysozyme crystals has been studied [18–20] and the adsorption of the formazan by alcohol dehydrogenase crystal is a related phenomena. The binding of another dye Cibacron Blue F3GA to alcohol dehydrogenase has been determined to be at the coenzyme binding site [21]. It is not surprising that these molecules bind to proteins even outside the active site and such binding has been detected [22].

In conclusion NADH bound to the enzyme may be oxidized to NAD bound to the enzyme. This opens now the possibility to study this enzyme and related enzymes in bypassing the cofactor association and dissociation steps. Beside the activity of alcohol dehydrogenase crystals may visualized by the color of the crystals and this may be extended to other dehydrogenases crystals and electron transferring systems. The binding of the formazan derivatives was unexpected and has to be explored in solution. Structural information on these complexes are awaited.

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