PEROXIDASE ACTIVITY OF HORSE LIVER ALCOHOL DEHYDROGENASE IN THE PRESENCE OF β-NAD$^+$ AND HYDROGEN PEROXIDE*

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Received 3 December 1974

1. Introduction

Horse liver alcohol dehydrogenase (LADH) has been extensively studied, mainly with respect to its catalytic ability to interconvert alcohols to aldehydes, but its main physiological function appears to be still unknown, since ethanol is not formed in the animal's body. During the last twenty years, other alternative activities have been found for LADH; the first, cronologically speaking, concerns the dismutation reaction of aldehydes [1,2]. An isomerase activity of LADH, concerning the isomerization of several aldehydes to ketones, was found first by Van Eys [3] and later allosterically correlated with the dehydrogenase activity [4].

In 1963 Waksman and Roberts [5] described a transaminase activity for yeast alcohol dehydrogenase and other dehydrogenases. A steroid activity of LADH, discovered in 1960 [6], could be solely ascribed to the more basic subfraction of the enzyme preparation only in 1966 [7] and later associated to an isoenzyme of LADH [8]. It has also been found that vitamin A is oxidized to retinene by LADH in the retina [9a,9b] and more recently that methanol can function as substrate [10].

In this paper we report evidence for a peroxidase activity of LADH, which originates when hydrogen peroxide is added to a solution containing β-NAD$^+$ and LADH.

The reaction occurs with consumption both of H_2O_2 and NAD$^+$ in a 1:1 stoichiometric amount without oxygen involvement and is not inhibited by 10^{-3} M EDTA. A NAD$^+$ derivative, which we call NADX, forms in this reaction; it differs from NADH and is inactive as coenzyme for the normal reaction of alcohol dehydrogenation. Evidence is also reported that this peroxidatic reaction occurs at the level of the same enzyme active site which is utilized for the normal alcohol oxidation reaction.

A simple chromatographic procedure for the isolation of NADX is also described.

2. Materials and methods

Horse liver alcohol dehydrogenase was purchased from Boehringer and Soehne and purified on a Whatman CM 52 column as previously described by one of us [11].

Alcohol dehydrogenase activity assays were performed in accordance with the methods of Theorell and Yonetani [12] and of Dalziel [13]. α-NAD$^+$, β-NAD$^+$ (grade V), NADH (grade III), ADPR, NMN, pyrazol and catalase, were obtained from Sigma. β-NAD$^+$ consumption was measured by a modified Dalziel's test at pH 9 (0.1 M pyrophosphate buffer) in the presence of 0.07 M semicarbazide. 30% hydrogen peroxide was obtained from BDH Chem.Co. (Peridrol) and assayed by the method of Eisenberg [14]. *N*-1-methyl nicotinamide iodide was a gift of Dr A. Mazzini. All other materials used were of analytical reagent grade of purity. The new enzymatic activity, exhibited by LADH in the presence of β-NAD$^+$ and H_2O_2, was

* A preliminary report on this work has been presented to the 1st Meeting of the 'Società Italiana di Biofisica Pura e Applicata' and published in the related Acta Volume, Camogli, December 1973.

North-Holland Publishing Company – Amsterdam

normally estimated following the increase in absorbance of the reaction mixture at pH 7.0, 0.05 M phosphate buffer at 300 nm. An LKB Uvicord II–Minirac fraction collector system has been utilized for the chromatographic procedure. A more detailed description of the experimental conditions used is given in the legend to the figures.

3. Results and discussion

When a solution containing LADH and NAD^+ is added of H_2O_2 it gives rise to an increasing absorbance around the 300 nm region, which can be recorded spectrophotometrically as a function of time, as shown in a typical experiment in fig.1.

Blank measurements performed on samples containing either NAD^+ and H_2O_2 without LADH, or LADH and H_2O_2 without NAD^+, or LADH and NAD^+ without H_2O_2, give no increase in absorbance at the same wavelength. Addition of traces of catalase to the reaction mixture completely blocks the reaction because of the rapid consumption of H_2O_2, as one expects if H_2O_2 is involved in the reaction.

Since many reactions of peroxides are catalyzed by metal ions, some experiments have been carried out at pH 7 in 1 mM EDTA, but no effect on the initial velocity at 300 nm has been observed.

The initial reaction velocity, recorded at 300 nm, is proportional to the enzyme active site concentration in the investigated range (0.5–15 μN). The net final increase of absorbance is however about the same, at constant initial concentration of NAD^+ independently of the concentrations of H_2O_2 and LADH used, if H_2O_2 is used in excess with respect to NAD^+.

These results indicate that the increase in absorbance observed at 300 nm is due to a reaction occurring between NAD^+ and H_2O_2 catalyzed by LADH.

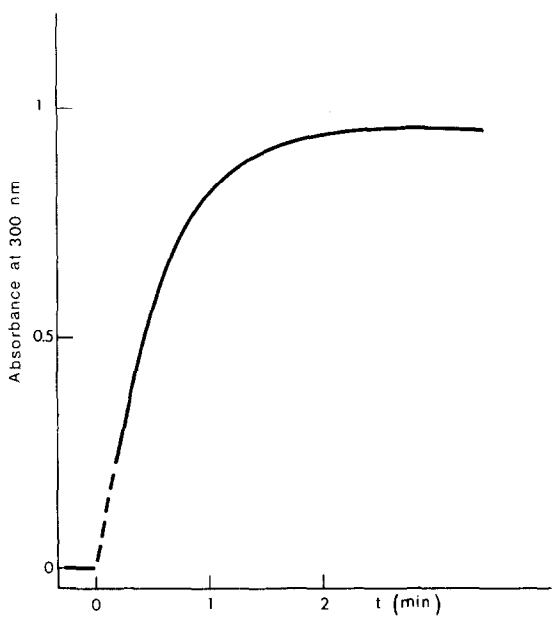

Fig.1. Kinetics of absorbance increase at 300 nm (A_{300}) as function of time, during the reaction between LADH, NAD^+ and H_2O_2. Conditions: Sample: LADH 6 μN; NAD^+ 66 μM; H_2O_2 88 mM. Reference: NAD^+ 66 μM; H_2O_2 88 mM. Cell path length 1 cm; total volume 1 ml; pH 7.0 0.05 M phosphate buffer.

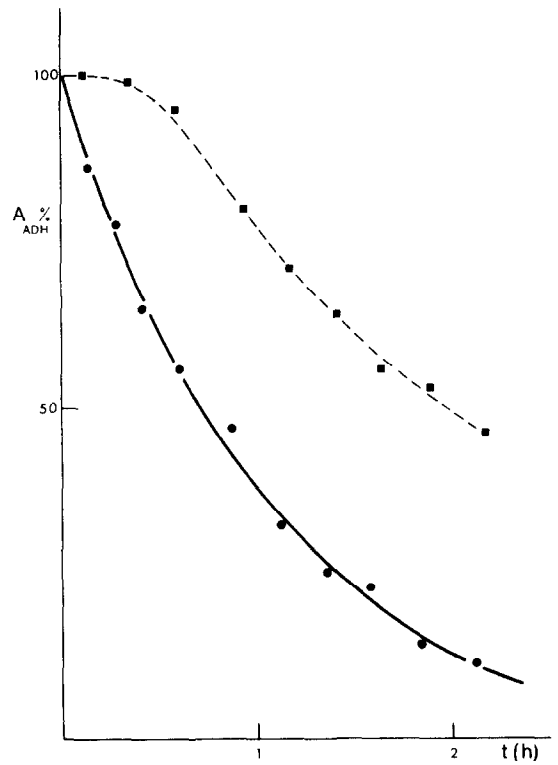

Fig.2. Alcohol dehydrogenase activity (A_{ADH}) curves of LADH as a function of preincubation time with H_2O_2 at pH 7.0 0.05 M phosphate buffer. Conditions: curve a: LADH 8 μN; H_2O_2 2 mM; curve b: LADH 8 μN; H_2O_2 2 mM; NAD^+ 0.68 mM. Activity measurements have been carried out according to Dalziel's method [13] by withdrawing 50 μl samples from the inactivation mixture at different times.

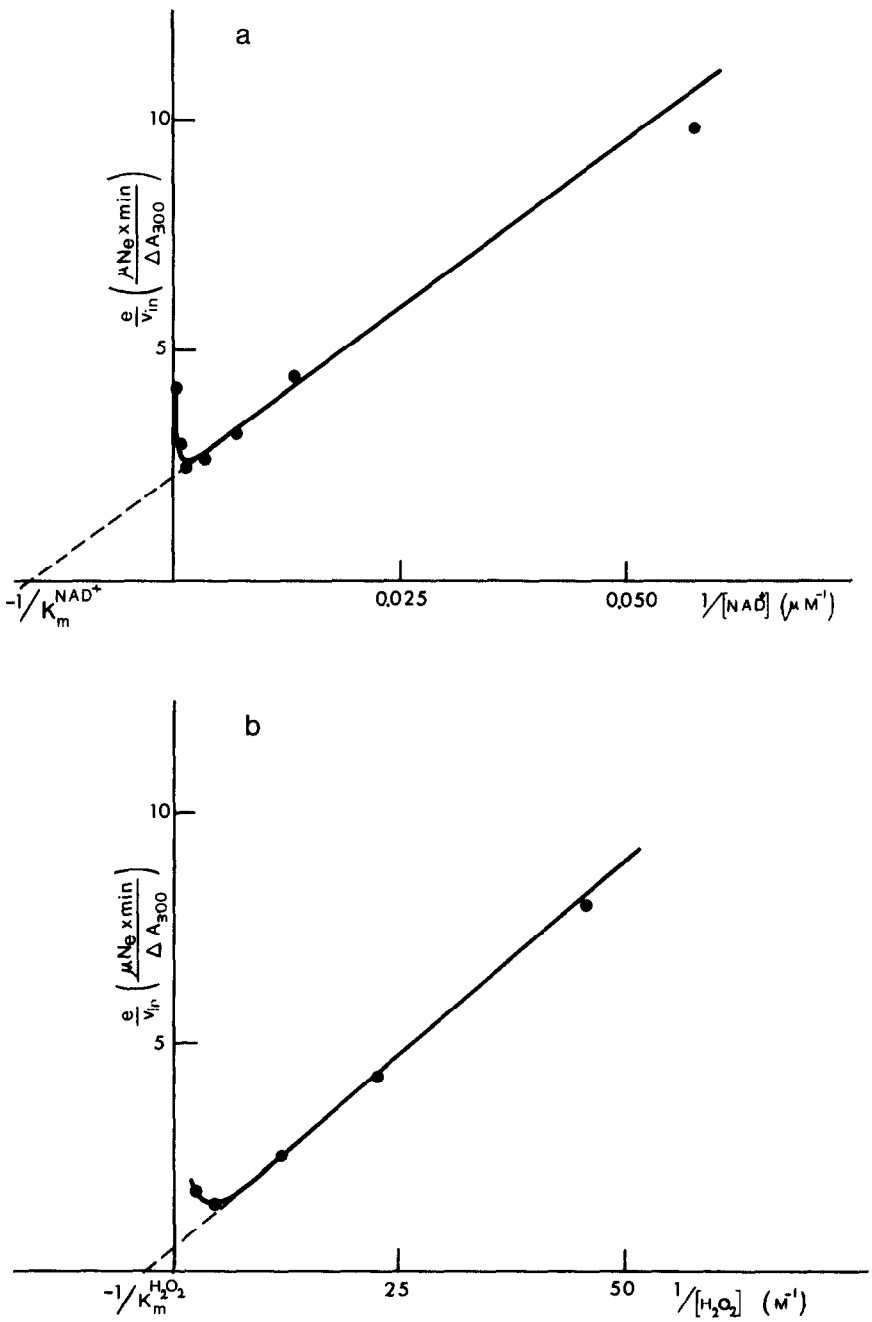

Fig.3. Lineweaver–Burk plots for the peroxidatic reaction of LADH at pH 7.0 in 0.05 M phosphate buffer. Conditions: curve a: 6 μN LADH, 88 mM H_2O_2, NAD^+ variable; curve b: 6 μN LADH; 0.625 mM NAD^+, H_2O_2 variable.

During the reaction, however, LADH becomes progressively inactivated by excess H_2O_2.
In fact, when LADH is treated with hydrogen peroxide, at pH 7 in 0.05 M phosphate buffer, an exponential decay of the alcohol dehydrogenase activity is observed with time as shown in fig.2 (curve a). Preincubation of LADH with NAD^+, before adding H_2O_2, partially protects the enzyme against inactivation (fig.2, curve b). Kinetic investigations on the new enzymatic reaction at pH 7 have furnished apparent K_m values of about 60 μM and 0.33 M for NAD^+ and H_2O_2 respectively. The K_m values have been obtained at not saturating levels of both substrates because a too high concentration of H_2O_2 (>0.2 M) very rapidly inactivates the enzyme while a too high concentration of NAD^+ (>1 mM) originates an autoinhibition effect, as shown in fig. 3a and 3b. From measurements on the consumption of NAD^+ and H_2O_2 during the enzymatic reaction we have seen that the reaction proceeds with the modification of one molecule of NAD^+ per molecule of H_2O_2, that is the stoichiometry is 1:1. The data obtained are corrected for the non-enzymatic consumption of NAD^+ and H_2O_2, as evaluated from measurements on a reference solution, containing only H_2O_2 and NAD^+ without LADH, in the same experimental conditions. When an excess pyrazol (e.g. 10^{-3} M) with respect to the enzyme active site normality is added to the reacting mixture containing LADH, NAD^+ and H_2O_2, no further increase with time is observed in the absorbance at 300 nm.

A similar effect is obtained if NADH (e.g. 200 μM), instead of pyrazol, is added to the reacting solution. These results clearly indicate that the two substrates, i.e. NAD^+ and H_2O_2, utilize the same enzyme active center, which is also utilized by NAD^+ and alcohol during the normal alcohol dehydrogenase reaction. In fact, in the case of addition of pyrazol it is the formation of a stable ternary complex among NAD^+, pyrazol and enzyme active site, which inhibits the enzymatic reaction between NAD^+ and H_2O_2; whereas in the case of addition of NADH it is the displacement of NAD^+ by NADH from the active center which prevents the reaction from proceeding.

Further support to this conclusion derives from the fact that the peroxidase and alcohol dehydrogenase activities are linearly correlated (fig.4).

As it is well known, cyanide reacts with NAD^+ to give an addition compound with an absorption maximum at 327 nm [15]; therefore we have added 1 M KCN both to a solution containing 22 mM H_2O_2 and 0.150 mM NAD^+ and to another solution, identical to the first one but containing 6 μN LADH, after the enzymatic reaction with H_2O_2 was completed and H_2O_2 removed with catalase. While the former solution gave rise to the expected absorption band centered at 327 nm, the latter solution showed a marked decrease in absorbance, in the same wavelength region.

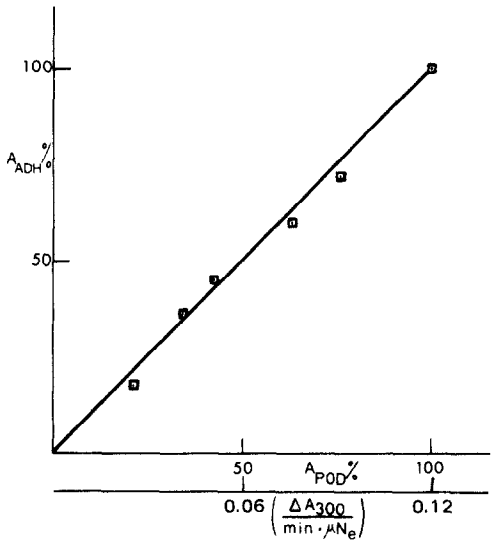

Fig.4. Peroxidase (A_{POD})–alcohol dehydrogenase (A_{ADH}) activities linear relationship. Each activity measurement has been carried out by withdrawing 50 μl samples from the inactivation mixture containing 8 μN LADH and 5 mM H_2O_2, in the absence of NAD^+, at different times. A_{ADH} has been tested according to Dalziel [13]. A_{POD} has been measured at pH 7.0 in 0.05 M phosphate buffer using the following experimental conditions: NAD^+ 750 μM; H_2O_2 22 mM. 100% A_{POD} has been taken as that corresponding to the initial reaction velocity at 300 nm, with LADH not yet incubated with H_2O_2.

Therefore this result indicates that the reaction between NAD^+ and H_2O_2 very probably leads to a chemical modification on the nicotinamide ring of NAD^+, such that the cyanide complex typical of *N*-1 substituted nicotinamide derivatives no longer forms.

In order to clarify the number of products obtained from the enzymatic reaction between NAD^+ and H_2O_2 a simple chromatographic procedure has been adopted, using a column filled with diethylaminoethylcellulose (Whatman DE 52). The results are shown in fig. 5.

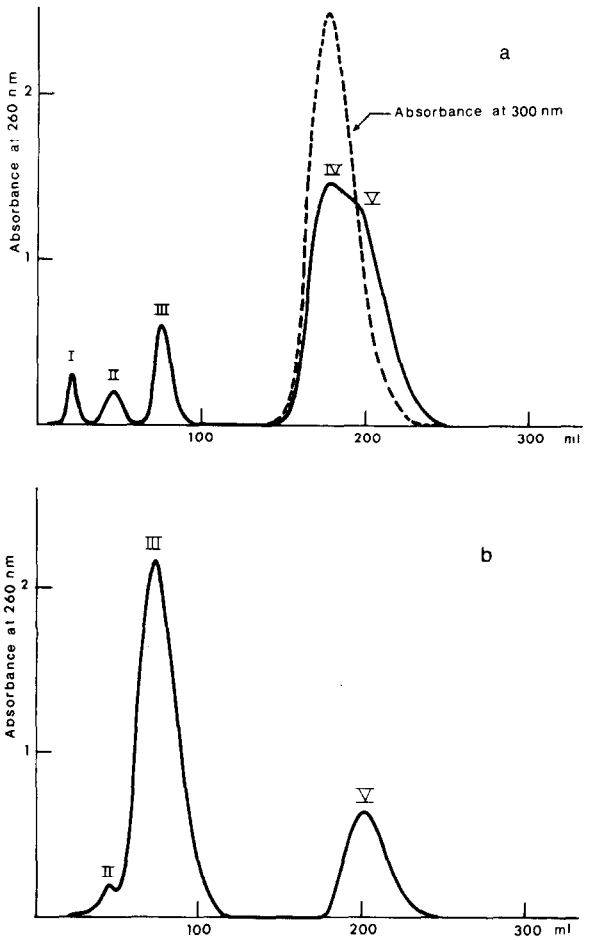

Fig.5. a) Elution pattern of a 1 ml solution, as obtained after 15 min of reaction between NAD^+ (750 μM), H_2O_2 (22 mM) and LADH (6 μN) (initial volume: 10 ml) and removal of excess H_2O_2 by addition of 10^{-8} M catalase. The chromatography has been carried out through a DE 52 (Whatman) column (cm 50 X 1.2) with 0.05 M pH 7.0 phosphate buffer as eluent, by monitoring the absorbance of the eluate at 260 and at 300 nm. b) Elution pattern of a 1 ml solution obtained after 15 min from the mixing of NAD^+ (750 μM) and H_2O_2 (22 mM) in the absence of LADH (initial volume 10 ml). The other experimental conditions are the same as those described at point a).

Analysis of the fractions has revealed that peak I in fig. 5a refers to partially denatured LADH, peak III refers to residual NAD^+ (about 10% with respect to the initial 5 mg of NAD^+ used) and peak IV to NADX, obtained enzymatically (65%).

Peak II and V are present in both patterns of fig.5 and refer to degradation products of NAD^+ (~25%) which form by the direct action of H_2O_2 on NAD^+ itself, without the catalysis by LADH.

As already observed by Burton and Lamborg [16], peak II probably refers to nicotinamide while peak V to a compound related to ADPR. Other conditions of pH and concentration of the eluent buffer have been tested in order to obtain a complete separation between peaks IV and V but without better success. For UV measurements only those fractions having a constant ratio A_{300}/A_{260}=1.7 have been utilized (fig.6).

In order to see if the reaction could also occur with analogues of β-NAD^+, experiments have been performed using α-NAD^+, adenosindiphosphoribose (ADPR), nicotinamide mononucleotide (NMN), *N*-1-methyl-nicotinamide iodide and nicotinamide, instead of β-NAD^+, but in no case increase in absorbance at 300 nm has been observed. In particular *N*-1-methyl nicotinamide iodide was interesting to be assayed, because this compound, when enzymatically oxidized by rabbit liver aldehyde oxidase, gives rise to pyridone derivatives with corresponding increase of absorbance at 300 nm, as firstly shown by Know and Grossmann [17] and later by Chaykin and coworkers [18].

The results, so far obtained at pH 7.0 in 0.05 M phosphate buffer, can then be rationalized assuming

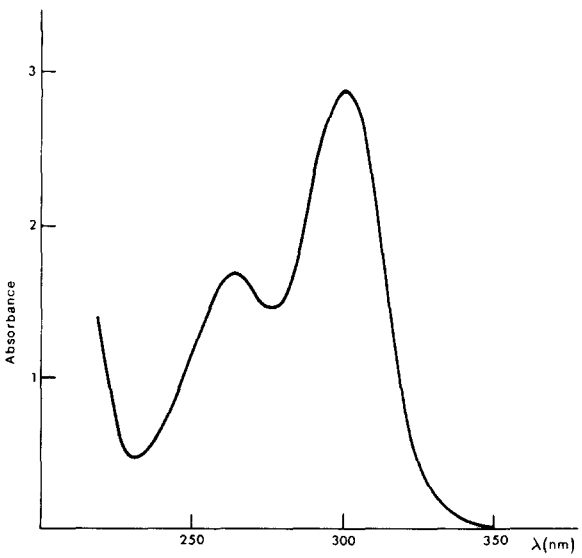

Fig.6. UV spectrum of NADX, as obtained after purification of the reaction mixture through DE 52 column chromatography.

that LADH exhibits a new catalytic activity, in the reaction occurring between NAD^+ and H_2O_2, which can therefore be classified as a peroxidase activity. This is the first time, to our knowledge, that H_2O_2 has been found to behave as substrate with this liver enzyme.

The most interesting aspect of this new reaction is, probably, the fact that NAD^+, which normally behaves as an oxidizing coenzyme, plays a role as a reducing substrate for H_2O_2 during the enzymatic process. The UV spectrum of NADX is similar to those of 4- and 6-pyridones of NAD^+, chemically prepared [19–21], except for a marked difference in the relative intensity of the two peaks.

At this stage, however, we are not yet able to give the exact chemical structure of the enzymatically formed NADX. An oxidized NAD^+ derivative was suggested to be an intermediate in oxidative phosphorylation [22], but experiments carried out firstly on NAD 2-pyridone and NAD 4-pyridone [23] and later on NAD 6-pyridone [20] by incubation with mitochondria have given negative results.

We are now dealing with experiments aimed to verify if NADX can play some role during the oxidative phosphorylation.

At the same time, we are investigating the pH effect and the mechanism with which LADH functions in this new enzymatic reaction as well as the behaviour of other enzymes, particularly NAD^+ dependent dehydrogenases, other peroxides and NAD^+ analogues, in order to establish whether this type of reaction is of some generality.

Acknowledgements

We wish to thank Dr A. Vecli and Professor G. Casnati for helpful discussions and continuous interest in this work. This work was supported by a Research Grant from C.N.R. (Italy).

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