Monitoring enzymic peroxidation of NAD$^+$ by capillary electrophoresis

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Abstract

NAD$^+$ is degraded by horse liver alcohol dehydrogenase in a reaction with p-methylbenzyl hydroperoxide, which acts as a pseudo-substrate. An unstable intermediate product (called compound I) is formed, and this is converted into the final product NADX. The reaction can be monitored by capillary electrophoresis. NADX migrates as two well resolved peaks (NADX$_1$ and NADX$_2$), which belong probably to the tautomers of the same substance. The intermediate product, compound I, has the same effective mobility as the conformer NADX$_2$ and it was impossible to separate compound I from NADX$_2$. The spectra taken during the separation show clearly that the "9-min" peak contains different compounds during the course of the reaction and the NADX$_2$ is not identical with compound I.

1. Introduction

Horse liver alcohol dehydrogenase (LADH) has long been known to degrade NAD$^+$ in the presence of hydrogen peroxide [1]. NAD$^+$ was found to protect the enzyme from inactivation by H$_2$O$_2$ [2]. The reaction product, which was given the name NADX, was shown to have a characteristic UV spectrum with the absorption maxima at 260 and 300 nm. A two-step reaction mechanism was proposed for this reaction: in the first step, catalysed by enzyme, a metastable intermediate called compound I is formed, which is followed by an enzyme-independent hydrolytic step leading to NADX. Compound I can react with the excess of H$_2$O$_2$ to form compound Y.

The UV spectrum of compound Y differs form that of NADX: it does not have the absorption maximum at 300 nm [3]. From the ^{1}H and ^{13}C NMR spectra, the structure of NADX was deduced to be
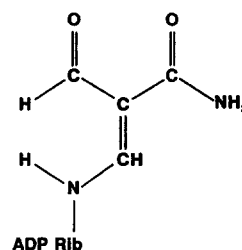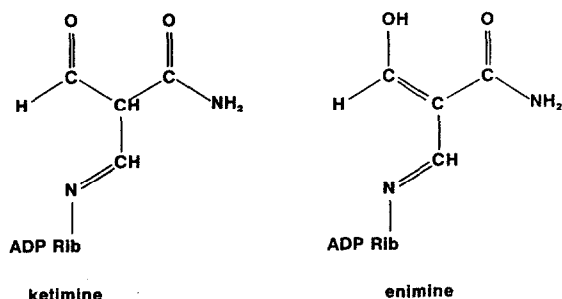

NADX

where ADP Rib = adenosine-5'-diphospho-5-β-D-ribosyl. However, the NMR spectra did not

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exclude the chemical structure of NADX from corresponding to enimine and/or ketimine [4,5]:

ketimine   enimine

The reaction of NADX with KCN and $NaBH_4$ led the authors to prefer the former structure [4].

LADH was demonstrated to be strongly inhibited by p-methylbenzyl hydroperoxide [6], but this inhibition is only transient [7]. In the presence of NAD^+, p-methylbenzyl hydroperoxide behaves as a pseudo-substrate of the enzyme, forming so far unidentified non-inhibiting products, while NAD^+ is converted into NADX [7].

The biological importance of NADX has not been studied extensively so far. Preliminary experiments showed that NADX increases the proliferation of fibroblasts in tissue cultures [8].

In this work, capillary zone electrophoresis was used to separate the reaction components of the enzymic peroxidation of NAD^+, using complex-forming equilibria [9], which enables a better separation to be achieved. The migration velocity of the reaction products is discussed in relation to the proposed structure.

2. Experimental

Horse liver alcohol dehydrogenase and adenosine diphosphoribose (ADPR) were purchased from Sigma (St. Louis, MO, USA). p-Methylbenzyl hydroperoxide was prepared by photooxidation of p-xylene and isolated as described [6]. NAD^+ was purchased from Imuna (Šárišské Michalany, Slovak Republic). Other chemicals were obtained from Lachema (Brno, Czech Republic).

The enzymic reaction was performed at room temperature and pH 7.0. The reaction mixture of total volume 250 μl contained 0.08 M phosphate buffer (pH 7.0), 25 mM p-methylbenzyl hydroperoxide and 15 mM NAD^+ and the reaction was started by adding LADH to the final concentration of 38 μM.

Fused-silica capillaries (75 μm I.D. × 360 μm O.D.) were obtained from J & W Scientific (Folsom, CA, USA). The total length of the capillaries used was about 40 cm. The capillaries were coated with linear polyacrylamide after the vinylation of the capillary surface with vinylmagnesium bromide [10]. The detection cell was made by cutting off the polyimide layer [11].

The in-house constructed instrumentation was similar to that described by Jorgenson and Lukacs [12] with the difference that a small chamber covering just the electrode vial, which contained the connected high-voltage electrode, was used instead of a Plexiglass protective box. A Spellman CZE1000R high-voltage power supply was used with an electric field strength applied of 300 V/cm. A Spectra 100 variable-wavelength detector (Spectra-Physics, Palo Alto, CA, USA) was used to monitor the course of analysis at 260 and 300 nm. To obtain spectra of the particular peaks, a Spectra Focus forward optical scanning detector (Spectra-Physics) was utilized in some experiments.

3. Results and discussion

As nucleotides can be easily separated at about pH 4 [13], an operational electrolyte containing 0.1 M ϵ-aminocaproic acid and 0.1 M adipic acid (pH 4.3) [14] was tested first to separate the ionizable reactant (NAD^+) and the reaction products (compound I and NADX). All compounds of interest were well resolved. Only the final reaction product, NADX, was shown surprisingly to migrate in two peaks, which were only partially separated (this observation was confirmed by analysis of NADX purified by LC). The pH of the operational electrolyte did not affect their resolution significantly (results not shown). When the pH optimization of electrophoretic separation fails, the application of com-

plex-forming equilibria is the next routine step in the optimization strategy [8]. Addition of Cu^{2+} ion to the operational electrolyte was unsuitable as it led to a very unstable baseline of the UV analytical signal. This may have been caused by complexation of polyacrylamide from the capillary coating with Cu^{2+} and its fluctuation.

In electropherograms of the reaction mixture, five peaks were separated (see below), which corresponded to adenosine diphosphoribose (ADPR), $NADX_1$, compound I, $NADX_2$, p-toluic acid (p-TA), which was delivered to the reaction mixture as an impurity from p-methylbenzyl hydroperoxide, and NAD^+. As NAD^+ is separated from the other compounds of interest more than sufficiently, only the separation of the remaining five compounds in four peaks (compound I and $NADX_2$ have never been separated) was optimized when the influence of Mg^{2+} as another complexing cation was tested. The plot of the effective mobility *versus* concentration of Mg^{2+} at a concentration of buffer (ϵ-aminocaproic acid–adipic acid) 40 mM is shown in Fig. 1. p-Toluic acid does not form obviously strong complexes with magnesium; its mobility is not affected substantially on increasing the concentration of Mg^{2+}. This does not apply for the remaining nucleotide derivatives.
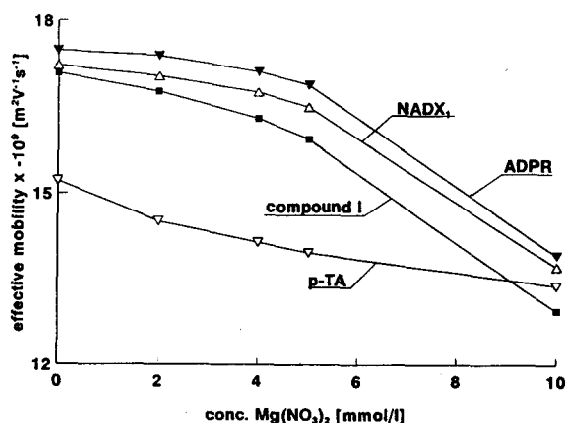

Fig. 1. Dependence of the effective mobilities of the compounds in question on the amount of $Mg(NO_3)_2$ in the operational electrolyte. Operational electrolyte, 40 mM ϵ-aminocaproic acid–40 mM adipic acid–0 to 10 mM $Mg(NO_3)_2$. Electric field strength, 300 V/cm. ADPR = adenosine diphosphoribose; p-TA = p-toluic acid.

An increase in the Mg^{2+} concentration helps to increase the resolution between the peaks of $NADX_1$ and compound I. The optimum concentration of Mg^{2+} was found to be 4 mM. A further increase of the Mg^{2+} concentration to 10 mM results in a change of the migration order when p-toluic acid migrates faster than compound I.

When the Mg^{2+}/ϵ-aminocaproic acid concentration ratio is kept constant, an increase in the total concentration of the operational electrolyte leads to a decrease in the effective mobilities of the separated compounds also (results not shown). This is not surprising and another operational electrolyte containing 90 mM ϵ-aminocaproic acid, 90 mM adipic acid and 10 mM magnesium nitrate was found to be suitable for the analysis of the particular mixture. Either this or an electrolyte containing 40 mM ϵ-aminocaproic acid, 40 mM adipic acid and 4 mM $Mg(NO_3)_2$ were used in further experiments to analyse the reaction mixture.

The course of the enzymatic reaction as monitored by capillary electrophoresis is shown in Fig. 2. Fig. 2a shows the composition of the reaction mixture before the reaction was started. Only peaks of ADPR, p-toluic acid and NAD^+ were found in the electropherogram. The significant difference in the migration velocity between ADPR and NAD^+ is caused primarily by the positive charge of the pyridine nitrogen in the case of NAD^+; this is lacking in the molecule of ADPR; 43 min after the start of the enzymatic reaction, the new peak of the intermediate product, compound I, appears in the electropherogram, whereas the peak of NAD^+ is significantly reduced (Fig. 2b). Two hours later, a small peak of NADX (denoted $NADX_1$), as the hydrolysis product of compound I, can be detected (Fig. 2c). Fig. 2d shows the composition of the reaction mixture 19 h after the start of the reaction: NAD^+ has disappeared completely and two peaks of NADX of approximately the same height are observed. By that time, their peak-height ratio was independent of time. The relative similarity of ADPR, $NADX_1$, $NADX_2$ and compound I in terms of effective mobility suggests that none of these compounds bears any
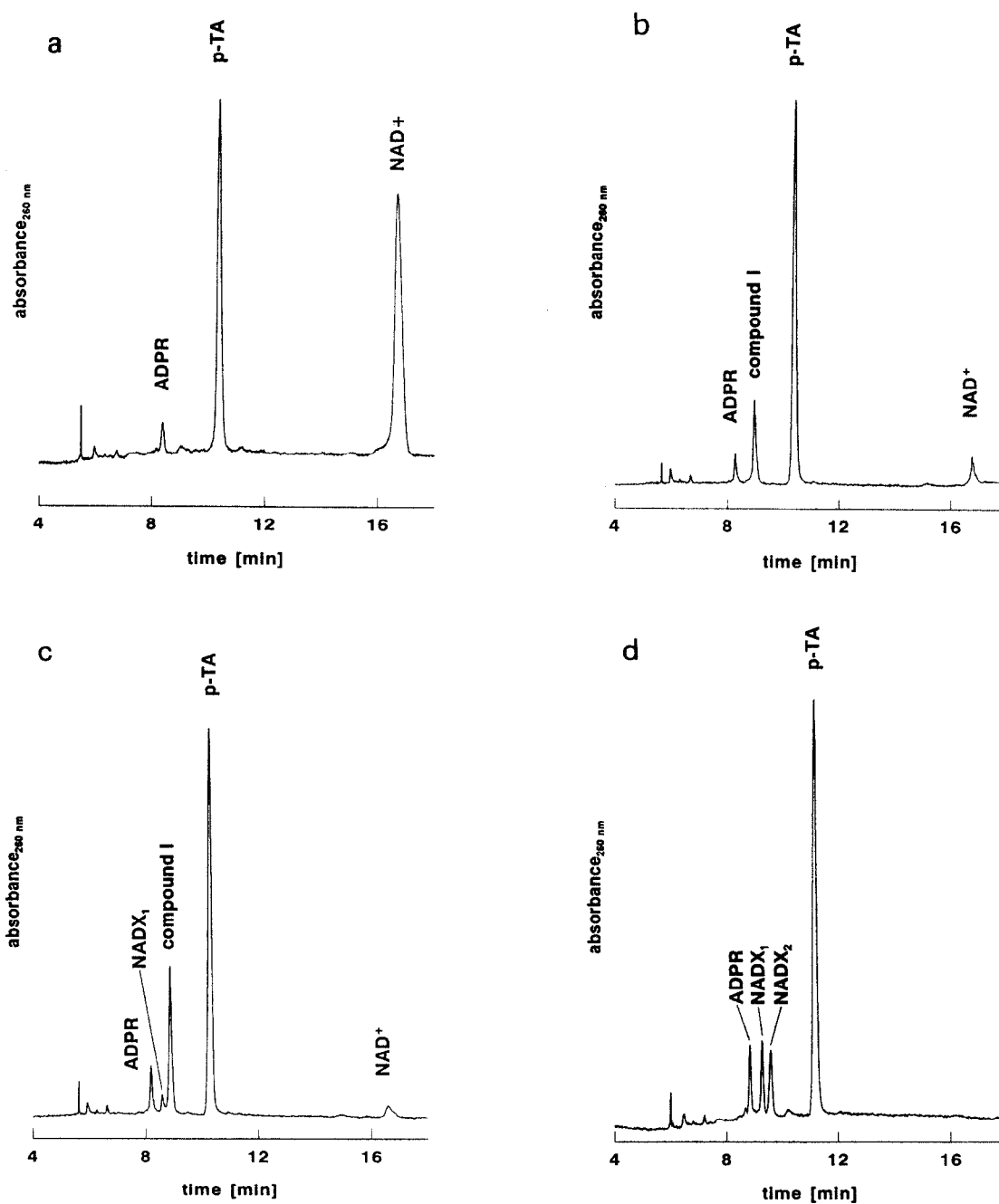
Fig. 2. Electrophoresis of the reaction mixture during the course of enzymatic peroxidation of NAD$^+$. (a) 0; (b) 43; (c) 163; (d) 1133 min. Separation conditions: coated capillary, 75 μm I.D., 360 μm O.D., total length 363 mm, effective length 263 mm; operational electrolyte, 90 mM ε-aminocaproic acid–90 mM adipic acid–10 mM Mg(NO$_3$)$_2$; voltage, 11 kV. Sampling by siphoning (3 s at 10-cm elevation). ADPR = adenosine diphosphoribose; p-TA = p-toluic acid.

positive charge on the residue from nicotin-amide. This is in a contradiction to the structure proposed for NADX [4]. The secondary amine of that structure would bear a positive charge at acidic pH, which would cause NADX to migrate in the vicinity of NAD^+. The position of the NADX peaks near ADPR suggests structures without an amine moiety. The same applies to the structure of the so far unidentified compound I. The existence of two peaks of NADX seems to support the hypothesis that NADX is formed by two tautomeric compounds.

It is possible that p-toluic acid is formed during the reaction. However, we were not able to verify this hypothesis because of the high content of p-toluic acid in the reaction mixture.

The scanning UV detector was very helpful in distinguishing two compounds migrating with the same mobility and differing in their spectra (Fig. 3). In the first experiments, we expected the peak with a migration time of ca. 9 min to be one of the NADX peaks as identified by analys-ing NADX preparations purified by LC. How-ever, the electrophoretic behaviour of the inter-mediate compound I was unclear. We therefore used a scanning detector to measure spectra of the individual peaks. The $NADX_1$ and NAD^+
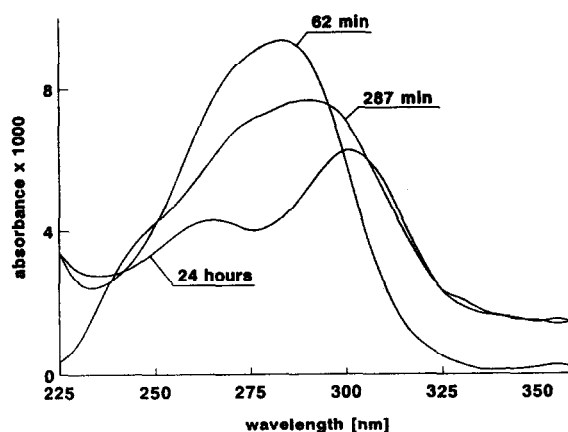

Fig. 4. Spectra taken at the maximum of the 9-min peak at different intervals from the start of the enzymatic reaction.

peaks provided spectra corresponding to the published data. Very interesting results were obtained for the peak with a migration time of ca. 9 min (Fig. 4). When the reaction mixture was analysed 62 min after the reaction was initiated, the absorption spectrum of the peak in question showed one peak with a maximum at ca. 285 nm, which is characteristic of compound I [1,7]; 225 min later, the maximum of the absorption peak was shifted slightly to longer
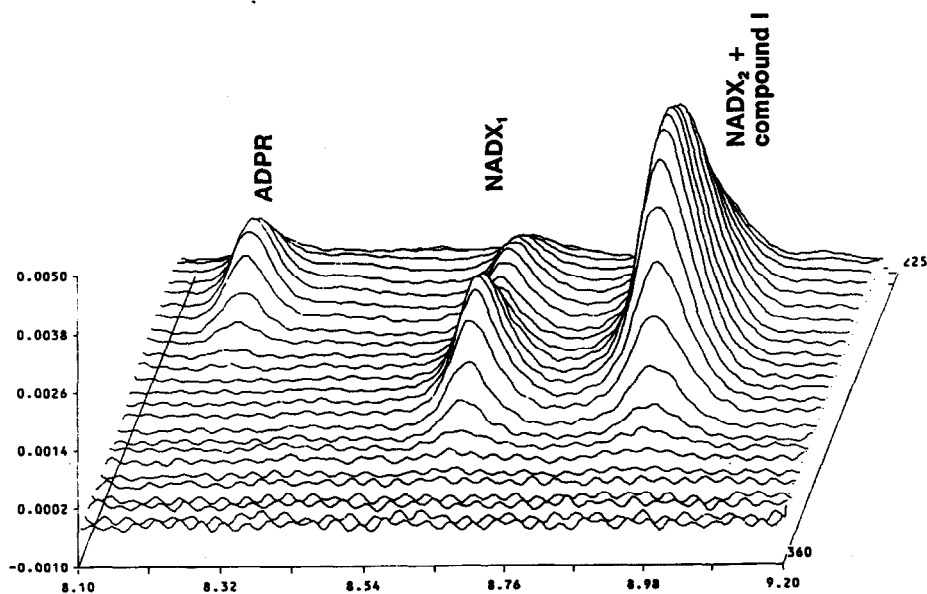

Fig. 3. UV scan of the electropherogram 287 min after the initiation of the enzymatic reaction. x-Axis: time (min); y-axis: absorbance; z-axis: wavelength (nm).

wavelengths and a shoulder appeared. Electro-phoresis of the reaction mixture after 19 h or more gave a peak at *ca.* 9 min, the spectrum of which exhibited two absorption maxima at 260 and 300 nm. This spectrum is typical of NADX [1]. This means that this peak can contain both compound I and $NADX_2$ at different intervals of the reaction. One hour after the reaction starts compound I dominates whereas 24 h later the peak contains $NADX_2$ exclusively. The spectrum taken at a reaction time of 287 min indicates the simultaneous presence of the both compounds. (The relatively short lifetime of compound I indicates its possible regulatory function in the organism.) It should be noted that absorption at wavelengths below 225 nm seemed to be in-fluenced by some factors other than just absorp-tion of the analyte in the detection cell and the spectral differences between the individual anal-yses were neglected.

Naturally, we were interested to establish whether compound I and $NADX_2$ can be at least partially resolved by capillary electrophoresis although they co-migrate in one peak. We made several time slices through the particular peak and compared the spectra. However, as shown in Fig. 5, the spectra from different time slices do not differ in absorption maxima, which dem-onstrates that compound I and $NADX_2$ cannot be separated under the given conditions.
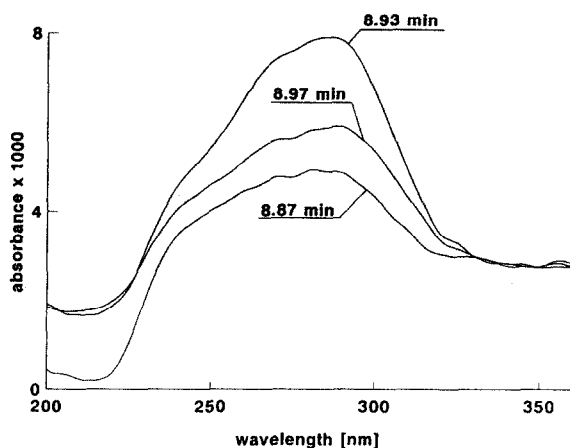
The existence of two peaks in purified prepa-rations of NADX indicates the possibility that two tautomers of NADX exist. The best way to verify this hypothesis would be micropreparative electrophoresis of NADX and collection of the fractions corresponding to NADX, followed by repeated electrophoresis of the particular frac-tions. The presence of the same peaks in the electropherograms from both fractions more than *ca.* 20 h after they were collected would be a clear confirmation of this hypothesis. Unfor-tunately, our efforts in this respect have not been successful so that as the concentration of NADX in the collected fraction was too low to give any measurable peaks in repeated electrophoresis.

4. Conclusions

Capillary electrophoresis resolves NADX, the final product of the enzymic peroxidation of NAD^+, into two peaks which we denote $NADX_1$ and $NADX_2$. An intermediate product called compound I can also be detected by capillary electrophoresis. Although it co-migrates with $NADX_2$, both compounds can be distinguished by their UV spectra. The reaction obviously starts by the formation of compound I, which is converted into $NADX_1$. $NADX_1$ generates $NADX_2$, probably as its tautomeric form. Since the detailed rection mechanism of the formation of NADX is not clear (or its biological function), capillary electrophoresis offers a valuable tool for such studies.

Fig. 5. Spectra from the time slices of the 9-min peak, as analysed 287 min after the start of the reaction.

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