Oxidation of NAD dimers by horseradish peroxidase

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Horseradish peroxidase catalyses the oxidation of NAD dimers, $(\text{NAD})_2$, to NAD^+ in accordance with a reaction that is pH-dependent and requires 1 mol of O_2 per 2 mol of $(\text{NAD})_2$. Horseradish peroxidase also catalyses the peroxidation of $(\text{NAD})_2$ to NAD^+ . In contrast, bacterial NADH peroxidase does not catalyse the peroxidation or the oxidation of $(\text{NAD})_2$. A free-radical mechanism is proposed for both horseradish-peroxidase-catalysed oxidation and peroxidation of $(\text{NAD})_2$.

Several NAD(P)H oxidases are known to use molecular O_2 to oxidize the reduced nicotinamide nucleotides (Sbarra & Karnovski, 1959). HRP has been reported to catalyse the oxidation of NADH in slightly acidic media (Akazawa & Conn, 1958; Yamazaki & Yokota, 1973; Halliwell & de Rycker, 1978), and a mechanism involving the formation of NAD^+ , which then reacts with O_2 to give NAD^+ , has been proposed (Halliwell & de Rycker, 1978).

We have previously conclusively determined the composition and the structure of products arising from electrochemical one-electron reduction of NAD^+ (Carelli *et al.*, 1980). The mixture contains three diastereoisomeric dimers, which have the tetrahydrobipyridine 4,4'-linked structure (I).

Since these dimers can be photolysed to NAD^+ , which then reacts with O_2 to give NAD^+ (Avigliano *et al.*, 1983), $(\text{NAD})_2$ would appear to be a promising substrate for HRP.

The aim of the present work is the study of the HRP-catalysed oxidation and peroxidation of $(\text{NAD})_2$, as compared with the same reactions of NADH.

Materials and methods

HRP (250 units/mg), *Streptococcus faecalis* NADH peroxidase (45 units/mg) and yeast alcohol dehydrogenase (240 units/mg) were obtained from Boehringer. Units are those reported in the Boehringer catalogue. $(\text{NAD})_2$ was prepared as described by Carelli *et al.* (1980), stored under vacuum at -30°C and dissolved just before use. NAD^+ and NADH were from Fluka or Calbiochem. All other reagents were analytical grade and used without further purification. The oxidation of $(\text{NAD})_2$ and NADH was followed either by the decrease of the absorbance at 340 nm, or by the O_2 uptake measured with an O_2 -sensitive electrode. Spectrophotometric measurements in anaerobiosis were carried out in a Thunberg-type cuvette that

Abbreviation used: HRP, horseradish peroxidase.

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was degassed and flushed with 99.9% Ar, freed from O_2 by an alkaline-dithionite trap. The specimens used in the present work had the following molar absorption coefficients ($M^{-1} \cdot cm^{-1}$) in water: NADH, $\epsilon_{260} = 14400$ and $\epsilon_{340} = 6200$; NAD^+ , $\epsilon_{260} = 17600$; $(NAD)_2$, $\epsilon_{260} = 31600$ and $\epsilon_{340} = 6400$ (Carelli *et al.*, 1980). H.p.l.c. analyses were performed on a Perkin-Elmer Series 3 liquid chromatograph equipped with an LC 55 B spectrophotometric detector, an LC 55 S digital scanner and a Hewlett-Packard 3390A integrating recorder, with a Merck Hibar RP 18 RT-250-4 Li-Chrosorb $10\mu m$ column. The experimental procedure has been described elsewhere (Avigliano *et al.*, 1983).

The presence of NAD^+ after the oxidation was established by h.p.l.c., on the basis of the retention time of its elution peak, as compared with that of authentic NAD^+ under identical chromatographic conditions, or by an enzymic test with alcohol dehydrogenase.

Results

HRP-catalysed oxidation

The pH-dependent kinetics of NADH oxidation in the presence of HRP are reported in Fig. 1. NADH is only slightly oxidized at pH 7.2, whereas a significant decrease of the absorbance at 340 nm as a function of time is apparent at pH 5.2. Fig. 1

Fig. 1. Oxidation and peroxidation of NADH and $(NAD)_2$ by HRP

The experiments were conducted in the presence of $0.25 mM-O_2$ at $25^\circ C$ in $50 mM$ -potassium phosphate buffer, pH 7.2 (curves A, B and C), or in $50 mM$ -sodium acetate buffer, pH 5.2 (curves a, b and c). The incubation mixture contained $0.3 mM$ -NADH (curves A and a) or $0.3 mM$ - $(NAD)_2$ (curves B and b). Curves C and c refer to solutions containing $0.3 mM$ - $(NAD)_2$ with $0.5 mM-H_2O_2$ added. HRP (final concn. $4 \mu M$) was added at the arrow.

also shows the oxidation of $(NAD)_2$ catalysed by HRP at the same pH values; again the rate is higher at pH 5.2.

Fig. 2 shows the initial oxidation rates of $(NAD)_2$ and NADH in the presence and in the absence of HRP at pH values ranging from 5 to 8. The oxidation rates are higher at lower pH values (Table 1 and Fig. 2). However, although the oxidation rate of NADH falls almost to zero at $pH \geq 6.2$, $(NAD)_2$ is still oxidized at pH values higher than 7 in the presence of HRP.

O_2 -uptake experiments showed that 1 mol of O_2 is consumed per 2 mol of $(NAD)_2$ or NADH oxidized. In each case the main oxidation product was identified as NAD^+ . H.p.l.c. analysis showed that after 60 min of aerobic incubation in the presence of HRP about 70% of the initial $(NAD)_2$ was converted into NAD^+ (see Fig. 3b).

HRP-catalysed peroxidation

The rate of HRP-catalysed peroxidation of $(NAD)_2$ or NADH was significantly higher than that of the HRP-catalysed oxidation. The enzymic peroxidation of NADH takes place even at pH 7.2. Interestingly, the peroxidation rate is 2–3-fold higher when the reaction is performed in the absence of air, irrespective of pH (Table 1). Again NAD^+ is the main oxidation product (about 85%;

Fig. 2. pH-dependence of $(NAD)_2$ and NADH oxidation by HRP

The Figure shows the initial oxidation rates, as determined in $50 mM$ -potassium phosphate buffer by absorbance decrease at 340 nm, of $0.3 mM$ - $(NAD)_2$ (Δ and $\blacktriangle$) or $0.3 mM$ -NADH ($\circ$ and $\bullet$) in the absence (Δ and $\circ$) or in the presence ($\blacktriangle$ and $\bullet$) of $4 \mu M$ -HRP.

Fig. 3. H.p.l.c. profiles of 2mM-(NAD)₂ in 70mM-NH₄HCO₃, pH7.5, at 25°C, in the presence of 0.25mM-O₂ (a) after 1 h in the absence of HRP, (b) after 1 h in the presence of 0.5 μM-HRP and (c) after 1 h in the presence of 0.5 μM-HRP and 50mM-H₂O₂.

The eluates were monitored at 259nm. Peaks A, B and C correspond to the main isomers of (NAD)₂ mixture; peak D corresponds to NAD⁺; unlabelled peaks in (a) correspond to small amounts of 4,6'-linked dimers (Jaegfeldt, 1981).

Table 1. Initial oxidation and peroxidation rates of NADH and (NAD)₂ in the presence of HRP

The incubation mixtures contained 0.3 mM reduced nucleotide in 50mM sodium acetate buffer pH5.2 or 50mM potassium phosphate buffer, pH7.2 (1 ml). The reactions were started by the addition of HRP (final concn. 1 μM). The rates were measured at 25°C by the absorbance decrease at 340nm. Each value reported is the mean of at least three different measurements. The standard deviation was within 10%.

Oxidant	Nucleotide oxidized (nmol/min)	
	NADH	(NAD) ₂
O ₂ (0.25mM)		
pH5.2	45	22
pH7.2	0.5	7
H ₂ O ₂ * (0.5mM)		
pH5.2	160	120
pH7.2	8	23
O ₂ (0.25mM) + H ₂ O ₂ (0.5mM)		
pH5.2	66	37
pH7.2	4	10

* Experiments carried out in anaerobiosis.

Fig. 3c). The reaction showed a pH-dependence similar to that of the enzymic oxidation (Fig. 1).

The oxidation and peroxidation of (NAD)₂ and NADH are 3–5 times faster in the presence of Mn²⁺ (0.1mM) or NaN₃ (0.1M).

In contrast with HRP, bacterial NADH peroxidase does not catalyse the oxidation or the peroxidation of (NAD)₂ in the pH range 5–7. The rate of enzymic peroxidation of NADH at pH7.2 is considerably lower in the presence of (NAD)₂.

Discussion

The HRP-catalysed oxidation and peroxidation of (NAD)₂ display the same features as the corresponding reactions of NADH, i.e.: (a) the formation of NAD⁺ as the main oxidation product; (b) the dependence of the oxidation rate on the pH; (c) the catalytic role of Mn²⁺ and NaN₃. This evidence indicates that an analogous mechanism could be postulated for the HRP-catalysed oxidation and peroxidation reactions of both (NAD)₂ and NADH. Therefore the free-radical mechanism postulated for HRP-catalysed oxidation of NADH (Halliwell & de Rycker, 1978) might be operating also in the case of (NAD)₂.

Bacterial NADH peroxidase is unable to catalyse the peroxidation of (NAD)₂, which appears to compete with NADH, perhaps in the binding to the enzyme, as indicated by a lower NADH peroxidation rate in the presence of (NAD)₂.

The inability of $(\text{NAD})_2$ to be peroxidized by bacterial NADH peroxidase, which probably catalyses two-electron processes (Dolin, 1957, 1982), further supports the hypothesis that in the HRP-catalysed oxidation and peroxidation of $(\text{NAD})_2$ a one-electron mechanism is operating.

According to Yamazaki & Yokota (1973) and Halliwell & de Rycker (1978), the start of HRP-catalysed oxidation of NADH by O_2 requires small amounts of H_2O_2 , which, in fact, can be present in NADH solutions owing to a slow auto-oxidation process (Bernofsky & Wanda, 1982). Also, in the case of $(\text{NAD})_2$ oxidation, the reaction might be primed by H_2O_2 . In fact, some NAD^+ was found in solutions of $(\text{NAD})_2$ kept at slightly acidic pH in absence of HRP, indicating that a slow auto-oxidation process is going on in this case as well.

On the basis of the available data, the following schemes for both HRP-catalysed oxidation and peroxidation of $(\text{NAD})_2$ can be proposed:

Scheme I:

Scheme II:

Compounds I and II represent two forms of HRP, with respectively 2 and 1 oxidation equivalents more than the resting enzyme (Yamazaki & Yokota, 1973).

As shown in the schemes, NAD^+ can be formed by the interaction of $(\text{NAD})_2$ with the oxidized forms of peroxidase (Scheme I, reactions 2 and 3; Scheme II, reaction 2) and by the interaction of $\text{NAD}^\bullet$ with O_2 (Scheme I, reaction 4) or with an oxidized form of peroxidase (Scheme II, reaction 6). The last process can really occur only in the anaerobic peroxidation of $(\text{NAD})_2$, whereas in the aerobic reactions there are very few chances of reaction between $\text{NAD}^\bullet$ and HRP, since the O_2 concentration is much higher than the HRP concentration.

Furthermore, we have found that the peroxidation rate is lower in the presence of air (Table 1). In our opinion, this indicates that, under these conditions, reaction (4) of Scheme I gives significant contribution to maintaining the $(\text{NAD})_2$ oxidation. The $\text{O}_2^{\bullet -}$ anion thus generated could react with HRP, giving rise to oxyperoxidase, a much-less-reactive species (Yamazaki & Yokota, 1973).

It is conceivable that other haem peroxidases may catalyse the oxidation of $(\text{NAD})_2$. Thus, for instance, the oxidation of $(\text{NAD})_2$ by plant extracts observed by Burnett & Underwood (1968) and by Fricks *et al.* (1973) might be due to a peroxidase.

The ability of $(\text{NAD})_2$ to undergo enzymic oxidative and peroxidative processes yielding NAD^+ provides experimental support to the hypothesis of a biological $(\text{NAD})_2/\text{NAD}^+$ redox cycle.

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