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PHOTOCATALYZED OXIDATION OF NAD DIMERS

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A mixture of dimers of nicotinamide adenine dinucleotide, largely 4,4′-linked, obtained by electrochemical reduction of NAD^+, can be photooxidized back to NAD^+ in the presence of oxygen. Oxygen is consumed during the photooxidation process with the production of hydrogen peroxide. The oxidation is almost pH independent and is stimulated by light whose wavelength exceeds 300 nm. Lactate dehydrogenase and alcohol dehydrogenase added to the solutions under irradiation increased the oxygen uptake by the NAD dimers in a concentration-dependent way. These observations suggest that light induces the homolytic cleavage of NAD dimers to NAD radicals which in turn are oxidized to NAD^+ by oxygen.

Introduction

A mixture of isomeric tetrahydrobipyridine dimers, $(NAD)_2$ largely 4,4′-linked, can be obtained by electrochemical reduction [1] of NAD. The back oxidation of the dimers to NAD^+, accomplished by chemical [2,3], electrochemical [4] or enzymatic [1,5,6] methods is biologically relevant, since it indicates the possibility of an 'in vitro' redox cycle $NAD^+/(NAD)_2$ analogous to that of the NAD^+/NADH couple. This explains the current interest in this aspect of tetrahydrobipyridine dimer chemistry.

Recently, the photocatalyzed oxidation of the dimers to NAD^+ under anaerobic conditions has been reported [7]. Since, however, several of our own experimental observations were at variance with the suggested reaction pathway, we found it necessary to investigate the photooxidation process in greater detail.

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Materials and Methods

$(NAD)_2$ was prepared according to Ref. 1, stored under vacuum at −30°C and dissolved just before use. NAD^+ was purchased from Merck, NADH from Fluka, bovine heart lactate dehydrogenase and yeast alcohol dehydrogenase were from Boehringer; all other reagents were analytical grade and used without further purification.

HPLC analyses were performed on a Perkin-Elmer Series 3 liquid chromatograph, equipped with an LC 55B spectrophotometric detector, an LC 55S digital scanner and a Hewlett-Packard 3390 A integrating recorder, using a Merck Hibar RP18 RT 250-4 LiChrosorb 10 μm column at 1.0 ml/min flow rate. The mobile phase had the following composition (v/v): (solvent A) redistilled water (30%), LiChrosolv ethanol (12%) and 0.1 M NH_4HCO_3 (58%); (solvent B) 0.07 M NH_4HCO_3 in redistilled water. The following solvent program was used: (1) a linearly increasing gradient of solvent A from 15 to 50% (12.5 min); (2) an isocratic step at 50% of solvent A (5 min);

(3) a purge step (50% of solvent A for 5 min); (4) an equilibration step (10 min) at the initial percentage of A. Helium was used to remove oxygen from solvents A and B. The effluent was monitored at 259 or 340 nm. The irradiation experiments were carried out in buffered solutions at pH 9.1, since $(NAD)_2$ exhibits its maximum stability at this pH value. $(NAD)_2$ samples were irradiated with a TQ 718 high-pressure Hg Hanau ultraviolet lamp (600 W), placed at 10 cm from the sample and equipped with a pyrex cooling apparatus. Quartz cuvettes equipped with a Thunberg device, or vacuum-sealed pyrex vessels were used. Anaerobiosis was obtained by three to four cycles of freeze/vacuum/inert gas (Ar or N_2 99.99%) flush/thaw. Nevertheless, a residual amount of oxygen (1–5 μM) was always present in solution. The oxygen uptake was measured at 25°C with a Gilson Oxygraph, equipped with a Clark electrode. The inside of the oxygraph cell was irradiated by means of a 150 W xenon lamp. The Clark electrode was found to be light sensitive, as described for other electrodes [8,9].

Results

A typical elution profile of an aqueous solution of $(NAD)_2$ analysed via HPLC is shown in Fig. 1a; its features were analogous to those recently reported by Jaegfeldt [10]. Irradiation of this solution in the absence and in the presence of O_2 modifies the elution profile as reported in Fig. 1b and c, respectively. In the absence of oxygen, only a different distribution of dimers is observed as shown by the variation of the area under the corresponding peaks, while in the presence of oxygen the area of dimer peaks is significantly reduced and a new peak appears. This has been identified as NAD^+ on the basis of the retention time as compared to that of an authentic NAD^+ sample under identical conditions. Further evidence on the formation of NAD^+ was provided by the ability of the irradiated solutions to oxidize ethanol in the presence of alcohol dehydrogenase. The oxygen uptake by aqueous solutions of $(NAD)_2$ buffered at pH 9.1 is extremely low in the dark.

When the solutions were irradiated with a xenon arc inside the polarographic cell, significant oxygen

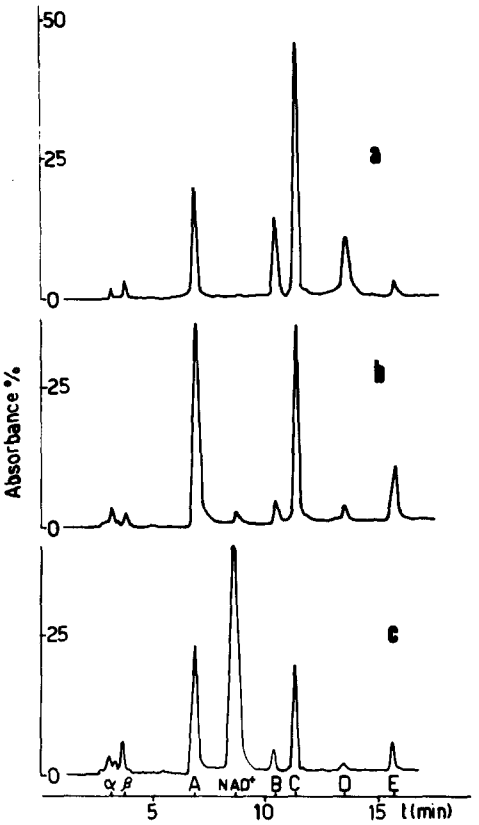

Fig. 1. HPLC elution profile of $(NAD)_2$ solutions. 1 mM $(NAD)_2$ in 100 mM carbonate buffer pH 9.1. (a) Solutions kept in the dark; (b) solutions irradiated in the absence of air for 10 min; (c) solutions irradiated in the presence of air for 10 min. The eluate was monitored at 259 nm. Peaks A–E correspond to different isomers of $(NAD)_2$, as indicated by ultraviolet scanning; Greek letters indicate unidentified products.

consumption was observed (Fig. 2). The interposition of a 4-mm thick glass sheet in the light path did not appreciably reduce the uptake of oxygen, showing that the reaction does not require ultraviolet radiation below 300 nm. The rate of the light-stimulated oxygen uptake was a function of the $(NAD)_2$ concentration (Fig. 3). Only a 15–20% increase in the rate of oxygen consumption was observed when the pH was changed from 9.1 (carbonate buffer) to 6.4 (phosphate buffer). Hydrogen peroxide was produced during the reaction as demonstrated by the oxygen development obtained after the addition of catalase to the solutions (Fig. 2). Catalase appreciably reduced the consumption rate of oxygen as well, while the

presence of superoxide dismutase had no effect. Addition of lactate dehydrogenase to a dimer solution under irradiation increased the oxygen uptake proportionally to the protein concentration (see Table I). It is essential in these experiments to reduce the amount of ultraviolet irradiation with a glass filter, otherwise significant oxygen consumption takes place due to protein alone, as shown by control experiments. Alcohol dehydrogenase gave essentially the same results.

The reactivity of $(NAD)_2$ toward superoxide and hydrogen peroxide was also tested. Solid KO_2 added to an aqueous solution of the dimers buffered at pH 9.1 was not effective. Hydrogen peroxide did not react with $(NAD)_2$ at least within the time scale of the above experiments.

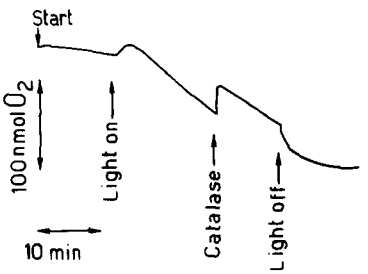

Fig. 2. Oxygen uptake by $(NAD)_2$ solutions under irradiation. 200 μM $(NAD)_2$ in 100 mM carbonate buffer pH 9.1 containing 1 mM EDTA, irradiated in a Clark-type electrode cell. At the times indicated light was turned on or off and catalase (0.4 μM) was added.

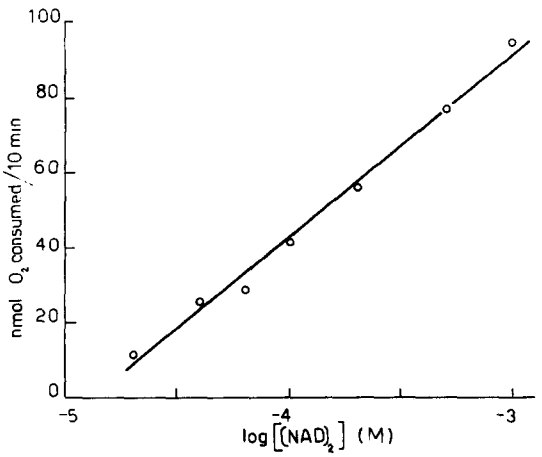

Fig. 3. Dependence of the oxygen consumption rate on the initial $(NAD)_2$ concentration. Experimental conditions as in Fig. 2.

TABLE I

OXYGEN UPTAKE BY IRRADIATED SOLUTIONS OF $(NAD)_2$

Oxygen uptake: oxygen consumed in 10 min by 3 ml of 100 μM $(NAD)_2$ in 100 mM carbonate buffer at pH 9.1 containing 1 mM EDTA. Each value is the mean of three determinations.

Addition	Oxygen uptake (nmol)
None	42.0
Catalase (0.4 μM)	29.4
Superoxide dismutase (1 μM)	42.0
Catalase (0.4 μM) and superoxide dismutase (1 μM)	29.4
Lactate dehydrogenase (33 μM)	45.0
Lactate dehydrogenase (66 μM)	47.4
Lactate dehydrogenase (100 μM)	49.5
Alcohol dehydrogenase (100 μM)	51.6

Discussion

It has been claimed that photochemical oxidation of $(NAD)_2$ takes place in the absence of oxygen through a pathway leading to the formation of dihydropyridinyl radicals. The radicals formed are said to interact with the solvent, but it was not immediately obvious as to how the solvent might act as an electron acceptor in this reaction [7]. Our results clearly show a dependence of this process on O_2 concentration, pointing to oxygen as the oxidizing agent.

The involvement of radicals in the oxidation reaction is supported (i) by the changes in the composition of the dimer mixture observed under irradiation in the absence of oxygen and (ii) by the increased oxygen uptake observed in the presence of alcohol and lactate dehydrogenases under aerobic irradiation.

As to point i, the photoinduced changes in the dimer mixture composition can be traced to their dissociation into dihydropyridinyl radicals and to the subsequent recombination of the latter, which

coincides with previous reports on the 1-benzylnicotinamide dimers [11].

Concerning point ii, it must be noted that Bielski and Chan [12] reported a stabilizing effect of several dehydrogenases on the NAD radicals, e.g., NAD· binds to alcohol dehydrogenase ($K_d = 2.4 \cdot 10^{-6}$ M) and to lactate dehydrogenase ($K_d = 7.6 \cdot 10^{-7}$ M). The protein-bound radicals undergo dimerization at a rate which is 2–3 orders of magnitude lower than that of the free radicals [12]. In our case, the longer lifetime of the protein-bound radicals can account for the increased oxygen uptake. On the above grounds, and since the product of $(NAD)_2$ photooxidation is NAD^+, the overall stoichiometry of the process appears to be:

$$(NAD)_2 + O_2 + 2H^+ \rightarrow 2NAD^+ + H_2O_2$$

and in the presence of catalase:

$$(NAD)_2 + \tfrac{1}{2}O_2 + 2H^+ \rightarrow 2NAD^+ + H_2O$$

The following steps should be involved:

$$(NAD)_2 \xrightarrow{h\nu} (NAD)_2^* \quad (1)$$

$$(NAD)_2^* \rightarrow 2NAD \quad (2)$$

$$NAD\cdot + O_2 \xrightarrow{k_{ox}} NAD^+ + O_2^{-} \quad (3)$$

$$NAD\cdot + NAD\cdot \xrightarrow{k_{dim}} (NAD)_2 \quad (4)$$

$$2O_2^{-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (5)$$

Step 1 describes the absorption of light by $(NAD)_2$, producing an excited species which may dissociate to give NAD radicals. These radicals react either with oxygen to yield NAD^+ and superoxide radicals (step 3), probably with the intermediacy of peroxy radicals ($NAD\cdot + O_2 \rightarrow NADOO\cdot \rightarrow NAD^+ + O_2^{-}$) or recombine to give back the dimer $(NAD)_2$ (step 4). The branching ratio between steps 3 and 4 depends on the relative rate constants, which have been found to be $k_{dim} = 5.6 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ [13] and $k_{ox} = 1.9 \cdot 10^9$ $M^{-1} \cdot s^{-1}$ [14]. Superoxide radicals from step 3 then undergo spontaneous dismutation to H_2O_2 and O_2.

In conclusion, the photoinduced homolytic cleavage of $(NAD)_2$ to NAD· followed by rapid reaction with O_2 appears to represent a reasonable pathway for photooxidation. In our view, dimeric NAD species can actually be involved in those biological processes whenever the intervention of dihydropyridinyl radicals may occur, as in the photosynthetic process where the formation of NADP radicals has been postulated [15]. There, the dimerization of NADP· could be made reversible by the subsequent photoinduced oxidation further stimulated in the presence of dehydrogenases.

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