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ESR signals of NADH and NADPH under illumination

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ESR method was applied to investigate the formation of NADH and NADPH free radicals. It was shown that under the action of light (340 nm) in water and other solutions of these compounds a reaction occurred resulting in the formation of free radicals having the typical ESR spectrum. The analysis of the temperature dependence showed the light-induced ESR signals to be registered at −30°C to −120°C, the most intensive ones being observed at −50°C. It was concluded that the observed ESR signals belonged to the products of one-electron oxidation of the coenzymes – free radicals NAD$^\bullet$ and NADP$^\bullet$.

ESR; NADH; NADPH; Light-induced free radical

1. INTRODUCTION

In the functioning of the universal coenzymes, NAD(H) and NADP(H), the intermediate formation of the products of their one-electron reduction or oxidation is possible [1–3].

By means of pulse radiolysis the one-electron NAD reduction [4] and one-electron NADH oxidation [5] were oberved; in both processes the same product, a free radical NAD$^\cdot$ was detected, capable of dimerization [5]. In the above experiments the oxidative-reductive transformations of the coenzymes were the result of NAD(H) reaction with the free radicals formed under the action of ionizing radiation on the compounds present in the medium.

In the pulse photolysis experiments, light quanta affect directly the molecule of NADH. As a result, an electron is removed from NADH with the formation of the oxidized form, NAD$^\cdot$, and a solvated electron (e^-aq.), which can be detected by the typical absorption spectrum in the region 600–700 nm [6,7].

Electrochemical oxidation of NAD(H) and a number of other compounds modelling the nicotinamide and adenine components of NAD molecule, led to the formation of several products of the oxidative-reductive transformation. One of these products corresponded to a NAD$^\cdot$ radical in the dimeric form [8]. In several works from our laboratory, the photochemical reactions involving NAD and NADP and their reduced forms were studied [9–12] in which NAD reduction and NADH oxidation photosensitized by photosynthetic pigments were revealed. We also studied the reactions proceeding as a result of the direct light activation of NADH and NADPH active intermediate products being formed in the course of this process [9].

The aim of the present study was to apply ESR method to observe the free-radical products of NADH and NADPH oxidation in solution.

2. MATERIALS AND METHODS

ESR spectra were measured on modified radiospectrometer RE-1301 with an attachment for sample irradiation in the resonator and a thermostating attachment which permitted one to carry out measurements at +100°C to −196°C. Samples were illuminated with a 250 W xenon lamp, absorption filters transmitting UV-radiation at 320–380 nm at the absorption band of NADH were used. Light intensity was 10^5 erg·cm^2. Preparations of NADH, NADPH, NAD, NADP (Reanal, Hungary and Serva, FRG) containing 80–90% of the compound were used without purification. The solvents were distilled water and ethanol. Oxygen was removed with a vacuum pump, and samples were exposed to a repeated freeze-thawing. The reaction was carried out in glass ampoules (3–4 mm diameter), one ampoule containing 0.03 ml of the solution.

3. RESULTS

Illumination of NADH (10^{-2} M) solutions in distilled water, frozen at a temperature below −30°C, in vacuum, caused the appearance of a light-induced signal with a typical spectrum exhibiting complex structure (fig.1). In fig.1 the results of the experiments performed at −50°C are shown. Under these conditions ESR signal appears at the first seconds of illumination, its intensity is increased to reach the maximal value at about 30 min. The yield of the paramagnetic particles was approximately 10^{14}, i.e. about 5% of the initial amount of NADH 10^{16}. If one takes into consideration that in the frozen snow-like matrix, only the surface molecules are photoactive, then, it is clear that the ac-

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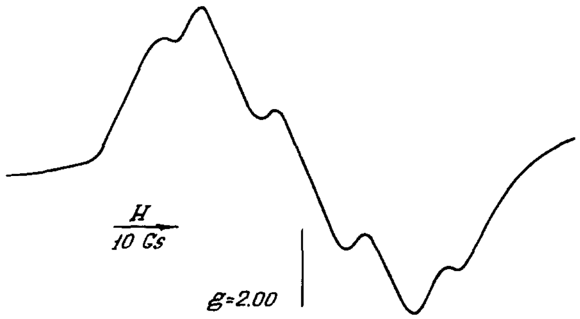

Fig.1. ESR spectrum observed for NADH (10^{-2}M) water solution under the action of light. Vacuum, −50°C, 30 min illumination through UFS-I and SZS-23 filters.

tual yield of the photooxidized pyridine nucleotide molecules is much higher. After the UV-light is switched off, the signal is maintained in the dark for a long period (for hours) at this temperature, and disappears only under heating of the samples to room temperature. The appearance of the ESR signal under the action of UV-radiation and its disappearance in the dark after thawing was many times observed with one and the same sample which testifies to the reversibility of the light-induced process.

To reveal the favourable conditions for the effective reactions, we investigated the temperature dependence of ESR signal appearance in NADH water solution. It is seen from fig.2 that the light-induced signal may be observed only at the temperature above −140°C. At lower temperatures the reaction is practically inhibited. The increase above −140°C leads to an increase of ESR signal which is the most pronounced within the temperature range −60°C to −40°C. The further increase of temperature leads to the decrease of ESR signal observed under illumination and its disappearance in the dark. The slower increase of the light-induced signal at a temperature higher than −40°C is, perhaps, accounted for by an increase in the rate of the back dark reaction or the involvement of side-photoprocesses. Based on the data obtained, we can see that the optimal temperature for the reaction is about −50°C because at this temperature the signal is most effectively increased under UV-radiation and stable in the dark after the light is switched off.

The light-induced appearance of ESR signal in the degassed water solutions of NADPH (10^{-2} M) was as efficient as in NADH solutions, their spectra being identical. In the control experiments with the oxidized forms of pyridine nucleotides in vacuum, or in the presence of air, the light-induced signal was absent at all the temperatures investigated.

We tested the action of HCl and NaOH on the light reaction in water solutions of NADH. It is known that a NADH molecule is unstable in the acidic and stable in the alkaline medium. Indeed, at pH below 4 no photo-induced ESR signal was observed apparently, as a result of the destruction of NADH molecule, while in the alkaline solution (pH 12) the light-induced ESR signal was the same as at pH 7.

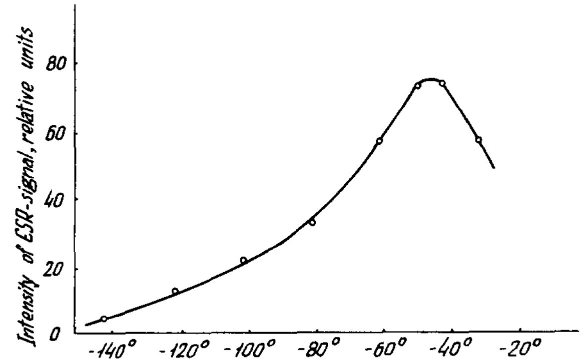

Fig.2. Temperature dependence of ESR light-induced signal in NADH (10^{-2}M) water solution. Vacuum, the relative values of ESR signal intensity are recorded 30 min after illumination through UFS-I and SZS-23 filters.

The photo-induced formation of free radicals in NADH solutions was measured in water, 50% ethanol and a 1% water micellar solution of Triton X-100. In all the media investigated the same typical ESR spectrum appeared, and the rates of the increase of ESR signal in ethanol solution and in the micellar solution were lower than those in water.

The effect of the oxidants (oxygen and methyl viologen (MV^{2+}) on the photo-induced formation of the ESR signal with a complex structure was investigated. Oxygen produced practically no influence on the course of photochemical reaction. MV^{2+} produced varied effects on the photo-induced formation of free radicals in NADH (10^{-2}) water solutions depending on its concentration. At MV^{2+} concentration 10^{-5} M an ESR signal with the typical complex spectral structure was recorded characteristic of NADH water solutions (fig.3), the rate of the signal being lower than

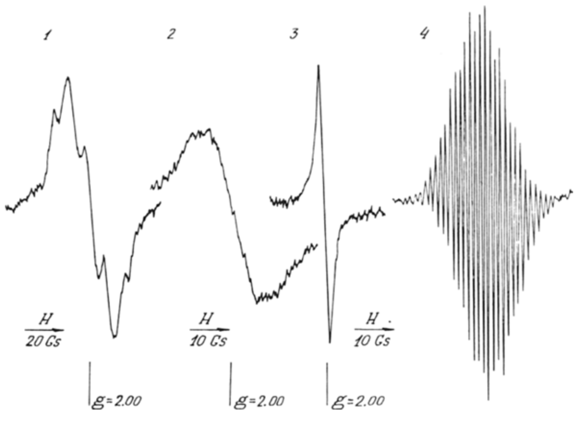

Fig.3. ESR spectra of water solutions of: (1) NADH (10^{-2}M) and MV^{2+} (10^{-5}M); (2) NADH (10^{-2}M) and MV^{2+} (10^{-3}M); (3) and (4) MV^{2+} alone (10^{-3}M). (1), (2), (3) at −50°C; (4) at −20°C. Illumination through UFS-I and SZS-23 filters for 30 min, water, vacuum.

without MV^{2+}, probably as a result of the interaction of the free radical observed in NADH solutions with MV^{2+} cation-radicals formed in the reaction [12]. With MV^{2+} at a concentration of 10^{-3} M in NADH (10^{-2} M), an intensive singlet ESR signal was induced with the width 14–15 Gauss and $g = 2.00$. The shape of the spectrum and the rate of the signal increase differed from those observed in water solution of NADH alone (typical spectrum with superfine structure; fig.1) and from ESR spectrum recorded at the illumination of MV^{2+} alone under the same conditions. The results of these experiments show the interactions of an excited pyridine nucleotide molecule with a MV^{2+} molecule in which a free radical is formed. A special study is required to elucidate the nature of the free radical.

4. DISCUSSION

Based on the data obtained, we may conclude that the typical ESR signal with a complex spectral structure belongs to the free radical oxidized forms of pyridine nucleotides, NAD$^{\bullet}$ and NADP$^{\bullet}$. Some assumptions may be advanced concerning the mechanism of ESR signal formation. Free radicals are formed in the system studied only under the action of radiation absorbed by NADH and NADPH (340 nm). The reaction can involve the singlet and/or triplet excited state of pyridine-nucleotides as revealed in our laboraroty [12,13]. One of the ways of NAD$^{\bullet}$ formation is electron removal from the excited molecule with the appearance of pyridine nucleotide free radical in the ionic or neutral form and a solvated electron. We cannot exclude that an excited NADH molecule interacts with the electron-accepting impurities present in the coenzyme preparations, oxidized forms of coenzymes, NAD and NADP included. Traces of oxygen are apparently present in the system, dissolved in or bound to the coenzymes (oxygen was shown not to inhibit the photo-reaction in our experiments).

Accumulation of the free radical oxidized form of pyridine nucleotides was registered only in the frozen matrix, where the secondary reactions of the radical transformations requiring energy activation are inhibited. Under heating below $-40°$C a fast decrease of the light-induced signal occurs up to its complete disappearance. One of the possible reactions of NAD$^{\bullet}$ radicals disappearance may be that of dimer formation: $2\ \mathrm{NAD} \rightarrow (\mathrm{NAD})_2$. Also possible is the reaction with oxygen resulting in the formation of the oxidized NAD^{+} and O_2^-. For NAD$^{\bullet}$ radicals no disproportionation reaction was observed [5]. Apparently, the electron is removed from the pyridinyl component of the coenzyme molecule; in several studies [14–16] the formation of the free pyridinyl radicals was shown by ESR method. There is evidence in favour of the fact that in the case when an electron is removed from the adenine component of the coenzyme molecule, the unpaired electron is rapidly transferred to the pyridinyl component [17].

Thus, it is shown that under the action of the exciting light (340 nm) on solutions of NADH and NADPH, a reaction occurs resulting in the formation of free radicals having the ESR signal with the typical spectrum. A conclusion is made of the observed ESR signals belonging to the products of one-electron oxidation of coenzymes, free radicals NAD$^{\bullet}$ and NADP$^{\bullet}$. The experiments described are connected with the problem of light activation of the enzymatic reactions.

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