Photoinitiated oxidation of NADH catalyzed by horseradish peroxidase studied by chemically induced dynamic nuclear polarization

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The photoinitiated oxidation of β-NADH catalyzed by horseradish peroxidase (Per^{3+}) was studied by time-resolved photoinitiated chemically induced dynamic nuclear polarization (CIDNP). The polarization observed on protons at the C(4) atom of the β-NADH molecule is evidence for the reversible one-electron transfer between the radical cation $NADH^{+\bullet}$ and the ferroperoxidase intermediate (Per^{2+}). A new approach based on electron transitions in the ($NADH^{+\bullet}$ Per^{2+}) pair was proposed to describe the formation of CIDNP effects in systems including quartet (Q)—doublet (D) electron transitions.

Key words: β-NADH, horseradish peroxidase, photoinduced one-electron transfer, chemically induced dynamic nuclear polarization.

Considerable interest in detailed mechanisms of chemical transformations of the NADH cofactor is caused by its participation in many enzymatic processes. The mechanism of NADH oxidation to NAD^+ and the role of one-electron transfer steps in this reaction have been studied in detail.[1] However, there are no sufficient proves for the involvement of NAD^+ and NADH in reactions of one-electron or non-synchronous two-electron transfer reactions, whose probability should be taken into account when the energetics of the one-electron transfer is not affected by the energetics and kinetic mechanism of the reaction. Detection of free radicals and radical ions in biological systems is difficult and, therefore, model reactions involving synthetic analogs of NADH are often used to elucidate the role of one-electron transfer in the transformations of NADH into NAD^+.

Radical mechanisms of the photochemical reactions of NADH with flavin[2] and photooxidation of model compounds, *viz.*, 1,4-dihydropyridines (synthetic analogs of NADH),[3—6] with quinones have been studied to the present time by the method of chemically induced dynamic nuclear polarization (CIDNP). The oxidation of NADH and its synthetic analogs was shown to proceed *via* the electron-chemical-electron (ECE) mechanism, and the one-electron transfer is the initial step for the oxidation of both flavin and quinones. Data in favor of radical steps in the chemically activated enzymatic oxidation of NADH by horseradish peroxidase have recently been obtained[7] by another spin chemistry technique: investigation of magnetic field effects (MFEs). A study of the magnetic field effect on the effective rate constants of the catalytic cycle of Per^{3+} and theoretical simulation of the experimental MFEs suggested[8] that the MFEs could originate in a radical pair consisting of the reduced form of peroxidase, *viz.*, ferroperoxidase (Per^{2+}), and the $NADH^{+\bullet}$ radical cation (Per^{2+} $NADH^{+\bullet}$).

Therefore, it seems reasonable to use the CIDNP method for studying one-electron transfer steps in the oxidation of NADH by horseradish peroxidase. The chemically activated reaction of NADH and peroxidase has been studied earlier; however, since the steady-state concentrations of paramagnetic species in enzymatic processes are very low, we used photochemical activation to study the reaction between NADH and Per^{3+}. The reaction of photochemically activated NADH with Per^{3+} has been described for the first time in Ref. 9, where the oxidized moiety of NADH (NAD^+) was shown to form according to Scheme 1. In addition, the one-electron transfer was assumed[9] to be the initial step of the studied process of NADH oxidation affording Per^{2+}.

Scheme 1

$$NADH^* + Fe^{3+} \longrightarrow NAD^\bullet + Fe^{2+} + H^+$$

$$Fe^{3+} + NAD^\bullet \longrightarrow Fe^{2+} + NAD^+$$

$$2\,NAD^\bullet + H^+ \longrightarrow NAD^+ + NADH$$

In the present work, to prove the one-electron transfer step in NADH oxidation catalyzed by horseradish peroxi-

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dase, we used the CIDNP method, which makes it possible to apply a new approach to studying selective steps of electron transfer in multispin systems including heme iron. We also proposed a new theoretical approach to describe CIDNP effects that appear in such multispin systems.

Experimental

Horseradish peroxidase (DIA-M, 250 U mg^{-1}, R/Z > 3.0) and β-NADH (US Biochemicals, Inc.) were used. The concentrations of peroxidase and β-NADH were determined spectrophotometrically on a Hewlett Packard HP 8453 optical spectrophotometer using the corresponding molar absorption coefficients ($\varepsilon_{403} = 1.02 \cdot 10^5$ L mol^{-1} cm^{-1} and $\varepsilon_{340} = 6.3 \cdot 10^3$ L mol^{-1} cm^{-1}). The concentrations of the enzyme and substrate were 10^{-4} mol L^{-1} and $1.5 \cdot 10^{-3}$ mol L^{-1}, respectively. Experiments were carried out in a 0.05 *M* phosphate buffer, which was prepared by the titration of a solution of KH_2PO_4 (Aldrich) with a solution of K_2HPO_4 (Aldrich) to pH 7.1. In all solutions D_2O (99.9%, Aldrich) was used as the solvent.

In CIDNP experiments, a solution of NADH and horseradish peroxidase was irradiated directly in the probe of the NMR spectrometer at room temperature. A Lambda Physik EMG 101 MSC excimer laser (λ = 308 nm, average pulse energy 100 mJ) was used as the light source. Time-resolved CIDNP spectra were accumulated at a radio-frequency detection pulse of 4 μs and two time delays between each laser flash and registration: 0 and 100 μs. ^{1}H NMR spectra of solutions before and after the reaction were recorded on a Bruker DPX200 spectrometer (operating frequency 200 MHz).

Results and Discussion

The photoinduced CIDNP spectrum of NADH in the presence of Per^{3+} (Fig. 1) contains two lines: the emissive signal at δ 2.75 attributed to β-NADH and the signal at δ 4.75 belonging to HDO.

The polarized line with the shift δ 2.75 becomes visible after 16 scans and reaches the maximum intensity after 128 scans. Longer irradiation decreases considerably the relative intensity of the polarized signal of NADH that is probably caused by suppression of the enzymatic reaction due to peroxidase inactivation by radicals.[10] The further irradiation does not increase the signals of NAD^+ in the ^{1}H NMR spectra of the reaction mixture. The structure of the polarized line at δ 2.75 in the region of the protons at the C(4) atom of a β-NADH molecule differs from the equilibrium signal of these protons (see Fig. 1). Hence, we assume that one of the factors giving this difference can be averaging of signals from the two protons at the C(4) atoms due to the acceleration of conformation transitions (induced by laser heating) in the dihydropyridine moiety of the NADH molecule. The model experiment showed that for thermal heating of a NADH solution in the absence of horseradish peroxidase in a temperature range of 22—50 °C the shape and width of the equilibrium

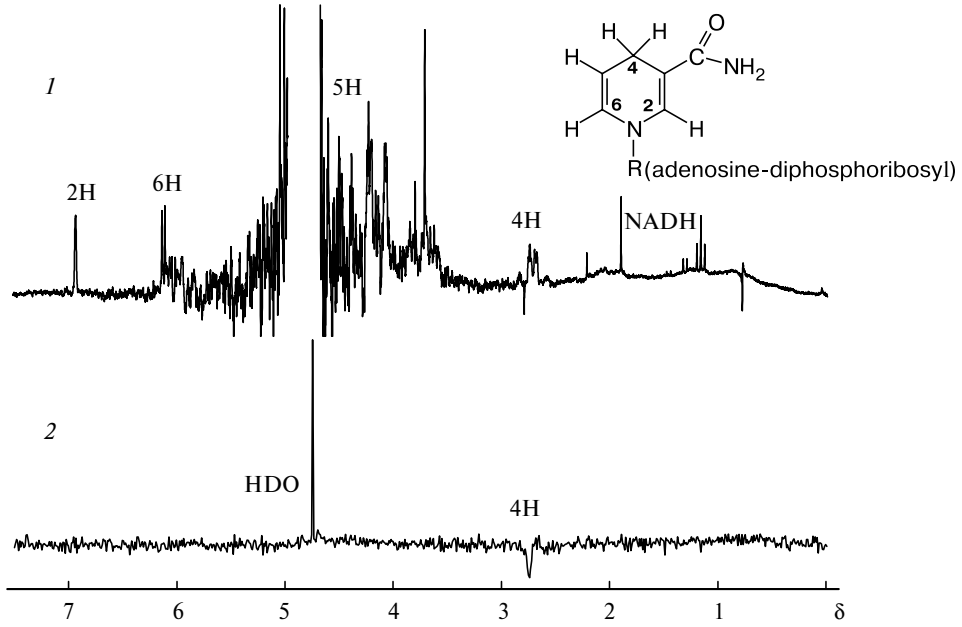


Fig. 1. NMR spectrum of a solution of β-NADH ($1.5 \cdot 10^{-3}$ mol L^{-1}) and horseradish peroxidase (10^{-4} mol L^{-1}) in a phosphate buffer (pH 7.1, 0.05 *M*, KH_2PO_4) (*1*) (only a part of the NMR spectrum of β-NADH, belonging to the nicotinamide moiety, is shown); (*2*) the time-resolved photoinduced CIDNP spectrum for β-NADH and horseradish peroxidase recorded under the same conditions (delay between the pulse and registration 0 μs, 128 scans, radio-frequency pulse 4 μs). Signal assignment, δ: 2.75, emission (protons at the C(4) atom of the β-NADH molecule); 4.75 (HDO).

signal of the protons at the C(4) atom at 50 °C coincide with these parameters for the polarized signal.

We attempted to reveal whether the signal of HDO appeared as a result of the incomplete suppression of the equilibrium signal of water or due to the polarization of HDO. For this purpose, an experiment without laser irradiation was carried out. The signal of HDO with the incomplete suppression of the equilibrium signal was observed in the ^{1}H NMR spectrum. Thus, it is impossible to demonstrate if the HDO polarization is indeed genuine.

No chemical polarization is observed for other NADH protons and the protons of the NAD^+ cation. The absence of nuclear polarization of the protons at the C(2), C(5), and C(6) atoms in the NADH molecule can be related to the low hyperfine coupling (HFC) constants (0.26, −0.62, and 0.2 mT, respectively) compared to the HFC constants on the protons of the C(4) atom (4.6 mT).[2] The absence of CIDNP on the protons in NAD^+ can be explained by the mechanism of cation formation in several sequential steps (see Scheme 1) and by spin-lattice relaxation processes in the polarized $NADH^{+\bullet}$ radical cation. The latter occur before the step of NAD^+ generation, whose rate is much lower than the rate of spin-lattice relaxation of $NADH^{+\bullet}$.

The results and explanation proposed agree well with the CIDNP data obtained[2–6] by studying the model photoinitiated reactions of NADH with flavin and the photooxidation reactions of synthetic analogs of NADH, *viz.*, 1,4-dihydropyridines, with electron acceptors. However, the observed CIDNP enhancement coefficient in this work is much lower than the corresponding coefficient used in Refs 2—6, where polarization was observed on all protons of the initial NADH and/or 1,4-dihydropyridines.

To reveal the reason for this discrepancy, one should check first whether the detected polarized signal belongs to the "in-cage" product (initial NADH). For this purpose, time-resolved experiments were carried out with two delays between each laser flash and registration: 0 and 100 μs. In these experiments, the spectra obtained under the same conditions but with different delay times coincided completely, which suggests that the observed CIDNP effect is formed in the step of geminal recombination. Thus, it can be asserted with a high probability that polarization is observed for the "in-cage" product (initial NADH) and formed in the primary radical pair including the reduced form of native peroxidase Per^{2+} and radical cation $NADH^{+\bullet}$, *i.e.*, in the (Per^{2+} $NADH^{+\bullet}$) radical pair.

This conclusion coincides with published results,[9] except for the fact that in Ref. 9 the $NADH^{+\bullet}$ radical cation was not considered as a kinetically independent intermediate because of fast deprotonation producing the $NAD^{\bullet}$ radical (see Scheme 1). More recently, based on the fact of polarization of the protons of NADH formed in the step of back electron transfer, it was suggested[2] that the lifetime of $NADH^{+\bullet}$ exceeds considerably the lifetime necessary for spin evolution (several nanoseconds). The deprotonation rate is thus lower than the rate of back electron transfer, which makes it possible to observe the CIDNP effect of the initial NADH cofactor.

Thus, NADH is polarized in the back electron transfer step:

$$NADH^{+\bullet} + Per^{2+} \rightarrow NADH + Per^{3+}.$$

Another reason for the low CIDNP enhancement coefficient observed is possibly related to the mechanism of polarization formation.

For analysis of the observed CIDNP effect, it is important to know the multiplicity of the paramagnetic pair in which spin evolution processes occur. Upon the photoexcitation of NADH, native Per^{3+} is reduced to Per^{2+} *via* one-electron transfer from the peroxidase to the triplet-excited NADH.[9] Therefore, the initial state of the (Per^{2+} $NADH^{+\bullet}$) pair is quartet (Q) with the total spin 3/2. Intersystem crossing transforms the quartet state of the pair into the doublet state (D) with the total spin 1/2 in which recombination (back electron transfer) can occur to form the initial peroxidase and polarized NADH (Scheme 2; $\overset{*}{H}$ are the polarized protons at the C(4) atom). The electronic structures of native peroxidase (Per^{3+}) and ferroperoxidase (Per^{2+}) presented in Scheme 2 were obtained using the crystal field theory.

Thus, spin evolution in the (Per^{2+} $NADH^{+\bullet}$) pair includes the quartet (Q)—doublet (D) transitions, unlike the known[11] singlet (S)—triplet (T) transitions. In the present work, we propose the theoretical description of CIDNP effects appeared in multispin systems using as an example the system including the heme-containing enzyme (horseradish peroxidase, Per^{3+}) and substrate (NADH).

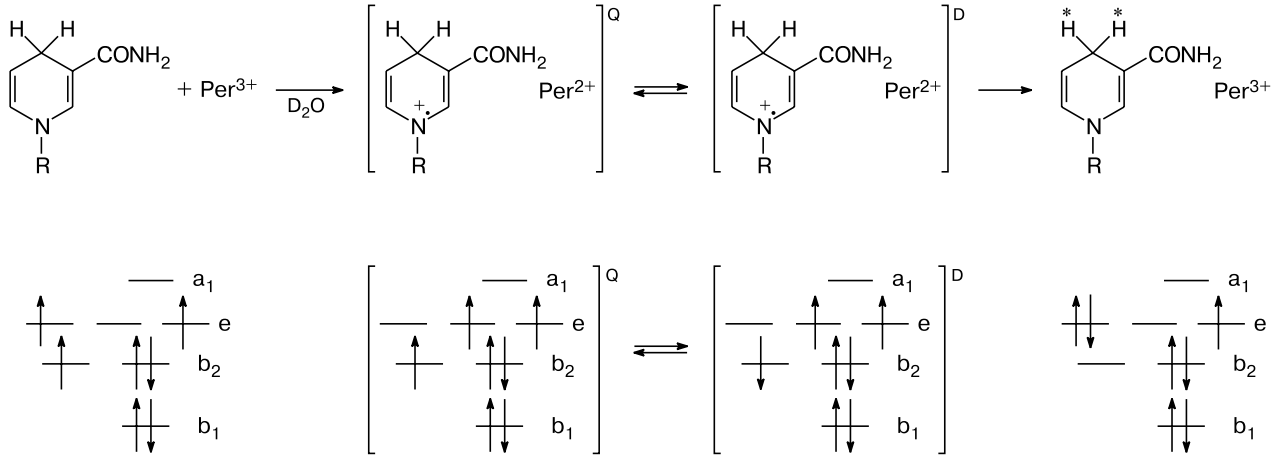
Spin evolution in the radical pair under study can be presented as the energy diagram showing the qualitative dependence of the energy of terms Q and D on the distance between the radicals in an external magnetic field (Fig. 2). According to this diagram, the main contribution to the formation of the CIDNP effect can be made by the non-adiabaticity zones, which can be divided into crossing (see Fig. 2, zones *1*, *2*, *3*) and closing zones (see Fig. 2, zones *4*, *5*).[12]

Let us consider the efficiency of spin transitions in the closing zones. In a constant high magnetic field (in this case, 4.7 T), the main contribution to the spin evolution is made by spin projection-conserving Q—D-transitions, such as $Q_{-1/2} \rightarrow D_{-1/2}$ and $Q_{+1/2} \rightarrow D_{+1/2}$. The efficiency of these transitions can be estimated by the formula:

$$p = 2V^2\tau^2/(\hbar^2 + 4(\Delta E^2 + V^2)\tau^2), \quad (1)$$

where V is the matrix transition element determined by the Zeeman and hyperfine interactions; ΔE is the

Scheme 2



Q—D-splitting determined by exchange interaction; τ is the average time of radical pair residence in the zone with ΔE splitting. In our case, the efficiency of spin transitions increases due to a large difference between the *g*-factors of ferroperoxidase ($g = 3.2$)[13] and the $NADH^{+\bullet}$ radical cation ($g = 2.0032$). Thus, if the lifetime of the radical pair ranges from 10^{-9} to 10^{-7} s, then the efficiency of p-transitions for α- and β-spins differs by approximately 10^{-6}—10^{-10}. In addition, changes in the matrix element due to the hyperfine interaction do not considerably change the p value. Therefore, the resonance Q—D-transitions do not contribute to CIDNP effect formation.

Taking into account that NADH oxidation catalyzed by horseradish peroxidase occurs in an enzyme—substrate complex, as the most part of enzymatic processes, we have to analyze the efficiency of spin transitions in the crossing zones of the terms, which can considerably contribute to the formation of CIDNP effects. In this case, a strong exchange interaction[14] in a paramagnetic pair can achieve 10^{12} Hz. Thus, the probability of transitions between the terms in the crossing zone can be described as follows:

$$W = 2\pi\chi^2 P\tau/E, \qquad (2)$$

where χ is the matrix transition element, τ is the lifetime of the radical pair, *P* is the part of the lifetime of the radical pair in the crossing zone, and $E = g_1\beta B_0 + g_2\beta B_0$ is the Zeeman energy term splitting.

Since the HFC constants of the $NADH^{+\bullet}$ radical cation, except for those on the protons at the C(4) atom, are rather low,[2] the Hamiltonian for the system (with allowance for the interaction only between the unpaired electron of the proton at the C(4) atom of $NADH^{+\bullet}$ with the magnetic nuclear spin 1/2) can be presented in the following form:

$$\hat{H} = a\hat{S}\hat{I} = a\hat{S}_Z\hat{I}_Z + (\hat{S}_+\hat{I}_- + \hat{S}_-\hat{I}_+)a/2. \qquad (3)$$

The solution of the Schrödinger equation with Hamiltonian (3) and wavefunctions for the Q and D states gives only two non-zero matrix elements

$$\left\langle Q_{-3/2}\alpha_I \middle| \hat{H} \middle| D_{-1/2}\beta_I \right\rangle = a/\sqrt{6}$$

$$\left\langle Q_{1/2}\alpha_I \middle| \hat{H} \middle| D_{-1/2}\beta_I \right\rangle = -a/2\sqrt{3}, \qquad (4)$$

where α_I and β_I are the projections of the nuclear spin equal to 1/2 and −1/2, respectively.

Therefore, the transitions in the crossing zones (see Fig. 2) result in the negatively polarized NADH, which is formed in the step of back electron transfer between Per^{2+} and $NADH^{+\bullet}$.

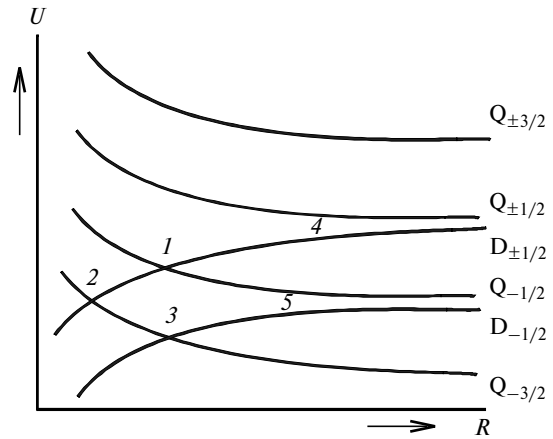


Fig. 2. Qualitative dependence of the energy levels in the paramagnetic pair $(Per^{2+}\ NADH^{+\bullet})^{Q,D}$ on the distance between the radicals in the pair in a constant magnetic field. $Q_{\pm3/2}$, $Q_{\pm1/2}$, and $D_{\pm1/2}$ are the spin states of the pair $(Per^{2+}\ NADH^{+\bullet})$; *1*, *2*, and *3* are the crossing zones; *4* and *5* are the closing zones of the terms.

Thus, in this work we demonstrated the formation of the CIDNP effect in the (Per^{2+} $NADH^{+\bullet}$) radical pair formed in the step of back electron transfer between photoexcited NADH and horseradish peroxidase. The differences in CIDNP effects observed in the reactions of NADH with the enzyme and organic electron acceptors can be explained by the mechanism of CIDNP formation in multispin systems and binding of the partners in the radical pair. This study fills a gap in the experimental application of the CIDNP method for studying enzyme-catalyzed processes and develops the theoretical description of the CIDNP effects observed in multispin systems.

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