ELECTRON PARAMAGNETIC RESONANCE STUDY OF THE INTERACTION OF A SPIN-LABELED ANALOG OF ADENOSINE DIPHOSPHORIBOSE WITH PARAMAGNETIC COBALT(II) LIVER ALCOHOL DEHYDROGENASE

H.R. DROTT

Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19174, USA

and

D. SANTIAGO and J.D. SHORE

Edsel B. Ford Institute for Medical Research, Henry Ford Hospital, Detroit, Michigan 48202, USA

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1. Introduction

Structural and dynamic information is manifested in the magnetic properties of biological macromolecules, and is usually extracted by magnetic resonance techniques or susceptibility methods. In particular, EPR* studies of enzymes have been recently expanded to include the detection and utilization of magnetic dipolar interaction between two unlike spin systems [1]. A workable theory has been developed in order to permit angular and displacement dependences of the dipolar interaction to be drawn from experimental findings [2].

Liver alcohol dehydrogenase is a dimer containing four zinc atoms per molecule, which are exchangeable with cobalt [3]. Two of the zinc atoms are rapidly exchangeable while the remaining two exchange at slower rates [4]. Since a spin-label analog of ADPR has been demonstrated to bind to the active center of ADH [5], an EPR investigation of ADPR· bound to cobalt derivatives of ADH should enable determination of the average distance of the spin label from the paramagnetic ion. These results, obtained with enzyme in solution, may be compared with the crystallographic model [6].

2. Materials and methods

The preparation of totally substituted cobalt alcohol dehydrogenase and cobalt hybrid alcohol dehydrogenase in which only the rapidly exchanging zinc atoms have been replaced by cobalt has been reported [3, 4]. From atomic absorption measurements for zinc and cobalt in the totally substituted cobalt enzyme and cobalt hybrid enzyme, the incorporation of the cobalt atoms for zinc atoms was found to be 95 ± 5% and 47 ± 5%, respectively. The concentration of the enzyme was determined either by spectrophotometric titration with pyrazole and NAD^+ [7] or by spectrofluorometric titration with isobutyramide and NADH [8]. The ADPR· has been prepared according to a literature procedure [5]. The concentration of ADPR· was measured spectrophotometrically‡. All experiments were carried out in 0.1 μ phosphate buffer at pH 7.0 at 23°C. EPR measurements were performed with a Varian 4502 spectrometer using a quartz capillary sample cell placed in a variable temperature Dewar regulated to 23°C.

* *Abbreviations*: EPR, electron paramagnetic resonance; ADH, liver alcohol dehydrogenase; ADPR, adenosine diphosphoribose, ADPR•, adenosine-5-diphosphate-4-(2,2,6,6-tetramethyl-piperidine-1-oxyl); PRR, proton relaxation rate.

‡ From experimentally determined stoichiometry of 1:2:1 for adenine, phosphorous, and spin label, respectively, an extinction coefficient for ADPR· was calculated to be ϵ_{260} = 15.4 $mM^{-1} \cdot cm^{-1}$ (unpublished data, H.R. Drott).

3. Results and discussion

In fig. 1, the EPR spectra of the ADPR· in the presence of either native LADH or totally substituted cobalt alcohol dehydrogenase clearly demonstrates that the nitroxide moiety of the ADPR· is moderately immobilized and that the rotational correlation time of the binary complex is of the order 10^{-8} to 10^{-9} sec. Such a degree of immobilization supports the crystallographic model that ADPR is bound in such a manner that terminal ribose points into the cleft containing the active site zinc atom [6] because the nitroxide moiety of ADPR· approximates the position of the terminal ribose moiety of ADPR [9]. From the unbound, highly mobile component of the EPR signal at high magnetic field strength, an estimation of the degree of binding can be made and approximately 95% of the ADPR· is bound for a molar ratio of ADPR· to ADPR·-binding sites of 1:2 [5]. Furthermore, comparison of the unbound components of the EPR spectra suggests that the binding capacity of the enzyme for ADPR· is unaltered by the substitution of the cobalt atom for the zinc atom.

If attention is focused upon the immobilized components of the EPR spectra, it is clearly seen that the amplitude of the binary complex with the totally substituted cobalt enzyme is less than that with the native enzyme. It was found, moreover, that the amplitude of immobilized component in the cobalt enzyme relative to the zinc enzyme was independent of the molar ratio of ADPR· to ADPR·-binding sites for molar ratios of 1:1, 1.5:1, and 2:1 and had a variation of less than 5% with three different enzyme preparations. Furthermore, an equal molar mixture of the cobalt enzyme and the zinc enzyme gave an EPR spectrum with ADPR· that lies between the two limits. A similar, but smaller, effect on the EPR signal is also noted when the experiment is repeated using the cobalt hybrid enzyme in place of the totally-substituted enzyme. Thus, the experimental evidence strongly implies that the difference in the amplitude of the immobilized species is due to the presence of the cobalt atom.

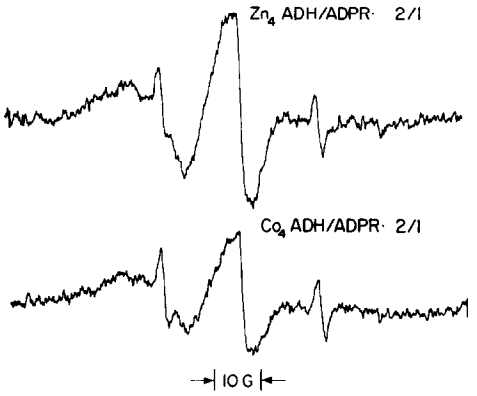

Fig. 1. EPR spectra of ADPR· in the presence of native ADH (ZnADH) and totally substituted cobald ADH (CoADH) in 0.1 μ phosphate buffer, pH 7.0 at 23°C. Identical instrumental settings were employed for recording the spectra. The sample concentration for the enzyme and the ADPR· were 100 μN and 50 μM, respectively.

The decrease in amplitude of the EPR spectrum for a nitroxide spin label in the presence of a paramagnetic ion can be accounted for by a magnetic dipolar interaction [1]. A theoretical treatment of dipolar interaction between two different spins in a rigid matrix provides not only an explanation for loss of amplitude of the EPR spectrum, but also a formulation for obtaining structural information [2]. The following equation results for the linewidth of the nitroxide EPR spectrum:

$$\delta H = \frac{g\beta\mu^2\tau}{r^6\hbar}\,(1-3\cos^2\theta_R)^2 + \delta Ho\ , \qquad (1)$$

where g, β, and $\hbar$ have the usual meanings, τ and μ are the electron spin relaxation time and magnetic moment of the paramagnetic cobalt ion, r is the distance between the cobalt ion and the spin label, θ_R is the angle between the dipole position vector and the magnetic field direction and δHo is the linewidth in the absence of dipolar broadening. In order to determine r, it is necessary to know the linewidths (δH, δHo) and τ. One can determine values for δH and δHo from the amplitudes of the EPR spectra of the bound ADPR·, 67 ± 4% for the totally substituted enzyme and 87 ± 3% for the cobalt hybrid enzyme.

The value of τ is not directly available, but a reasonable estimation of the correlation time for dipolar interaction can be ascertained from a PRR study of water with the cobalt-substituted enzymes. The correlation time, τ, for the dipolar interaction between cobalt and the protons of water was found by temperature dependence studies of the PRR to be controlled

by the electron spin relaxation time, T_{1e}, of the cobalt. The values of $1/T_{1p}$, the paramagnetic contribution to the PRR of water protons, were different for the two cobalt substituted enzymes, e.g., 0.048 sec^{-1} for the totally substituted enzyme compared to 0.010 sec^{-1} for the cobalt hybrid enzyme at 23°C, probably because of a difference in the number of water molecules coordinated to the cobalt atoms and certainly further demonstrating the difference in the metal binding sites. From the Solomon–Bloembergen equation [10, 11] for $1/T_{1M}$ with r taken as 2.8 Å, neglecting the hyperfine interaction term since it was found to be negligible for Co(II) and assuming a single water molecule of coordination for the totally cobalt substituted enzyme, a value for τ was calculated to 2.3×10^{-12} sec. This finding is in keeping with the EPR spectrum for cobalt being observed only at low temperatures (~10°K) [12], and the reported value for T_{1e} for $Co(H_2O)_6^{2+}$ of 1.2×10^{-12} sec at room temperature [13].

With the necessary data at hand, the distance between the spin label and the cobalt ion can be calculated upon substitution into eq. (1). A distance of 5.8 ± 0.3 Å was calculated for the binary complex with the totally substituted cobalt enzyme, while a value of r for the binary complex with the cobalt hybrid enzyme was calculated to be 7.4 ± 0.4 Å. Since the nitroxide radical of ADPR· approximates the position of the terminal ribose of ADPR [9], the calculated distance from the catalytic cobalt atom to the nitroxide spin is in excellent agreement with the 6–8 Å reported by the X-ray crystallographic data [6]. On the other hand, the distance from the structural cobalt atom to the spin label does not agree with the crystal data. Although it could be argued that the solution data does not necessarily have to match the crystal data, the discrepancy could be accounted for by several other sources such as a small contamination of the totally substituted cobalt enzyme in the cobalt hybrid enzyme, a difference in the electron spin relaxation time, or angular variation in the magnet moment due to two different types of coordination of the enzyme to the cobalt atoms. The possibility that the structural cobalt on the partner subunit is responsible has been considered, but based on the X-ray data, it would appear to be too distant to produce this degree of interaction. Further experimental studies are in progress to eliminate these potential error sources and establish the basis for the discrepancy.

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