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THE PROSTHETIC GROUP OF METHANOL DEHYDROGENASE FROM HYPHOMICROBIUM X: ELECTRON SPIN RESONANCE EVIDENCE FOR A QUINONE STRUCTURE

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SUMMARY: Partial reduction of the isolated prosthetic group of methanol dehydrogenase yields a free radical with the same characteristics as the one contained in the enzyme. The Electron Spin Resonance spectrum in alkaline aqueous solution displays hyperfine structure and is interpreted in terms of an isotropic g-value, hyperfine coupling constants and nuclear spins. The magnitudes of these parameters indicate that the prosthetic group is a quinone containing two nitrogen atoms.

Methanol dehydrogenase (alcohol: (acceptor) oxidoreductase, EC 1.1.99.8) is an enzyme that is found in methylotrophic bacteria grown on C_1-carbon substrates. It is a NAD(P)-independent enzyme. Several artificial one-electron acceptors can be used to detect its methanol-oxidation activity (1). As all the reported methanol dehydrogenases from methylotrophic bacteria have an identical absorption spectrum, the prosthetic group is probably the same for all of them. Although the nature of the prosthetic group is unknown, there is a general belief that it is a pteridine derivative (2,3,4). However, recently it was found that the methanol dehydrogenase from Hyphomicrobium X contains a free radical with characteristics that are incompatible with a pteridine derivative (1). The purpose of the present study is to show that the radical is derived from the prosthetic group and that Electron Spin Resonance can be used as a tool in the structure-elucidation of the prosthetic group.

Abbreviation: ESR - Electron Spin Resonance.

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MATERIALS AND METHODS: The purification of methanol dehydrogenase and the isolation of the prosthetic group were described previously (1).

ESR spectra were recorded at room temperature (20-22°) with a Varian E-4 X-band spectrometer. For g-value measurements the ESR spectrometer was calibrated with an anaerobic solution of Wurster's blue in ethanol (g=2.00305) (5).

The ESR spectrum depicted in Fig. 2a was interpreted in terms of hyperfine coupling constants and nuclear spins using the "pattern-search" method described earlier (6,7). Gaussian lineshapes were employed throughout. The final simulation (Fig. 2b) was performed with a top-to-top linewidth of 0.012 mT.

Preparation of samples for ESR:

a. Lyophilized methanol dehydrogenase.

40 mg of lyophilized enzyme was placed in a cylindrical quartz tube of 2 mm internal diameter and the ESR spectrum of Fig. 1 was recorded.

b. Methanol dehydrogenase in neutral aqueous solution.

40 mg of lyophilized enzyme was dissolved in 1 ml of 0.02 M potassium phosphate buffer (pH=7.1). The solution was transferred to a standard flat ESR sample-cell.

c. Methanol dehydrogenase in alkaline salicylate buffer.

0.5 ml of a freshly prepared 4M sodium salicylate ($pK_{a,2}$=13.4) solution was mixed with 0.5 ml of 2M sodium hydroxide. This solution and 40 mg of lyophilized enzyme were deoxygenated separately by flushing with argon for 15 minutes. The enzyme was dissolved in the alkaline salicylate buffer and transferred under argon to a standard flat sample-cell. The ESR spectrum of Fig. 2a was then recorded.

d. The partially reduced prosthetic group in alkaline salicylate buffer.

0.5 ml of a freshly prepared 4M sodium salicylate solution was mixed with 0.5 ml of 2M sodium hydroxide. The resulting solution was deoxygenated by flushing with argon for 15 minutes. A solution of 10 γ of the prosthetic group in 20 μl of water and 20 μl of a 1% solution of mercaptoethanol in water were added rapidly to the alkaline salicylate buffer. The solution was flushed with argon for 5 minutes and transferred anaerobically to a standard flat sample-cell.

e. The partially reduced prosthetic group in deuterated alkaline salicylate buffer.

The experiment was carried out in the same way as experiment d, but using deuterium oxide instead of water and NaOD instead of sodium hydroxide.

RESULTS AND DISCUSSION.

The X-band ESR spectrum of lyophilized methanol dehydrogenase from Hyphomicrobium X is shown in Fig. 1. The slightly unsymmetrical signal has a top-to-top linewidth of 0.7 mT.

The g-value (g=2) and linewidth of the spectrum indicate that it originates from an organic free radical. The same "powder-spectrum" is observed when the lyophilized enzyme is dissolved in water. This behaviour is quite normal for a radical that is tightly linked to a high molecular-weight protein. The anisotropy cannot

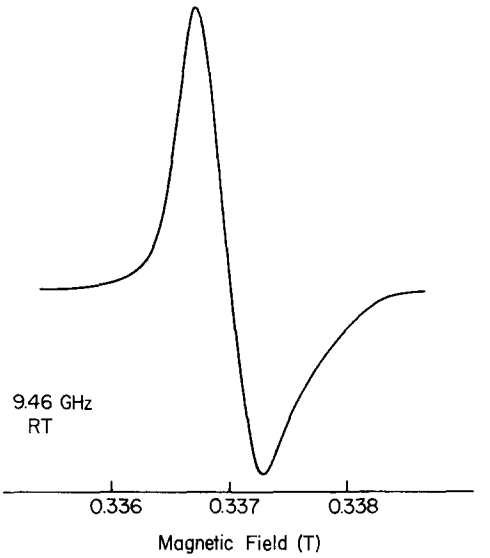

Fig. 1. The X-band ESR spectrum of methanol dehydrogenase.

average out as it does when a low molecular-weight organic radical is rotating freely in a solution.

The main disadvantage of powder-spectra of organic radicals is that the hyperfine structure is often obscured. Indeed no hyperfine structure is visible in the ESR spectra of lyophilized enzyme and enzyme solution. In order to observe hyperfine interactions we must disconnect the radical from the enzyme, so that it can rotate freely in solution. We found that this can be accomplished by dissolving lyophilized enzyme in a concentrated alkaline salicylate buffer under anaerobic conditions. The ESR spectrum of the resulting solution is shown in Fig. 2a.

As expected the ESR spectrum (Fig. 2a) has hyperfine structure. The isotropic g-value of the radical in salicylate buffer ($g=2.0046$) is essentially the same as the one of the enzyme-linked radical (1,8).

In order to prove that the radical of methanol dehydrogenase is derived from the prosthetic group, an experiment with the isolated prosthetic group (1) was carried out.

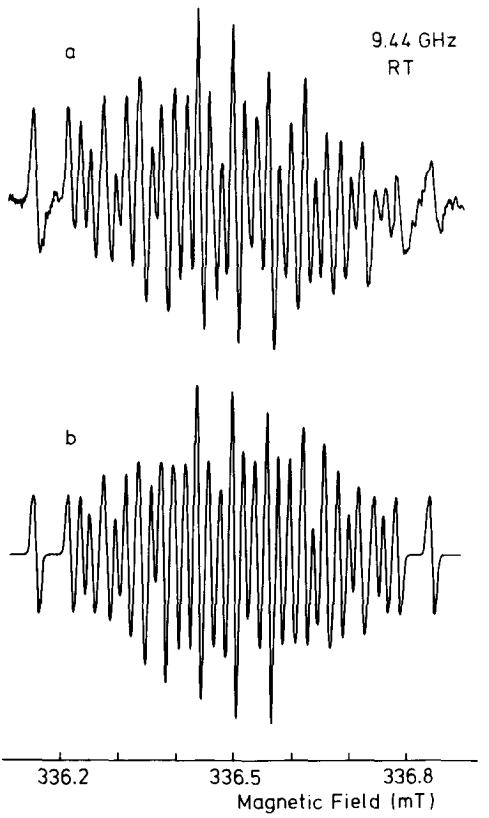

Fig. 2. a) X-band ESR spectrum of the free radical after release from the enzyme. b) Simulation.

The isolated prosthetic group is in the oxidized state and must be reduced in order to form the radical-state which can be detected by ESR. The ESR spectrum obtained after reduction of the prosthetic group in alkaline salicylate buffer turns out to be identical with the spectrum in Fig. 2a. Extraction of the enzyme-bound radical with alkaline salicylate buffer and reduction of the isolated prosthetic group in alkaline salicylate buffer both result in the same ESR spectrum (Fig. 2a). This means that the enzyme-bound radical is derived from the prosthetic group.

The spectrum shown in Fig. 2a has a number of interesting features which shed light on the chemical structure of the radical.

The g-value (2.0046) is too large for a pterin or lumazine radical, but it is quite compatible with a quinone radical (1).

The total width between the outer lines of the spectrum is 0.69 mT. The widths of the ESR spectra of those pterin and lumazine radicals that are known range from 3 to 10 mT (6,7,9,10,11). For quinone radicals the widths range from 0.6 to 2 mT (12-18). As in the case of the g-value, the width of the spectrum (Fig. 2a) is not compatible with a pterin or lumazine structure, but it is in excellent agreement with a quinone structure.

The experimental spectrum (Fig. 2a) was interpreted in terms of hyperfine coupling constants and nuclear spins with which the spectrum could be simulated (Fig. 2b) accurately. A detailed description of the methods used for ESR spectrum interpretation has been published (6,7). Their reliability has been demonstrated in the interpretation of ESR spectra of pterin and lumazine radicals.

Two nuclei with spin=1 (^{14}N) and three nuclei with spin=1/2 (^{1}H) have interaction with the unpaired electron. The corresponding hyperfine coupling constants are:

a_{N1}=0.060 , a_{N2}=0.081 , a_{H1}=0.097 , a_{H2}=0.126 and a_{H3}=0.184 (mT).

The proton with the smallest hyperfine coupling constant (H1) is replaced by deuterium if the ESR experiment is carried out with a deuterated solvent.

A complete elucidation of the structure of the radical by ESR is not possible at this stage.

The semiquinone radical that is described in this study can be generated by reduction of the isolated prosthetic group. We therefore propose that the prosthetic group of the methanol dehydrogenase from Hyphomicrobium X is a quinone containing two nitrogen atoms. Further investigations on the structure of the prosthetic group are in progress.

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