A STABLE FREE RADICAL INTERMEDIATE IN THE REACTION OF 2-OXOACID:FERREDOXIN OXIDOREDUCTASES OF *HALOBACTERIUM HALOBIUM*

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1. Introduction

Two enzymes have been detected in the extremely halophilic bacterium *Halobacterium halobium* [1] which catalyze reactions of the type:

$$R{-}CO{-}COO^- + 2\ Fd_{ox} + CoA \longrightarrow$$

$$R{-}CO{-}CoA + 2\ Fd_{red} + CO_2$$

where Fd is the [2 Fe-2 S] ferredoxin of this organism [2].

The enzymes have now been isolated [3]. 2-Oxoglutarate:ferredoxin oxidoreductase (EC 1.2.7.2), here referred to as 'enzyme I', catalyzes the oxidative decarboxylation of 2-oxoglutarate only. Pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1), 'enzyme II', catalyzes the oxidative decarboxylation of 2-oxobutyrate, pyruvate and 2-oxoglutarate in decreasing order of effectiveness. Both enzymes have relative molecular mass (M_r) ~250 000. Analysis indicated two [4 Fe–4 S] clusters and 1.1–1.6 molecules thiamine diphosphate/molecule enzyme. No other cofactors have been detected. In these respects the enzyme resembles the pyruvate:ferredoxin oxidoreductase of *Clostridium acidi-urici* [4].

Electron paramagnetic resonance (EPR) spectroscopic investigations were undertaken to study the iron–sulphur clusters in these enzymes. During these studies we observed free radical signals presumed to arise from intermediates in the enzyme reaction. Another type of signal was assigned to a spin–spin interaction between the radical species and the reduced iron-sulphur cluster. These signals are of great assistance in elucidating the mechanism of action of these enzymes.

2. Materials and methods

The methods of isolation of enzymes I and II are described in [3]. Enzyme samples were prepared in 3 M KCl + 0.1 M Tris–phosphate buffer, (pH 8.0). For the preparation of EPR samples the solutions of enzymes and reagents were purged with argon, so that the oxygen concentration in the solution was diminished.

EPR spectra were recorded on a Varian E4 spectrometer with an Oxford Instr. ESR9 liquid helium flow cryostat. Averaging of spectra and other manipulations were carried out on a Data Lab. DL4000 microprocessor system.

3. Results

3.1. *Free radical signals from the enzymes*

Treatment of enzymes I and II with their respective 2-oxoacid substrates induced free radical signals which were stable at room temperature. Spectra of samples frozen in this state are shown in fig.1. The signal of enzyme I is centred at $g = 2.006$ with an overall linewidth of 1.6 mT. That of enzyme II is somewhat broader and shows hyperfine splitting. The second derivative spectrum (fig.1c) suggests an anisotropic g-tensor and splitting by at least one nuclear spin. Spectra measured at room temperature (not shown) were similar to those in the frozen state. From this conservation of anisotropy we conclude that the radical species are immobilized within the enzyme proteins. The stability of these radicals is shown by the fact that the enzymes, as prepared, showed variable amounts of them. They could be increased by treatment of the enzymes with 2-oxoacid.

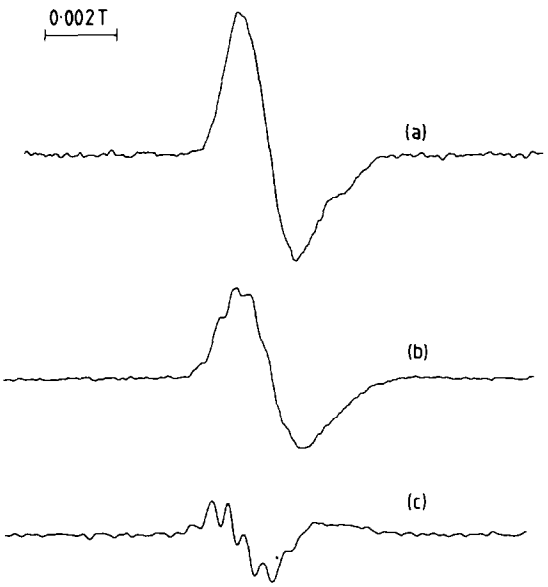

Fig.1. EPR spectra of: (a) enzyme I + 2-oxoglutarate; (b) enzyme II + pyruvate; (c) is the second derivative obtained by digital differentiation of (b). The enzyme samples (2 mg/ml) were mixed with substrate (0.5 mM) at 20°C for 1 min before freezing the samples. EPR spectra were recorded at 92 K with the following instrument settings: microwave power, 0.2 mW frequency 9.26 GHz; modulation amplitude 0.2 mT; frequency 100 kHz.

The excess substrates could subsequently be removed by gel filtration, without any change in the radical concentration. The signals were removed by treatment with coenzyme A.

3.2. *Spectra of the reduced enzymes*

On reduction with dithionite, a spectrum typical of a reduced iron–sulphur cluster appeared, with principal apparent g-values: 1.866, 1.940, 2055 for enzyme I, and 1.835, 1.933, 2.049 for enzyme II (fig.2a). The signals were observed over a lower range of temperature (6–16 K) than for most (4 Fe–4 S]$^{1+}$ clusters. This is in accord with the well-known relationship between g-value anisotropy and electron-spin relaxation rate [5]. The same spectrum was seen on reducing the enzyme with dithionite in the presence of the 2-oxoacid substrate.

A brief treatment of the enzymes with the 2-oxoacid substrate and coenzyme A, induced a different reduced species of the enzyme. This is illustrated in fig.2(b) for enzyme II; the corresponding spectrum for enzyme I was less well-defined. At >20 K only

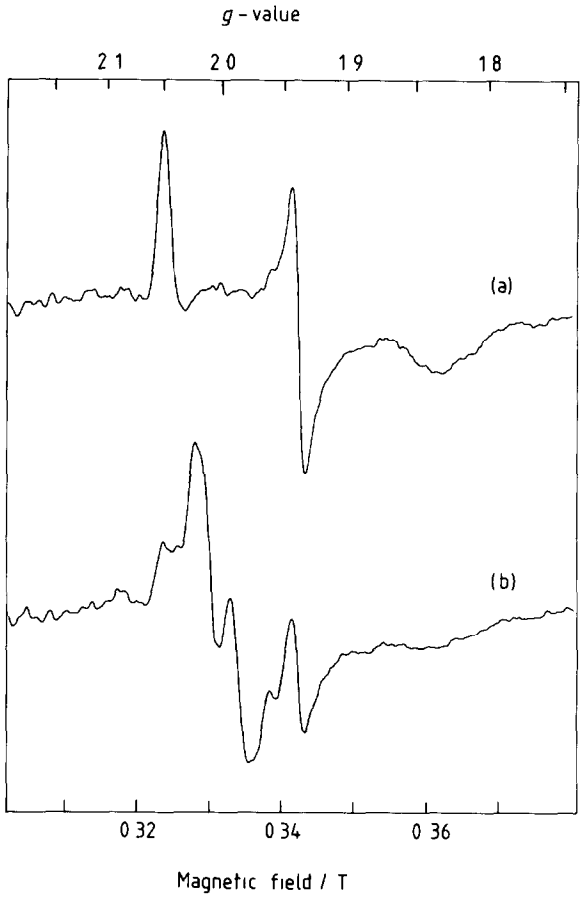

Fig.2. Spectra of enzyme II: (a) reduced with 2 mM $Na_2S_2SO_4$ for 3 min at 20°C; (b) treated with 0.5 mM pyruvate + 50 μM coenzyme A, for 5 min at 20°C before freezing in liquid nitrogen. Conditions of measurement: temp. 12 K; microwave power, 20 mW; modulation amplitude, 1 mT.

the radical signal was observed from this sample, but at 12 K a more complex spectrum was seen. This spectrum appears to contain at least two components: one identical to the [4 Fe–4 S]$^{1+}$ signal of the dithionite-reduced enzyme (fig.2a); and one that we interpret as due to a spin–spin interaction between the reduced [4 Fe–4 S]$^{1+}$ cluster and the radical species. This interpretation implies that both groups are close together in the active site. In enzyme II this interaction appears to give a narrow signal around $g = 2$, though broad outer lines may also be present. At >20 K, where the electron-spin relaxation rate of the reduced [4 Fe–4 S] cluster is so rapid that its signal is broadened out, the effect of the spin coupling is lost and the radical signal is seen.

4. Discussion

It is excluded that the free radical signals arise from contaminants, for after gel filtration they are only observed in the enzyme-containing fractions. Furthermore, increase in signal intensity after addition of 2-oxoacid and disappearance of the radicals on addition of coenzyme A indicate that they are intermediates of the catalytic cycle. Therefore 2-oxoacid:ferredoxin oxidoreductases differ from the well-studied pyridine nucleotide-dependent 2-oxoacid dehydrogenase complexes [6]. This difference presumably reflects the requirements for electrons to be withdrawn by ferredoxin one at a time. We assume that on formation of the free radical one electron is transferred to an enzyme-bound [4 Fe–4 S] cluster, which in turn donates it to ferredoxin or, under our experimental conditions, to molecular oxygen. Transfer of the second electron occurs, when after addition of coenzyme A the catalytic cycle is completed. If 2-oxoacid and coenzyme A are present in excess, catalysis continues until oxygen is consumed and the [4 Fe–4 S] clusters remain in the reduced state. Since part of the enzyme molecules also contain the free radical, the complex EPR spectrum shown in fig.2b is observed. On adding dithionite, the radical presumably undergoes reduction, leaving the spectrum of a reduced iron–sulphur cluster as usually observed (fig.2a).

Acknowledgements

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