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Mössbauer and EPR Evidence for Nickel and 3Fe Cluster in the Hydrogenases of *D. desulfuricans* and *D. gigas*

B. H. HUYNH

Emory University, Atlanta, Ga., U.S.A.

J. LEGALL, D. V. DERVARTANIAN, H. D. PECK, Jr., H.-J. KRÜGER

University of Georgia, Athens, Ga., U.S.A.

I. MOURA, J. J. G. MOURA and A. V. XAVIER

Universidades de Lisbon, Portugal

Hydrogenases from *Desulfovibrio desulfuricans* (27774) and from *Desulfovibrio gigas* have been studied by EPR and Mössbauer spectroscopy. As isolated, hydrogenase from *D. gigas* exhibits an 'isotropic' EPR signal at g = 2.02 and a rhombic EPR signal at g = 2.31, 2.20 and 2.02. Isotopic substitution of ^{61}Ni (I = 3/2) proves that the rhombic signal is due to Ni. A similar EPR spectrum was observed for *D. desulfuricans* hydrogenase, indicating that both enzymes contain an EPR active nickel center. Combining the Mössbauer and EPR data, the isotropic g = 2.02 EPR signal was shown to originate from a 3Fe cluster which may have oxygenous or nitrogenous ligands. In addition, the Mössbauer data also revealed two [4Fe–4S] clusters in each molecule of hydrogenase.

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Core Extrusion of Iron–Sulfur Proteins with Benzenethiol in Aqueous Urea/Triton X-100 Solutions: A Study on the Reactivity of Different Conformational States of Succinate Dehydrogenase

FRANCO BONOMI, SILVIA PAGANI and PAOLO CERLETTI

Department of General Biochemistry, Faculty of Agriculture, University of Milan, Milan, Italy

The aqueous DMF/Triton X-100/benzenethiol extrusion procedure developed by Kurtz [1] and investigated in detail in [2] has been modified by substituting urea for DMF as the protein-denaturing agent. This replacement allows for shorter reaction times, lower sensitivity to ionic strength [3] and for lower dilution factors. Preliminary experiments with spinach ferredoxin [4] demonstrated the identity of the extruded thiolate with respect to the synthetic analogue, and the feasibility of an easy and ready separation of the products of the reaction by ion-exchange chromatography. The thiolate does not bind to a cellulosic anion exchanger, whereas the bound apoprotein is eluted with high-ionic strength buffer and can be chemically [5] or enzymatically [6] reconstituted to its native form.

TABLE I. Relationships between the Conformational State, E_m^{S1}, and the Rate Constant of the Urea/Triton/Benzenethiol Extrusion Reaction for Succinate Dehydrogenase. E_m^{S1} is determined by potentiometric titration of the EPR signal at g = 1.94 at liquid nitrogen temperature. Reaction rates for the extrusion reaction are determined by monitoring the increase in absorbance at 474 nm resulting from the addition of the protein to a suitable reaction medium, whose final composition is: 9×10^{-6} *M* protein, 0.096 *M* benzenethiol, 4.73 *M* urea, 4.77 (% v/v) Triton X-100, 0.05 *M* Tris-HCl and enough effector or NaCl to give a final ionic strength of 0.0365 *M*.

Effector	E_m^{S1}, mV	Pseudo-first-order rate constant of the extrusion reaction, min^{-1}
None	−14	0.0769
Oxaloacetate	+80	0.2631
Succinate	+108	0.4444

We used this technique in our investigation on the molecular basis of the modulation of the redox potentials of the electron-transferring groups in the complex iron–sulfur–flavoprotein succinate dehydrogenase. This protein exists in three different conformational states, or conformers, depending on the binding of different effectors to a site different from the catalytic one [7]. We thus distinguish between: the active, ligand-free enzyme; the active, succinate-stabilized enzyme, and the inactive complex between the protein and oxaloacetate. Different catalytic activities of the conformers depend on the different midpoint potential of the covalently bound flavin, which is high in the active conformers and low in the inactive one [8].

Also the redox midpoint potential of the tetrahedral Fe–S center S1 (E_m^{S1}) is affected by ligand binding. This parameter is related in Table I to the reaction rates measured for the extrusion of Fe–S structures from different conformers. Although a possible non-selectivity of the extrusion must be considered, rate constants for different conformers exactly parallel the figures obtained for E_m^{S1}, increasing as this latter increases. The concentrations of both the denaturing agent (urea) and of the extruding one (micellar benzenethiol) being the same, the reaction rate only depends on the accessibility of the cluster to the hydrophobic extrudant. Thus binding of effectors which increase E_m^{S1} also increases the hydrophobicity of the environment of the cluster. This extends to different conformational states of the

same protein previously inferred by comparing the redox properties of the same cluster in different proteins of known structure [9].

Since several recent reports present modifications of the thermodynamic properties of Fe–S centers in a number of proteins and relate these modifications to more or less subtle conformational changes, the approach we introduced may be of some interest.

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NMR Studies of Porcine Uteroferrin: Evidence for a Spin-coupled Binuclear Iron Cluster

L. QUE, R. B. LAUFFER

Baker Laboratory, Department of Chemistry, Cornell University, Ithaca, N.Y. 14853, U.S.A.

B. C. ANTANAITIS and P. AISEN

Departments of Physiology and Biophysics and Medicine, Albert Einstein College of Medicine, Bronx, N.Y. 10461, U.S.A.

Uteroferrin is an iron-containing acid phosphatase of molecular weight near 35,000 isolated from either the allantoic fluid of pregnant sows or the uterine flushings of pseudopregnant sows [1–3]. The protein can exist in two forms – a purple (λ_{max} ~ 570 nm), enzymatically inactive, oxidized form and a pink (λ_{max} ~ 510 nm), enzymatically active, reduced form [2, 3]. The former is EPR-silent, while the latter exhibits a novel EPR signal centered near g = 1.74 [4–6], reminiscent of signals observed for the semimethemerythrins [7].

We have undertaken a ^{1}H NMR study of porcine uteroferrin focusing on paramagnetically shifted resonances in order to elucidate the active site structure and magnetic properties of the protein. Figure 1 shows the 300 MHz ^{1}H NMR spectrum of pink two-iron uteroferrin in sodium acetate buffer (D_2O), pH 4.9, at 30 °C. Well-resolved features spanning 160 ppm are observed with linewidths ranging from 300–2000 Hz. In buffered H_2O, additional resonances are observed near 89, 43, and –25 ppm.

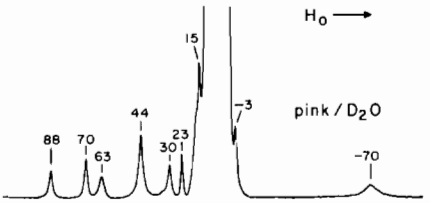

Fig. 1. 300 MHz ^{1}H NMR spectrum of pink uteroferrin.

The similarity of the EPR signal exhibited by pink uteroferrin to those of the semimethemerythrins [7] suggests the involvement of antiferromagnetically coupled Fe(III)–Fe(II) centers. Estimates for the value of the antiferromagnetic coupling constant J ($H = -2JS_1 \cdot S_2$) can be made from the temperature dependence of the isotropic shifts, since the shifts are proportional to magnetic susceptibility, assuming a temperature-invariant Fermi contact term [8]. Based on data obtained from 0–50 °C, we conclude that $-J < 20$ cm^{-1} for pink uteroferrin, in agreement with the estimate of J (-7 cm^{-1}) obtained from the temperature dependence of the intensity of the EPR signal [6].

Some of the metal ligands in pink uteroferrin can be identified by comparing the observed shifts to those of synthetic complexes. Based on our model studies, histidine is found in the coordination environments of both the ferrous and the ferric centers, while tyrosine is coordinated only to the ferric center. Other ligands including the bridging group have yet to be identified.

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