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EVIDENCE FOR A TWO PROTON DEPENDENT REDOX EQUILIBRIUM IN AN ARCHAEAL RIESKE IRON-SULFUR CLUSTER

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The recently detected Rieske iron - sulfur center in the membrane of the thermoacidophilic archaeon *Sulfolobus acidocaldarius* (Anemüller et al., 1993, FEBS Lett. 318, 61 - 64) was further characterized by EPR spectroscopy, coupled to redox - potentiometry and functional studies. The reduction potential is pH - dependent above pH 6, revealing the influence of two ionization equilibria in the oxidized form, with pK_a^{ox} - values of 6.2 and 8.5. Above pH 9, the slope of the curve is - 120 mV/pH - unit. A partially purified fraction exerted a ubiquinol - cytochrome *c* oxidoreductase activity. To our knowledge, for the first time, in a membrane bound Rieske iron - sulfur protein, unequivocal evidence for a two proton dependent redox equilibrium is presented.

Rieske iron - sulfur clusters are found in electron transfer chains responsible for energy transduction, as part of membrane - bound protein complexes (e.g. the cytochrome bc_1 or b_6f - complexes) playing an important role in electron transport from reduced quinones to cytochrome *c* or plastocyanin in a process coupled to proton translocation [1,2]. Rieske - type centers have also been found in bacterial mono - or dioxygenase systems catalyzing the oxidative degradation of aromatic carbon compounds [3]. Both types of enzymes were exclusively isolated from eucaryotic and bacterial species, whereas until recently no archaeal Rieske center had been detected. The biophysical investigation of the Rieske and the Rieske - type iron - sulfur centers showed a large similarity in the cluster structure and ligation [4-6]. However, both classes differ in two major aspects: the redox - potentials of the Rieske iron - sulfur clusters are in the range from + 150 to + 300 mV and display a strong pH - dependence [7], whereas the respective values for the Rieske - type proteins vary between +5 and -150

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mV and are pH - independent [8-10]. So far, all studies of the Rieske iron -sulfur proteins in the membrane bound state revealed only one clearly defined pK_a- value [11]. The possible existence of a second pK_a- value was indicated by a steeper slope of the redox - titration curve of about 80 mV/pH - unit. In the following, we present unequivocal evidence for the presence of two ionization equilibria in the oxidized form of a membrane bound archaeal Rieske iron - sulfur center from *S .acidocaldarius*.

MATERIALS AND METHODS

Part of the *S. acidocaldarius* cells used in this study were grown at the "Gesellschaft für Biotechnologische Forschung mbH", Braunschweig, F.R.G.

S. acidocaldarius membranes were prepared as described [12]. EPR redox titrations were performed under anaerobic conditions as previously described [13] in 10% (v/v) ethylene - glycol using the following buffers: 0.2 M MES (pH 5.4), 0.8 M KH_2PO_4 (pH 5.9, 6.5) and 0.2 M glycine (pH 8.44, 8.9, 9.44). Membrane protein concentration were 14 - 26 mg/ml.

Preparation of a Rieske iron-sulfur protein enriched membrane protein fraction: Membranes isolated from *S. acidocaldarius* cells grown under conditions of strong aeration (approx. 1.2 m^3/h) in a 50 l fermenter were resuspended at a protein concentration of 7.5 mg/ml in a buffer containing 20 mM sodium diphosphate and 50 mM potassium phosphate adjusted to pH 7.5. The suspension was gently stirred for 1 h at 4°C and the membranes were spun down for 45 min. at 100 000 * g. The pellet was resuspended in half of the previous volume of a buffer containing 50 mM Tris HCl, pH 7.5 and 20 mM dodecyl maltoside (DM) at 4°C. This solubilization mixture was gently stirred for 90 min. at 4°C and subsequently the membranes spun down for 1 h at 100 000 * g. An equal volume of saturated ammonium sulfate (AS) solution was slowly added to the supernatant and the solution loaded at a flow rate of 0.3 ml/min. onto a propyl agarose column (r=0.75 cm, height=6 cm) (Sigma Chemical Co.) equilibrated with 25 mM Tris HCl, pH 7.5 (4°C), 1 mM DM in a 50% saturated AS - solution. The column was washed with 60 ml of the equilibration buffer followed by 50 ml of 25 mM Tris HCl, pH 7.5, 0.2 mM DM at 40% AS saturation and eluted with 50 ml of 25 mM Tris HCl, pH 7.5, 0.2 mM DM, 20% AS. This protein fraction was desalted on a Sephadex G - 25 column equilibrated and eluted with 25 mM Tris HCl, pH 7.5, 0.2 mM DM, and concentrated by diafiltration on a PM 10 membrane (Amicon).

Membrane protein concentration was determined by a modified Biuret reaction [14]. EPR spectra were recorded with an X - band Bruker ESP 380 spectrometer equipped with an ESR 900 continuous flow helium cryostat from Oxford Instruments.

RESULTS

The reduction potential of the Rieske iron - sulfur cluster and its pH dependence were determined by a series of EPR redox titrations of membrane preparations, performed in the pH 5.4 to 9.5 range. All individual titration curves could be simulated with simple n=1 Nernst equations, as shown previously [15]. The reduction potential is pH dependent above pH 6 and, in the above range, is clearly affected by more than one protonation equilibrium. (Figure 1). The data could be well matched assuming two proton dependent redox equilibria of the oxidized protein, described by the following equation [16]:

$$E_m = E_m(\text{low pH}) + (RT/nF) * \ln([H^+]^2/([H^+]^2 + K^{ox}_1 * [H^+] + K^{ox}_1 * K^{ox}_2))$$

The fit of this equation to the data points yields an E_m (low pH)=+400 mV and pK_a^{ox} values of 6.2 and 8.5 (Fig.1, solid line). Above pH 9 the slope is -120 mV/pH unit, indicating the

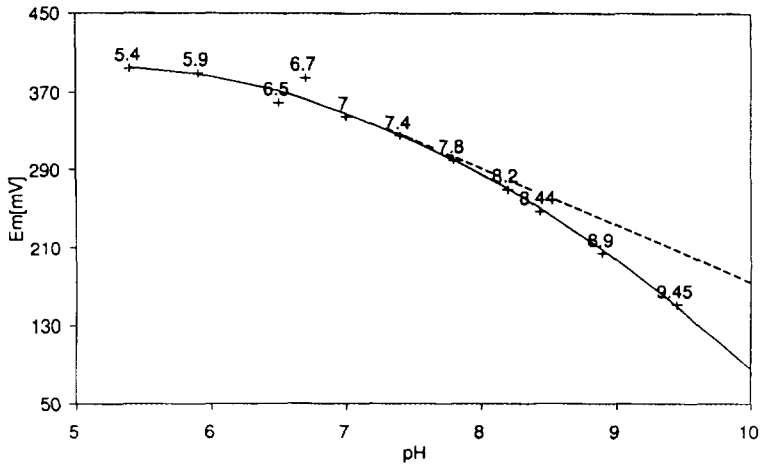

FIG. 1. pH - dependence of the Rieske iron - sulfur center reduction potential. The data points for pH 6.7 to 8.2 were taken from reference [15]. The solid line was calculated assuming two ionization equilibria with pK_a- values of 6.2 and 8.5 and E_m (low pH) = + 400 mV. The dashed line was calculated using only one ionization equilibrium with pK_a =6.2 and E_m = + 400 mV.

involvement of two protons. Moreover, the data analysis excludes the presence of a further proton dependent equilibrium with a pK_a-value lower than 9.5. No evidence for a pK_a^{red}-value was found, in agreement with the fact that no EPR spectral changes were observed in this pH range. For comparison, a curve with just one pK_a^{ox}-value is depicted in figure 1 (dashed line), which obviously does not correctly describe the pH dependence of the reduction potential.

The Rieske iron - sulfur protein enriched membrane protein fraction was found to catalyze the electron transfer between the soluble ubiquinone analogue n-decyl-ubiquinol and horse - heart cytochrome *c* with a catalytic activity of 15 nmol cytochrome *c* reduced/min. mg. Figure 2 illustrates the redox changes of the Rieske iron - sulfur center in an EPR experiment. The initially oxidized center (top trace) becomes reduced upon addition of the quinol (second trace). Subsequent addition of an excess of cytochrome *c* leads to a complete reoxidation of the Rieske iron - sulfur cluster (third trace). The bottom trace shows the Rieske iron - sulfur protein completely reduced by ascorbate.

DISCUSSION

In a previous publication [15], we have already shown a pH-dependence of the reduction potential of the archaeal Rieske iron-sulfur center in the range from pH 6.7 to 8.2. Although these data suggested the involvement of at least one dissociable proton from the oxidized cluster, no unambiguous information about the exact number of protons and the pK_a - values could be obtained. The successful determination of these parameters was now possible after extending the study over a wider pH - range. The pK_a^{ox}-values for the archaeal

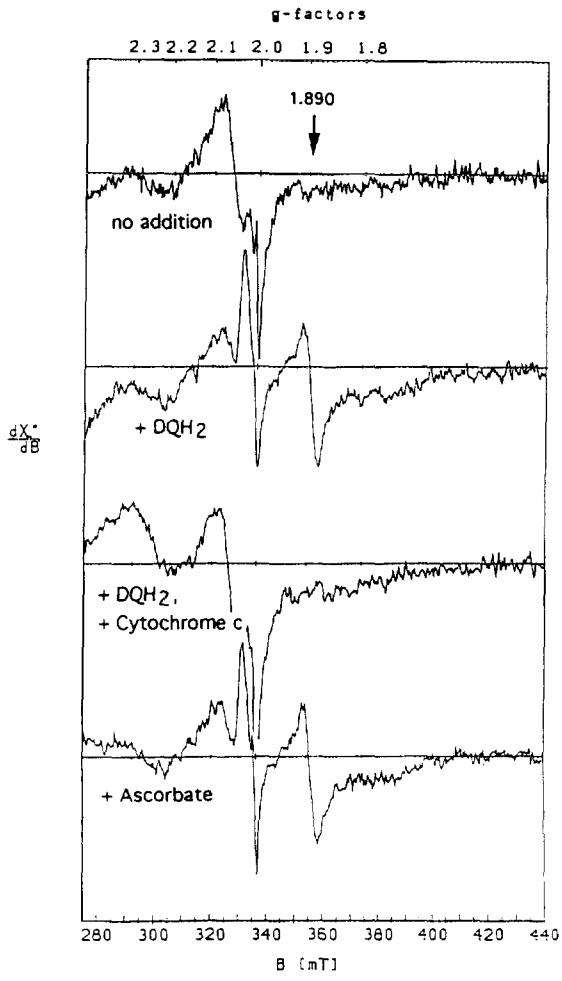

FIG. 2. Changes of the redox state of the Rieske iron - sulfur center upon addition of n - decyl - ubiquinone and horse - heart cytochrome *c*.
Upper trace: control, second trace: addition of n - decyl - ubiquinone (DQH_2), final concentration 23.5 μM, incubation at 37°C for 2 min. third trace: horse heart cytochrome *c* final concentration 70 μM, was added to a sample identical to the one in the second trace and incubated at 37°C for 5 min. bottom trace: complete reduction by ascorbate, final concentration 5 mM, and incubation at 37°C for 5 min. All samples were subsequently transferred to EPR tubes and frozen in liquid nitrogen. Protein concentration was 0.45 mg solubilized membrane protein in 155 μl 60 mM bis - Tris - propan HCl buffer, pH 8.0. EPR conditions: temperature: 15 K, microwave power: 2 mW, modulation amplitude: 1 mT.

iron - sulfur cluster (6.2 and 8.5) turned out to be significantly lower than those reported for the water soluble fragment of the Rieske iron - sulfur protein from the bovine heart mitochondrial cytochrome bc_1 - complex (7.6 and 9.2) [17] and for other bacterial sources [11]. Since the internal pH in *S. acidocaldarius* cells, approx. 6.5 [18], is also clearly lower than the corresponding value in mitochondria, approx. 7.4, the first pK_a^{ox}- values of the

archaeal (6.2) and the mitochondrial (7.6) proteins are very close to the relevant physiological pH - values. The present study also provides a clear evidence for the presence of two redox - dependent pH equilibria of the Rieske protein in the membrane bound state, showing that the redox equilibrium is coupled to the release (uptake) of protons.

The E_m - value of + 400 mV is unusually high for a Rieske iron - sulfur protein, when compared to the + 312 mV observed for the mitochondrial protein [17]. However, the redox titrations were performed at room temperature, whereas the physiological temperature for the archaeon is about 80°C. Assuming a temperature dependence of the reduction potential of about -1.5 mV/K, as determined for the mitochondrial Rieske protein [17], the E_m - value for the archaeal FeS - cluster would decrease to about +320 mV nicely coinciding with the mitochondrial protein redox potential.

In addition to the physiological reductants NADH and succinate, which have been shown to reduce the membrane bound Rieske iron-sulfur cluster [15], the FeS center can also interact with cytochrome *c* as shown by the observation of a ubiquinol - cytochrome *c* - oxidoreductase activity of partially purified iron - sulfur protein fractions. The results presented in figure 2 clearly demonstrate this activity and also the participation of the Rieske iron - sulfur protein in the catalytic process. Even though the activity is relatively low as compared to that of the well characterized cytochrome bc_1 - complexes [2], it has to be considered that neither the electron donor, nor the electron acceptor, nor the temperature at which this activity was measured are comparable to the physiological conditions of *Sulfolobus*. Caldariella quinone, most likely to be the physiological electron donor, is relatively unstable and difficult to handle compared to ubiquinone or the analogue used here. The physiological electron acceptor is as yet unknown. However, the applied model system provides valuable insights into the function of this first archaeal Rieske iron - sulfur center. Even though the biophysical properties of the Rieske protein are almost identical to those of the respective eucaryotic proteins it must be part of a structure clearly different from the known bc_1/b_6f - complexes. Since the archaeon *Sulfolobus* is located at the deepest aerobic branch of the evolutionary tree [19], further studies of this system are expected to provide a key to the role of Rieske proteins in the evolution of respiratory electron transport.

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