THE OXIDATION-REDUCTION POTENTIALS OF IRON–SULFUR CENTERS IN THE SITE I REGION OF THE RESPIRATORY CHAIN IN *C. UTILIS* SUBMITOCHONDRIAL PARTICLES

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Received 22 December 1971

1. Introduction

Ohnishi et al. [1] have reported that in the NADH dehydrogenase region of the respiratory chain of *C. utilis* there are iron–sulfur proteins whose EPR absorbance is measurable only at temperatures below that of liquid nitrogen. More recently, similar EPR-absorbing species were independently observed in mammalian systems by Ohnishi et al. [2] and Orme-Johnson et al. [3], and confirmed by Albracht and Slater [4]. Orme-Johnson et al. titrated the anaerobic NADH-UQ reductase [5] prepared from beef heart with reducing equivalents and identified 4 separate iron–sulfur centers. In the present paper, we report on the resolution of the iron–sulfur centers in *C. utilis* by the method of Dutton and co-workers [6, 7] as adapted for the use of EPR measurements [8]. In addition, the values of the half-reduction potentials of these iron–sulfur centers are presented and their relationship to energy conservation at site I is discussed.

2. Materials and methods

C. utilis mitochondria and submitochondrial particles were prepared as reported previously [2]. The oxidation–reduction potentials were measured potentiometrically by the method of Dutton and co-workers [6, 7] using the chamber designed to permit the transfer of aliquots directly into EPR sample tubes.

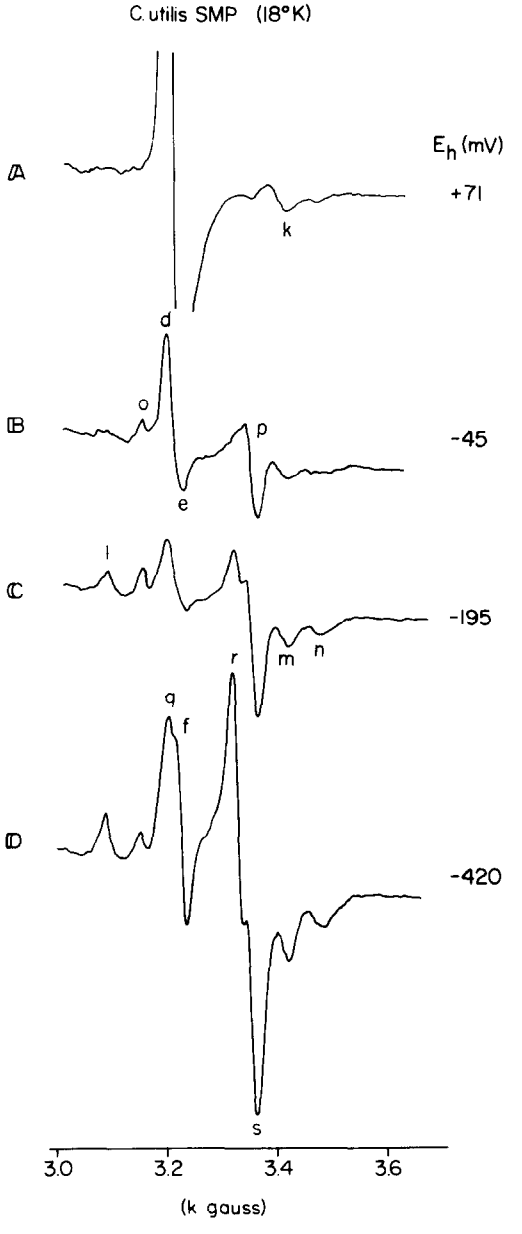
Oxidation–reduction mediators employed were phenazine methosulfate, phenazine ethosulfate, duroquinone, pyocyanine, resorufin, 2-OH-naphthoquinone, phenosafranine, benzylviologen and methylviologen.

EPR spectra were recorded with a Varian X-band spectrometer (V 4502) at temperatures $\leq 77^{\circ}$ K. Temperatures below 77° K were obtained by cooling samples with a stream of cold helium gas derived from boiling liquid helium. A thermocouple, gold–cobalt alloy-vs.-platinum, was used for temperature measurements.

3. Results and discussion

The oxidation–reduction mediators were added to an anaerobic suspension of *C. utilis* submitochondrial particles and reducing equivalents (NADH) were added to achieve the indicated oxidation–reduction potentials. The aliquots were then transferred anaerobically to EPR sample tubes and immediately frozen by immersion in liquid nitrogen. In fig. 1, the EPR spectra are presented for the samples at various oxidation–reduction potentials. The individual EPR signals and their responsible iron–sulfur centers are designated according to Orme-Johnson et al. [3]. At potentials more positive than +71 mV the central resonance (k) of an iron–sulfur protein in the same redox potential region as cytochrome b-c_1 [9] and a resonance principally from oxidized high-spin non-heme iron [10] are observed (spectrum A). Lowering the potential of the sample to −45 mV resulted in the

appearance of signals (*o*, *p*) due to the partial reduction of iron–sulfur Center 2 (spectrum B). On the further decrease of the oxidation–reduction potential to −195 mV, signals (*l*, *m*, and *n*) due to iron–sulfur Center 3 + 4 appeared, as shown in spectrum C. At −420 mV these resonance signals were further intensified and the signals (*q*, *r* and *s*) due to the reduction of iron–sulfur Center 1 were observed (spectrum D). The approximate field positions of the prominent peaks for the iron–sulfur centers in the site I region of the respiratory chain in *C. utilis* SMP are: Center 1 (*q*, *r*, *s*) 2.02, 1.94, 1.92; Center 2 (*o*, *p*) 2.05, 1.92; Center 3 + 4 (*l*, *m*, *n*) 2.10, 1.98, 1.85. These values are very similar to those of iron–sulfur centers in mammalian systems as reported by Orme-Johnson et al. [3].



In fig. 2, the height of the first derivative signals are plotted as a function of the oxidation–reduction potential of the sample, since first derivative signal is proportional to the concentration of the absorbing species in systems for which line shape of the absorption curve is constant. Measurements at 77° K show a higher potential component with an *n* value of 1 and a half-reduction of 0 mV at pH 7.2. This signal actually results from 2 separate iron–sulfur proteins associated with glycerol-1-phosphate and succinate dehydrogenase [11] whose half-reduction potentials differ by less than 40 mV [2]. The lower potential component measured at 77° K can also be measured at 18° K, and corresponds to Center 1. The *n* value is clearly less than 1, indicating that Center 1 consists of more than one component. The present data do not permit their resolution, but do suggest at least 2 components which differ in half-reduction

Fig. 1. EPR spectra of iron–sulfur proteins in *C. utilis* SMP at different oxidation–reduction potentials measured at 18° K. *C. utilis* SMP were suspended at approx. 25 mg of protein per ml in a medium containing 0.3 M mannitol, 5 mM K-phosphate buffer, pH 7.2, 1 mM $MgCl_2$, and 50 mM morpholinopropane sulfonic acid buffer, pH 7.2. Oxidation–reduction potentials were measured potentiometrically as described in the text. Oxidation–reduction mediators added were 40 μM phenazine methosulfate, 40 μM phenazine ethosulfate, 63 μM duroquinone, 6 μM pyrocyanine, 6 μM resorufin, 58 μM phenosafranine, 56 μM benzylviologen, 108 μM methylviologen, and 25 μM 2-OH-naphthoquinone. The oxidation–reduction potential of the suspension system was lowered by stepwise additions of small aliquots of 0.1 M NADH solution. Oxidation–reduction potentials shown in the figure are relative to the standard hydrogen electrode.

The EPR operating conditions were: modulation amplitude, 12 gauss; microwave power, 0.205 mwatt; microwave frequency, 9.02 GHz; time constant, 0.001 sec; scanning rate, 1 kgauss per min; temperature, 18° K.

The ordinate is the first derivative of the microwave absorption in an arbitrary unit. Small Roman letters are placed along the spectra vertically above or below the field position of the resonances typical for various components according to Orme-Johnson et al. [3].

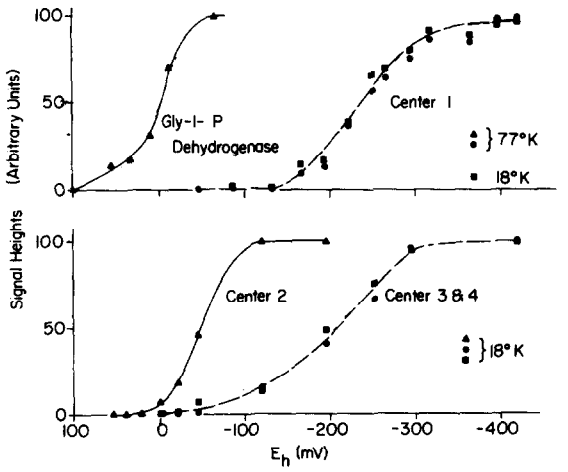


Fig. 2. The oxidation–reduction potential dependence of the EPR signals of the iron–sulfur proteins of *C. utilis* SMP. Experimental conditions as in fig. 1. At 77° K, the g = 1.94 signal was measured for both iron–sulfur proteins associated with glycerol-1-phosphate (and succinate) dehydrogenase and Center 1 in the NADH dehydrogenase. The principal absorption band (*r–sp*) was used at 18° K for the titration of both Center 1 and Center 2. Centers 3 and 4 were titrated using the signal heights of troughs *m* and *n*. The signal heights of different iron–sulfur proteins were normalized because of different temperatures and instrumental gains used.

potentials by approx. 60 mV. The half-reduction potential for the combined signal is approx. –240 mV more positive than the value –305 mV found for the mammalian and avian respiratory chains [8].

The titration of Center 2 was obtained by measuring signal (*p*) with the samples cooled to 18° K. The half-reduction potential of Center 1 is more negative than that of Center 2 and thus Center 1 is not reduced under these conditions and does not interfere with the titration of Center 2. Center 2 has a half-reduction potential at pH 7.2 of –50 mV and an *n* value of 1.0. This center is located on the substrate side of the site of piericidin A inhibition [2, 4, 13]. In contrast to the mammalian system, Center 2 in *C. utilis* is not reducible by glycerol-1-phosphate or by succinate. The EPR signal of Center 2 is very sensitive to the sample temperature and instrument power settings. The signal increases rapidly in size as the temperature is lowered and attains a maximum near 18° K. It is easily saturated as the

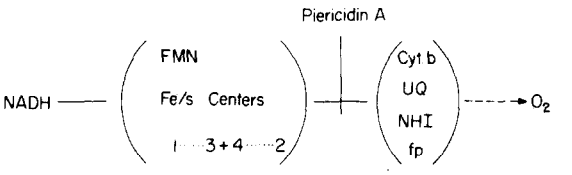


Fig. 3. Schematic representation of the respiratory chain in *C. utilis* submitochondrial particles in the region between NADH and cytochrome *b*. The half-reduction potentials of Centers 1, 3 + 4, and 2 are –240, –210 and –50 mV, respectively.

temperature is lowered below 18° K at 0.205 mW microwave power, the signal rapidly decreases in size.

As shown in the lower curve of fig. 2, Centers 3 and 4 are not readily separated in the potentiometric titration. The 2 have different half-reduction potentials, as indicated by an *n* value of less than 1, but the signal shapes and half-reduction potentials are so similar that separation is not possible. The half-reduction potential of the combined signal is approx. –210 mV.

As shown in our previous publication [2], all 4 iron–sulfur centers are located on the substrate side of the piericidin A inhibition site. As illustrated in fig. 3, the order of the half-reduction potentials of these iron–sulfur components is Center $1 < 3 + 4 < 2$ which is in agreement with the order found in mammalian systems of $1 < 4 < 3 < 2$, reported by Orme-Johnson et al. [3]. It is very interesting that there is a large gap, 190 mV, between the half-reduction potentials of iron–sulfur Centers 1 and 2. The same values for the half-reduction potentials of Centers 1, 2 and 3 + 4 were obtained in titrations of intact mitochondria from *C. utilis* as were found in titrations of the submitochondrial particles. In the respiration (state 4) of mitochondria with ethanol as a substrate, Center 1 is largely (approx. 80%) reduced while Center 2 is about 50% reduced, giving an oxidation–reduction potential difference of approx. 240 mV. This suggests that the energy conservation for site I phosphorylation occurs between Center 1 and Center 2.

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