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THE PROSTHETIC GROUPS IN SUCCINATE DEHYDROGENASE

NUMBER AND STOICHIOMETRY

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Summary

1. Succinate:Q oxidoreductase (EC 1.3.99.1) as present in beef-heart submitochondrial particles contains equal amounts of FAD, a [2Fe-2S] cluster and a [4Fe-4S] cluster. Both Fe-S clusters are reducible by succinate.

2. A second type of [2Fe-2S] cluster, called center S-2, that has been proposed to be present in purified preparations of succinate dehydrogenase and isolated Complex II (Ohnishi, T., Winter, D.B., Lim, J. and King, T.E. (1973) Biochem. Biophys. Res. Commun. 53, 231—237) is an artifact introduced by the purification procedure.

3. It is suggested that the 70 000 dalton subunit which is known to bind the flavin, accomodates also the [4Fe-4S] cluster whereas the 28 000 dalton subunit contains the [2Fe-2S] cluster.

Introduction

In 1954—1956 the first successful attempts to solubilize and purify the enzyme succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1) were published [1—3]. At the same time evidence was brought forward that tightly bound FAD and non-heme Fe are essential components of the enzyme. In these early preparations 4 Fe atoms were found per FAD molecule. In 1963 King [4] demonstrated that these preparations did not reconstitute with alkali-treated heart-muscle preparation in contrast to the enzyme extracted from membranes in the presence of succinate, and introduced the distinction between reconstitutively active and inactive preparations. The

reconstitution activity was found to be very labile towards oxygen and was improved by solubilizing the enzyme under anaerobic conditions [4].

It is now generally agreed that the reconstitutively active enzyme contains 8 atoms of Fe and acid-labile sulphur per mol flavin compared with 4—8 in reconstitutively inactive preparations [5—11]. In both types of preparations, the FAD is covalently linked to a histidyl residue of the protein via the 8α-CH_2 group of the isoalloxazine ring system [12].

In 1960 Beinert and Sands [13] discovered that in addition to a radical signal due to the semiquinone form of the FAD, the succinate-reduced enzyme exhibits a characteristic EPR signal that later was found to be typical for Fe-S clusters. Studies with ^{57}Fe ($I = 0.5$)-containing succinate dehydrogenase revealed that it is a $[2Fe\text{-}2S]^{1+(2+,1+)}$ cluster * [15]. Reports on the intensity of this signal initially stated that it correlates to a spin concentration equal to the flavin concentration [16] and that dithionite gives the same amount of signal [16—18]. More recent reports, however, show that although the signal intensity obtained with succinate agrees with the earlier observations, dithionite gives rise to a more intense signal in soluble preparations of the enzyme and Complex II [19—23]. The intensity of the dithionite induced signal is preparation-dependent. Whereas Ohnishi and coworkers [19,20] report values up to 2 for the intensity ratio of the signal induced with dithionite versus that induced by succinate, Beinert et al. [23] only find values of 1.4—1.5. The extra signal intensity, only obtained with dithionite, was ascribed to a second [2Fe-2S] cluster, cluster 2, by Ohnishi et al. [19,20] in addition to the cluster 1 that is reducible both by succinate and dithionite. The fluctuating quantities of cluster 2 and its low redox potential, which makes it useless as a redox carrier in the respiratory chain [5], led Beinert to question the status of this cluster [22,24].

A third EPR signal can be observed in the oxidized but not in the reduced form of Complex II and the reconstitutively active enzyme [21,25,26]. Its properties identify it as due to a $[4Fe\text{-}4S]^{3+(3+,2+)}$ cluster [15,26]. The concentration of this cluster 3 is at best equal to the flavin concentration [11,23, 26]. It is absent in reconstitutively inactive preparations [22,26].

The experiments reported in this paper deal with succinate dehydrogenase as present in isolated succinate:cytochrome *c* oxidoreductase [27] and beef-heart submitochondrial particles. It will be shown that in these preparations cluster 2 is absent and that the enzyme contains only one [2Fe-2S] cluster and one [4Fe-4S] cluster per FAD molecule. Part of this study has been presented in poster form at a symposium held in 1977 (Albracht, S.P.J. (1977) Abstr. No. 37, Int. Symp. Membrane Bioenerg., Spetsai, Greece).

Materials and Methods

Complex II-III (Succinate:cytochrome *c* oxidoreductase) was isolated from heart muscle preparation [28] essentially as described by Hatefi et al. [27]. Mg-ATP submitochondrial particles were prepared according to Löw and Vallin [29] and A-particles as described by Fessenden and Racker [30]. For the

* Nomenclature according to the NC-IUB recommendations [14].

samples used in Table II ten individual batches of submitochondrial particles were prepared from one batch of beef heart mitochondria suspended in 0.25 M sucrose and 50 mM Tris-HCl buffer (pH 8.0) by sonication for 2—4 min and differential centrifugation. When indicated the particles were incubated with 2 mM malonate for 30 min at 30°C to remove bound oxaloacetate from succinate dehydrogenase [6] and then washed twice. Hydrogenase from *Chromatium vinosum* was partly purified according to [31].

Covalently linked FAD was determined fluorimetrically exactly as described by Singer et al. [32] in a Perkin Elmer MPF-2A spectrofluorimeter using 452 nm light for excitation and monitoring the emission at 530 nm (band width 9 nm for both). The riboflavin concentration in the standard was determined using the extinction coefficients given by Koziol [33].

EPR spectra were recorded on a Varian E-9 EPR spectrometer connected to a HP 2100 computer via a data acquisition frontend septum based on a PDP 11-03 microprocessor. Spectra were stored on magnetic disc for later retrieval. Other experimental details were as described previously [34].

Signal quantitation was carried out by comparison of the experimental spectra with computer simulations. An example for the signals of the [2Fe-2S] and [4Fe-4S] clusters of succinate dehydrogenase in particles is given in Fig. 1.

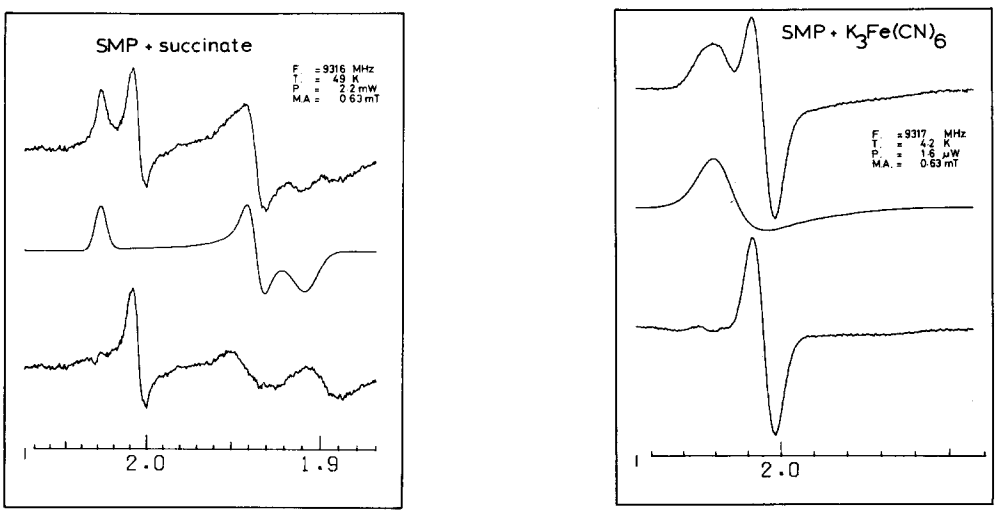

Fig. 1. Quantitation of the EPR spectra of the [2Fe-2S] and [4Fe-4S] clusters from succinate dehydrogenase as present in experimental spectra of submitochondrial particles. (A) (left panel) upper trace: particles in 0.25 M sucrose and 50 mM Tris-HCl buffer (pH 8.0) were incubated with 40 mM succinate for 15 min at 30°C and the EPR spectrum was recorded under the following conditions: microwave frequency (F), 9316 MHz; temperature (T), 49 K; incident microwave power (P), 2.2 mW; modulation amplitude (MA), 0.63 mT; scanning rate (SR), 10 mT/min. The modulation frequency for this and and other spectra was 100 kHz. The *X*-axis scale refers to *g* values. Middle trace: simulated spectrum for the [2Fe-2S] cluster of succinate dehydrogenase as reduced by succinate. The parameters used are in the legend of Fig. 2. Lower trace: difference spectrum. The criteria for a good subtraction are the absence of absorption peaks at the g_z position at 2.026 and at the g_x position at 1.91. (B) (right panel) upper trace: particles mixed with 0.4 mM $K_3FEe(CN)_6$ for 1 min at 0°C. EPR conditions: F, 9317 MHz; T, 4.2 K; P, 1.6 μM; MA, 0.63 mT; SR, 5 mT/min. Middle trace: computed spectrum for the [4Fe-4S] cluster of succinate dehydrogenase. For parameters see the legend of Fig. 6. Lower trace: difference spectrum. The criterium for a good subtraction is a flat base line on the left (low field) side of the radical signal.

The intensity of the simulation was varied until the difference spectrum no longer contained the characteristics of the signal considered. The double integral value of the simulations was used to compute the concentration of the clusters. A standard of 10 mM $Cu(ClO_4)_2$ was taken as a reference at both 49 K and 4.2 K. At the latter temperature an incident microwave power of only 250 nW had to be used to avoid saturation. It was experienced then that a 1 mM CU $Cu(ClO_4)_2$ solution, which is normally used as a standard, gave less reliable, scattering values for the double integral of its spectrum under these conditions, due to a poorer signal/noise ratio and base line. The intensity of the EPR signal of a copper perchlorate solution is proportional to the concentration in the region 1—20 mM.

All further manipulations with the spectra, including the quantitations, were carried out on a Tektronix 4010 computer display terminal connected to a HP 2100 computer. Simulation of EPR spectra was performed as before [34] on the HP computer.

Results

Succinate dehydrogenase in Complex II-III

The experiments reported in this paper were initiated by the observation shown in Fig. 2A: isolated Complex II-III reduced with succinate shows no enhancement of the EPR signal of cluster 1 of succinate dehydrogenase when dithionite is subsequently added. This is most clearly indicated by the area of

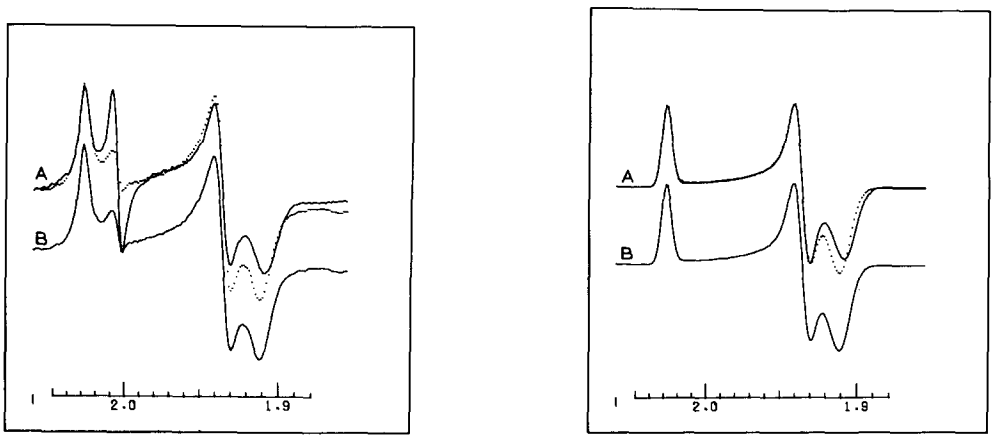

Fig. 2. Effect of dithionite on the EPR spectrum of Complex II-III reduced with succinate. A (left panel): Complex II-III (31 mg protein/ml) in 0.66 M sucrose and 50 mM Tris-HCl buffer (pH 8.0) was incubated with 60 mM succinate for 15 min at 30°C (trace A, solid line). A few grains of solid dithionite were then added and the incubation was continued for 5 min (trace A, dotted line and trace B). EPR conditions: F, 9130 MHz; T, 82 K; P, 20 mW; MA, 0.25 mT; SR, 10 mT/min. The gain is the same for both traces. B (right panel): computer simulations of the Fe-S signals of succinate dehydrogenase in the left panel. Parameters for trace A, solid line (corresponding to the analogous trace in the left panel): $g_{z,y,x}$ = 2.0264, 1.9351, 1.9074 and widths (z,y,x) = 1.16 mT, 1.4 mT, 2.43 mT. Parameters for trace A, dotted line and trace B (corresponding to the analogous trace in the left panel): $g_{z,y,x}$ = 2.0267, 1.935, 1.9105 and widths (z,y,x) = 1.16 mT, 1.51 mT, 2.27 mT. Both traces are adjusted to the same intensity (double intregral value).

the g_z line, that is independent of the reducing agent. The amplitude of the g_y line at 1.933 is increased slightly by addition of $Na_2S_2O_4$, probably because of reduction of the two Fe-S clusters [35,36] of contaminating outer-membrane fragments as indicated by the shape of the line at $g = 2.01$ in trace B of Fig. 2A. The slight increase in amplitude of the g_x line at 1.91 is due to several reasons. It is in part caused by the contribution of the signals of the outer-membrane Fe-S clusters and by a base line drift. In addition the position of the g_x line has shifted downfield and the line width is somewhat decreased, both of which effects cause the amplitude to increase at a constant signal intensity as can be seen in the simulated spectra in Fig. 2B.

The signals of the clusters 1 and 2 of QH_2:cytochrome *c* oxidoreductase [37] are very broad at the temperature used, but their contributions can be observed in the g_z region if one compares the simulations of the signals of succinate dehydrogenase with the experimental spectra (Fig. 2B versus Fig. 2A). The radical signal obtained with succinate is solely due to the semiquinone form of FAD as shown by the absence of any effect of extracting Q-10. Addition of dithionite without succinate gives the same spectrum as Fig. 2A, trace B. Less intense signals were obtained if succinate was mixed with the complex for only 30 s at 0—4°C, since no efforts had been made to previously activate the preparation.

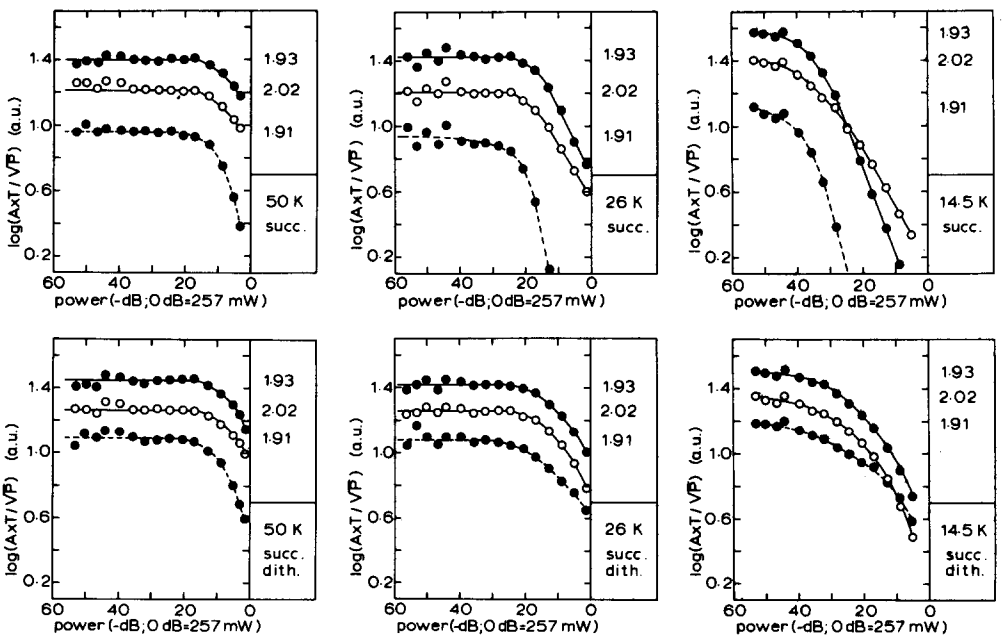

Fig. 3. Power saturation behaviour of the g_z, g_y and g_x lines of the EPR signal of the [2Fe-2S] cluster of succinate dehydrogenase and the effect of dithionite. The three panels represent power plots at 3 different temperatures: 50 K, 26 K and 14.5 K. Each panel contains two blocks displaying the behaviour of the signal of Fig. 2A, trace A, solid line (upper block) or Fig. 2A, trace B (lower block). Abbreviations and symbols: succ., succinate; dith., dithionite; A, amplitude; T, temperature in K; P, microwave power in mW; a.u., arbitrary units; ○———○, $g_z = 2.02$; ●———●, $g_y = 1.93$; ●- - - - -●, $g_x = 1.91$. A horizontal line means no saturation.

As Ohnishi et al. [5] reported that in reconstitutively active preparations the extra signal induced by dithionite could only be observed at $T < 20$ K, the temperature dependence of the line amplitudes of the signals in Fig. 2A was measured. This requires a knowledge of the power saturation behaviour of all lines at the various temperatures (Fig. 3), so that the temperature dependence can be measured at non-saturating microwave powers (Fig. 4). Especially from Fig. 3B and C it is obvious that with dihionite as reductant a greater microwave power is needed for saturation than with succinate as reductant, i.e. dithionite enhances the relaxation rate of the Fe-S cluster 1. This is in accordance with observations by other [5,23]. The enhancement of the spin-lattice relaxation time is not an artefact introduced by dithionite-reduction with hydrogen plus hydrogenase and benzylviologen has the same effect (Fig. 5). From Figs. 2A and 4 it is concluded that dithionite has no effect on the line amplitudes of the g_z and g_y lines of the signal of cluster 1, nor on the dependencies of these amplitudes on the temperature in the region 10-82 K. Even at 4.2 K no effect was seen when saturation was taken into account. Dithionite shifts the g_x slightly downfield and decreases its line width, but does not influence its temperature dependence. In fact this line behaves like a normal S = 0.5 system. There is thus no indication that cluster 2 is present in this type of preparation.

It becomes of interest then to know the absolute concentrations of the various prosthetic groups. For a determination of the concentration of the Fe-S clusters of succinate dehydrogenase in Complex II-III by the procedure described in Materials and Methods, a good fitting simulation of the signals of the Fe-S clusters is required. For spectra of the reduced enzyme the simulations displayed in Fig. 2B were used. The $g_{x,y,z}$ lines of the signal of cluster 1 of

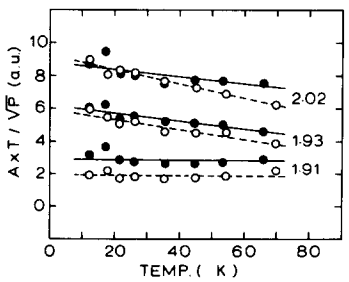

Fig. 4. Effect of temperature on the line amplitudes of the EPR signal of the [2Fe-2S] cluster of succinate dehydrogenase and the effect of dithionite. Line amplitudes were measured at 8 different temperatures at non saturating power, i.e. in the horizontal region of the plots of Fig. 3. Open circles refer to the behaviour of Fig. 2A, trace A, solid line, closed circles to that of Fig. 2A, trace B. Extra care was taken to measure the sample temperature in this case. A water-filled EPR tube was used in which two calibrated carbon resistors were placed one just below and one just above the measuring area of the EPR cavity. At each temperature this tube was inserted in the flow Dewar immediately after the sample tube had been remove and the mean value of the resistances of both resistors was used to compute the temperature difference between the sample and the temperature routinely read via a carbon resistor below the sample. The experimental conditions (helium flow speed) within the cavity were not changed during this readout, except that no microwave power was present. A S = 0.5 system will give a horizontal line in this type of plot if no line width changes take place due to relaxation broadening and no passage effects occur. The decrease in amplitude of the $g_z = 2.02$ and $g_y = 1.93$ lines towards higher temperatures is in part due to underlying lines of the clusters 1 and 2 of QH_2:cytochrome *c* oxidoreductase [37] that are known to broaden at these temperatures. Symbols used: A, amplitude:; T and temp., temperature in K; P, microwave power in mW; a.u., arbitrary units.

succinate dehydrogenase in Fig. 2A are so prominent that the parameters needed for the simulation (g values and widths of the lines) are easily obtained from and corrected to these experimental spectra. This is not possible for the spectrum of the [4Fe-4S] cluster. To ensure that maximal oxidation was obtained, $K_3Fe(CN)_6$ was added to the preparations, resulting in a relatively intense radical signal (see e.g. Fig. 1B). The radical is due to a semiquinone form of Q-10, as shown by the fact that it was absent in Q-10-free preparations. This signal made it impossible to obtain the simulation parameters from such a spectrum. The apparent width of the peak at $g = 2.02$ of the signal of the [4Fe-4S] cluster decreases noticeably on going from 13 to 4.2 K. The latter temperature was therefore always used to record spectra for quantitation of this Fe-S cluster. An experimental line shape, kindly provided by Dr. H. Beinert, of the [4Fe-4S] cluster as present in Complex II was used to obtain a

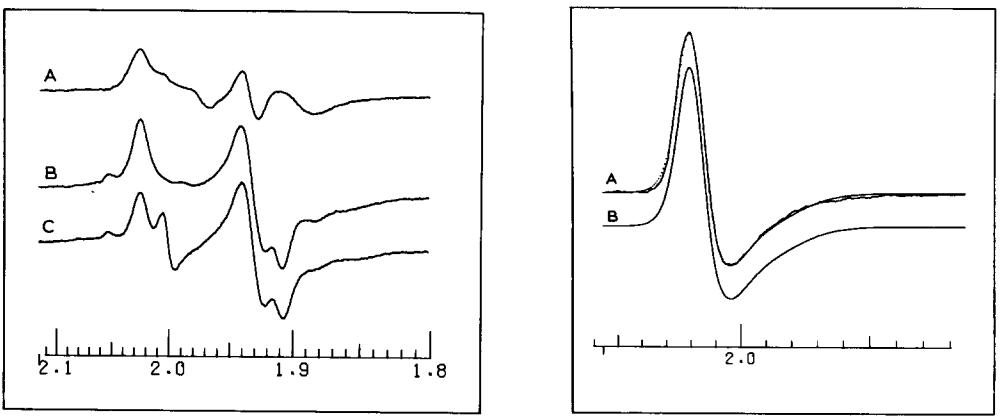

Fig. 5. Effect of low redox potentials on the spin-lattice relaxation of the [2Fe-2S] cluster of succinate dehydrogenase. Trace A: EPR spectrum of the same sample as used for Fig. 2A, Trace A, solid line. Trace B: same sample as used for Fig. 2A, trace B. Trace C: Complex II-III reduced with H_2, catalyzed by hydrogenase and 0.8 mM benzylviologen. Complex II-III was placed in a side arm of a H_2-filled Thunberg cell attached to an EPR tube. This arm was kept in ice-water. Another arm contained hydrogenase from *Chromatium vinosum* purified to a stage where no other EPR signals but that from its own Fe-S cluster could be detected. The amount of hydrogenase used gave an uptake of 27 μl H_2 gas/min at 30° C when assayed in a Warburg vessel with 5 mM methylene blue as acceptor. Benzylviologen was added to the hydrogenase and the mixture was kept at room temperature. After 10 min the hydrogenase was activated as seen from the purple colour of the reduced benzylviologen. After 1 h the contents of the two side arms were mixed, kept for 5 min at room temperature and then frozen in liquid nitrogen. The frozen mixture was still purple, indicating that its redox potential was equal to or less than that of benzylviologen (E'_0 = −350 mV). EPR conditions: F, 9331 MH_Z; T, 11 K; P, 151 mW; MA, 0.63 mT; SR, 50 mT/min. The gain for the traces A and B is the same. That for trace C is adjusted to correct for dilution. The radical signal in trace C represents reduced benzylviologen. The EPR conditions were chosen such as deliberately to saturate partly all the signals, so that relaxation differences are directly expressed in size and shape of the signals. Hydrogenase from *C. vinosum* exhibits no EPR signals in the reduced state [31].

Fig. 6. Computer simulation of the EPR spectrum of the [4Fe-4S] cluster of succinate dehydrogenase as present in isolated Complex II. (A) Trace A, solid line: experimental spectrum of Complex II. EPR conditions: F, 9206 MHz; T, 5.5 K; P, 0.3 μW; MA, 0.8 mT/SR, 10 mT/min. This trace was kindly provided by Dr. H. Beinert. (B) Trace A, dotted line and trace B: simulation. The parameters used were: $g_{x,y,z}$ = 2.01775, 2.01155, 1.99013 and widths (x,y,z) = 1.4 mT, 2.5 mT, 5.0 mT. No good fit could be obtained with $g_z > g_x$. The g values used are not necessarily the true g values for reasons explained in [52]. Each division on the g value scale is 0.01.

TABLE I

STOICHIOMETRY OF THE PROSTHETIC GROUPS OF SUCCINATE DEHYDROGENASE IN ISOLATED SUCCINATE-CYTOCHROME *c* OXIDOREDUCTASE

[2Fe-2S] represents the concentration of the clusters observed with succinate, [2Fe-2S] * the concentration observed with dithionite. Preparation No. 2 was used for the studies in Figs. 2—5.

Preparation No.	$\frac{[2Fe\text{-}2S]\ *}{[2Fe\text{-}2S]}$	$\frac{[4Fe\text{-}4S]}{[2Fe\text{-}2S]\ *}$	$\frac{[2Fe\text{-}2S]\ *}{FAD}$
1	1.1	1.0	1.3
2	1.1	1.0	1.1

good fitting simulation (see Fig. 6). Since Complex II contains no Q-10 [38,7] this shape is free from the disturbing radical signal.

The simulations of Figs. 2B and 6 were then used to quantitate the various signals of the clusters in the experimental spectra of Complex II-III and submitochondrial particles (see Materials and Methods). For two preparations of Complex II-III the relative concentrations of the prosthetic groups are given in Table I. All prosthetic groups appear to be present in the same concentration.

Succinate dehydrogenase in submitochondrial particles

Fig. 7 shows that also with particles no effect of dithionite was seen on the intensity of the signal of cluster 1 of succinate dehydrogenase as produced by succinate. The shift of the g_x to lower field is the same as noticed with Complex II-III as is the effect on the spin-lattice relaxation time. In Fig. 7 the amplitudes of the g_z at 2.025 and the g_y at 1.93 are most indicative for the absence of any effect of dithionite on the signal intensity. The Fe-S clusters of the mitochondrial outer membrane [39,36] are completely reduced by

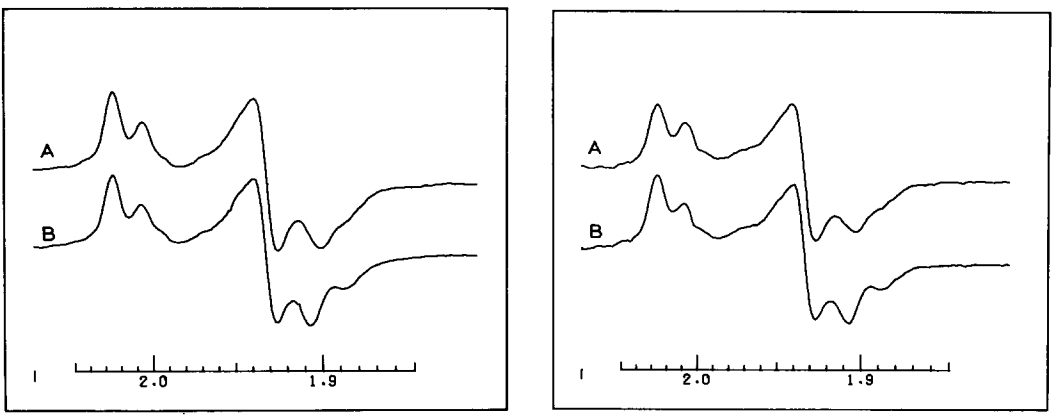

Fig. 7. Effect of dithionite on the EPR spectrum of succinate dehydrogenase in submitochondrial particles. A (left panel), trace A: Mg-ATP particles (45 mg protein/ml) in 0.25 M sucrose, 10 mM $MgCl_2$ were incubated with 60 mM succinate for 15 min at 30° C. Trace B: Mg-ATP particles reduced with dithionite for 1 min at 0° C. B (right panel) as A, but now for A-particles (37 mg protein/ml) in 0.25 M sucrose. EPR conditions: F, 9158 MHz; T, 81 K; P, 63 mW; MA, 1.25 mT; SR, 50 mT/min. The gain is the same for each pair of spectra.

TABLE II

STOICHIOMETRY OF THE PROSTHETIC GROUPS OF SUCCINATE DEHYDROGENASE AS PRESENT IN BEEF HEART SUBMITOCHONDRIAL PARTICLES

[2Fe-2S] represents the concentration obtained with succinate, [2FE-2S] * represents the concentration obtained with dithionite

Preparation No.	$\frac{[2Fe\text{-}2S]\ *}{[2Fe\text{-}2S]}$	$\frac{[4Fe\text{-}4S]}{[2Fe\text{-}2S]\ *}$	$\frac{[2Fe\text{-}2S]\ *}{FAD}$
1	0.95	1.02	0.99
2	1.06	0.95	0.93
3	1.20	1.00	1.04
4	1.21	0.97	1.02
5	1.24	1.04	0.88
6	1.15	0.92	1.33
7	1.21	0.99	1.10
8	0.92	1.11	0.95
9	1.00	1.22	0.91
10	1.34	1.00	1.07
Mean value *	1.13 (0.14)	1.02 (0.09)	1.02 (0.13)

* The number in parenthesis is the standard deviation of the data using N-1 weighting.

succinate under the conditions used as seen from the lines at 2.01 and 1.89. The clusters 1a and 1b of NADH dehydrogenase [34] are not reducible by succinate and their reduction by dithionite proceeds only very slowly at 0—4°C. It is noteworthy that dithionite had no detectable effect on the shape of the signal of succinate dehydrogenase in particles from the yeast *Candida utilis* (see Fig. 5 of Ref. 15). In the course of the present study the effect of dithionite on the relaxation behaviour of cluster 1 in these yeast particles was found to be similar to that of particles from beef heart and Complex II-III.

The concentrations of the prosthetic groups of succinate dehydrogenase has been determined in ten different preparations of submitochondrial particles. The results are given in Table II. It is clear that the total amounts of FAD, [2Fe-2S] cluster and [4Fe-4S] cluster are precisely the same and that cluster 2 is absent.

Attempts to induce cluster 2 and to understand the stoichiometry established for soluble preparations

Since it is clear that the ratio of the prosthetic group concentrations FAD/[2Fe-2S]/[4Fe-4S] is 1 : 1 : 1 in both particles and Complex II-III, the problem arises of correlating this with the many experimental data on soluble enzyme preparations of reconstitutively active succinate dehydrogenase [20—23] where a ratio FAD/[2Fe-2S]/[4Fe-4S] of 1 : 1.2—1.8 : $\leqslant$1 is found. Several possibilities are open:

(1) During the various purification procedures for the soluble enzyme, part of the FAD might be selectively destroyed along with the oxygen-labile [4Fe-4S] cluster.

(2) Part of the FAD might be solubilized in a form not precipitable by trichloroacetic acid, and would no longer be recognized as covalently linked FAD.

As with the first possibility, part of the [4Fe-4S] cluster might also be destroyed.

(3) The isolation procedure has no effect on the FAD, cluster 1 and the [4Fe-4S] cluster, but cluster 2 is EPR-invisible in preparations like the ones used in this paper and only shows up in EPR spectra once the enzyme is in a soluble form.

Undoubtedly the most drastic step in the various purification procedures for succinate dehydrogenase is the extraction of the enzyme from the mitochondrial inner membrane at pH values higher than 9 with or without the use of organic solvents or by treatment with KCN [4]. It was decided, therefore, to follow the behaviour of the prosthetic groups of the enzyme after treatment of malonate-activated particles in three different ways:

(a) Incubation of particles with 50 mM KCN at pH 8.0 and 30° C for 45 min. Under these conditions succinate dehydrogenase irreversibly dissociates from the membrane in a reconstitutively inactive form [40,41,18].

(b) Partial dissociation of the enzyme from particles at high pH in the presence of succinate and under exclusion of oxygen. This type of dissociation is reversed when the pH is lowered subsequently [4]. The soluble enzyme produced in this way is active in reconstitution.

(c) Dissociation of the enzyme from particles at high pH in air and in the absence of succinate. These conditions are known to severely inactivate the enzyme [4].

The results of these treatments on the FAD, the [2Fe-2S] clusters reducible

TABLE III

EFFECT OF KCN AND ALKALI ON THE PROSTHETIC GROUPS OF SUCCINATE DEHYDROGENASE IN SUBMITOCHONDRIAL PARTICLES

(B) KCN treatment: malonate-treated particles in 0.25 M sucrose, 50 mM Tris-HCl buffer (pH 8.0) were incubated in an EPR tube with 50 mM KCN (pH 8) for 45 min at 30°C. (C) Alkali treatment: particles were mixed with 40 mM succinate under a N_2 atmosphere. The suspension was then brought to pH 9.8 with 1 M NaOH and kept for 45 min at 0°C. Part of the suspension was then neutralized to pH 8.0 with 1 M HCl without precautions to exclude air. (D) Particles were treated as under (C) but in air and without added succinate. To determine the succinate-reducible [2Fe-2S] cluster concentration, samples were reduced with 40 mM succinate for 15 min at 30°C (B and D). The samples of (C) were directly used. The total amount of reducible [2Fe-2S] cluster, indicated in the table as [2Fe-2S] *, was determined after reduction of samples with a few grains of dithionate for 1 min at 0°C. To obtain the [4Fe-4S] cluster concentration, samples were mixed with 0.8 mM $K_3Fe(CN)_6$ for 1 min at 0°C. All concentrations given in the table are corrected for dilutions.

Mixture	Concentration (μM)				Ratio: [2Fe-2S] * / [2Fe-2S]
	[2Fe-2S]	[2Fe-2S] *	[4Fe-4S]	FAD	
A Particles, control	9.2	8.5	10.3	8.9	0.9
B(1) KCN, 0 min, 0°C	8.9	9.1	—	8.9	1.0
B(2) KCN, 45 min, 30°C	8.3	9.2	0	9.0	1.1
C(1) pH 9.8, 45 min 0°C, N_2	9.5	9.1	—	7.9	1.0
C(2) as C(1), then neutralized	6.5	6.9	—	8.2	1.1
D(1) pH 9.8, 45 min, 0°C, air	7.5	8.8	0	8.1	1.2
D(2) as D(1), then neutralized	4.0	6.3	0	8.0	1.6

by succinate or dithionite and the [4Fe-4S] clusters are summarized in Table III. All analyses were carried out on the total mixtures, so that any destruction of components or induction of new EPR signals can be observed directly.

The only effect that KCN brings about is a total destruction of the [4Fe-4S] clusters. Prolonged treatment of the enzyme at pH 9.8 seems slightly to affect the amount of detectable covalently linked FAD. In all cases a loss of about 10% was noticed. When succinate was present and oxygen was excluded, no effect was observed on the total concentration of the [2Fe-2S] clusters, nor on their reducibility by succinate. Neutralization of the mixture in air causes the loss of about 30% of these clusters, the remaining clusters still being fully reduced by succinate. The behaviour of the [4Fe-4S] clusters was not followed in this case since 40 mM succinate was present in the mixture. As expected exposure of particles to pH 9.8 in air completely destroyed the [4Fe-4S] clusters, but surprisingly the total amount of [2Fe-2S] clusters did not change and was nearly totally reducible by succinate. After neutralizing the mixture, considerable damage took place and only 70% was recovered. Since less than 2/3 of the [2Fe-2S] clusters could be reduced with succinate, the mixture D(2) from Table III indeed contained [2Fe-2S] clusters with the characteristics of cluster 2. From the absolute concentrations, however, it is easily seen that these are in fact clusters 1 that for some reason can no longer be reduced by succinate.

The effect of the critical first step of the Complex II preparation procedure [38] on the prosthetic groups was also studied (Table IV). Although the

TABLE IV

EFFECT OF THE FIRST STEP OF THE ISOLATION PROCEDURE OF COMPLEX II [38] ON THE PROSTHETIC GROUPS OF SUCCINATE DEHYDROGENASE IN SUBMITOCHONDRIAL PARTICLES

Particles were suspended in a supernatant, analogous to the 'Sharples effluent' of [38], originating from the first 15 000 × *g* centrifugation step in a routine isolation procedure of beef-heart mitochondria. After addition of 10 μM $CaCl_2$ the thick suspension was frozen at −20°C for 48 h. The suspension was then stored at 4°C overnight, a zero time control sample was withdrawn, 0.4 volume of 1 M potassium phosphate buffer (pH 7.4) was added and the mixture was incubated for 15 min at 38°C. Cholate was then added to a final concentration of 2.6% and at the indicated times samples were taken to measure the succinate-phenazinemethosulphate reductase reaction [6] at a fixed acceptor concentration (1 mM) and from a duplicate experiment samples were withdrawn for determination of the three prosthetic groups. The [2Fe-2S] clusters were determined after incubation with 40 mM succinate for 10 min at 30°C, the [2Fe-2S] * clusters after mixing with solid dithionite for 1 min at 0°C and the [4Fe-4S] clusters after mixing with 40 μM $K_3Fe(CN)_6$ for 1 min at 0°C.

Time (min)	Treatment	Activity (%)	Concentration (μM)			
			[2Fe-2S]	[2Fe-2S] *	[4Fe-4S]	FAD
0	control	100	—	—	—	9.0
0	+ phosphate	99	8.2	10.3	11.0	9.6
15	—	115	—	—	—	—
15	+ cholate	—	—	—	—	—
25	—	77	10.3	10.1	11.7	8.7
35	—	60	8.9	10.5	11.9	9.4
45	—	60	8.9	10.4	10.8	9.2
55	—	48	—	—	—	—
65	—	45	7.2	9.0	9.3	8.4

succinate-phenazinemethosulphate oxidoreductase activity dropped gradually to half the initial value, no change in the concentration of the three prosthetic groups, nor induction of cluster 2 was observed during the first 30 min in the presence of cholate. Since incubation for 15 min in the presence of cholate is sufficient for a successful isolation of Complex II [38], cluster 2 present in purified Complex II [20—23] must be induced later on in the procedure. It was not possible to check the effect on succinate dehydrogenase of the other drastic step in the Complex II isolation procedure namely extraction with organic solvents. Treatment of inner membrane preparations with 30% (v/v) ethanol and 50% (v/v) cyclohexane resulted in such a drastic effect on other metal-containing components of the preparation with the appearance of new intense signals, that quantitation of the EPR signals of succinate dehydrogenase was impossible.

The conclusion from the experiments in Tables III and IV is that FAD is neither destroyed nor solubilized. The [4Fe-4S] cluster is most labile and is easily destroyed by the KCN treatment or by oxygen at pH 9.8. The [2Fe-2S] cluster is much more stable and can survive at pH 9.8 in air for 45 min at 0°C. The possibility of an EPR-invisible [2Fe-2S] cluster can clearly be ruled out.

Solubilization of the enzymes thus gives no clue as to why the total amount of [2Fe-2S] clusters in the various purified preparations of succinate dehydrogenase is 1.2—1.8 times that of FAD. The procedures used in fact leave the ratio FAD/[2Fe-2S] = 1 : 1 intact. Separation of the soluble enzyme from the mixtures described in Table III was, therefore, undertaken. The results of an $(NH_4)_2SO_4$ fractionation of the material solubilized by KCN is shown in Table V. As expected [40,41,18] the 30—60% satd. $(NH_4)_2SO_4$ fraction contained the succinate dehydrogenase. The amounts of EPR signal induced by succinate and dithionite were the same, at both 50 and 11 K, with no sign of cluster 2. The flavin content of this fraction is about 50% higher than that of the [2Fe-2S] cluster, whereas the extracted particles contained 40% more [2Fe-2S] cluster than flavin. These ratios are more drastically changed in the fractions obtained after alkali treatment in air (Table V). In this case, the 0—30% satd. $(NH_4)_2SO_4$ fraction is particularly rich in FAD whereas the non-soluble fraction is rich in [2Fe-2S] clusters. In all fractions succinate could only partly reduce the [2Fe-2S] clusters and much of cluster 2 is induced. However, comparison with the results of the mixtures D(1) and D(2) of Table III shows that these clusters are in fact clusters 1 that can no longer be reduced by succinate, as the total amount of [2Fe-2S] clusters did not increase. The fraction precipitating between 30—50% satd. $(NH_4)_2SO_4$ has a solubility most closely resembling that of succinate dehydrogenase. Its composition is similar to that of the KCN enzyme, although less than 60% of the [2Fe-2S] clusters are reduced by succinate.

The third type of crude enzyme was obtained from heart muscle preparation in a way similar to the procedure described by King [4]. Again the total amount of [2Fe-2S] clusters (Table V, fraction C) was less than the FAD. Probably due to the presence of succinate and the absence of oxygen during the extraction of the enzyme and during subsequent neutralization of the extract, nearly all the [2Fe-2S] clusters remained reducible by succinate.

Table V shows that the firm attachment of the protein moieties to which the

TABLE V

THE STOICHIOMETRY OF THE PROSTHETIC GROUPS OF SUCCINATE DEHYDROGENASE IN VARIOUS $(NH_4)_2SO_4$ FRACTIONS OF KCN OR ALKALI TREATED SUBMITOCHONDRIAL PARTICLES

A and B: The KCN treatment was as in Table III(B), the alkali treatment as in Table III(D) but at pH 9.9, both now at a preparative scale. The extracted particles were precipitated by centrifugation (30 min, 100 000 × *g*) and washed once with 0.25 M sucrose, 50 mM Tris-HCl buffer (pH 8.0). The supernatant, that in the case of the alkali treatment was first neutralized to pH 7.5, was fractionated with solid $(NH_4)_2SO_4$. All fractions were taken up in the sucrose-Tris medium. C: heart muscle preparation [28] was washed 3 times with 50 mM potassium phosphate buffer (pH 7.5). Succinate (60 mM) was added and the suspension (about 10 mg protein/ml) was placed in a two-neck glass bottle under a helium atmosphere. It was heated to 30°C for 15 min under continuous stirring to activate succinate dehydrogenase and then cooled to 4°C. NaOH (1 M) was added until the pH was 9.95 and the suspension was centrifuged (30 min, 60 000 × *g*). The supernatant was neutralized to pH 7.7 under helium and then fractionated with solid $(NH_4)_2SO_4$ in air. The yellow fraction, precipitating between 30 and 50% satd. $(NH_4)_2SO_4$ was taken up in phosphate buffer. The incubations to determine the Fe-S cluster concentrations were as in Table III, except, for the $K_3Fe(CN)_6$ concentration which was 8 mM. The [4Fe-4S] cluster concentration in the 30—50% satd. $(NH_4)_2SO_4$ fraction was 3.3 μM.

Fraction	Concentration (μM)			Ratio	
	[2Fe-2S]	[2Fe-2S] *	FAD	$\frac{[2Fe\text{-}2S]^*}{[2Fe\text{-}2S]}$	$\frac{[2Fe\text{-}2S]^*}{FAD}$
A KCN solubilization					
A(1) Extracted particles	1.2	1.5	1.1	1.3	1.4
A(2) 0—30% $(NH_4)_2SO_4$	0	0	0.2	—	—
A(3) 30—60% $(NH_4)_2SO_4$	14.1	12.3	20.7	0.9	0.6
B Alkali solubilization, air					
B(1) Extracted particles	1.3	4.5	1.5	3.5	3.0
B(2) 0—30% $(NH_4)_2SO_4$	0.7	2.5	10.4	3.6	0.2
B(3) 30—50% $(NH_4)_2SO_4$	2.4	4.3	6.2	1.8	0.7
C Alkali solubilization, He					
C(1) 30—50% satd. $(NH_4)_2SO_4$	4.3	4.7	5.9	1.1	0.8

FAD molecules and [2Fe-2S] clusters are bound can be weakened under certain, rather drastic conditions, causing the appearance of fractions with a stoichiometry considerably deviating from FAD/[2Fe-2S] = 1 : 1. This probably explains the variable stoichiometries of these prosthetic groups reported in the literature for the purified soluble preparation of the enzyme.

Discussion

Some comments on the evidence for cluster 2 as described in the literature

The first detailed report on cluster 2 of succinate dehydrogenase [5] showed that reconstitutively inactive enzyme preparations exhibit 1.6—1.7 times more EPR signal with dithionite than with succinate, the total observable [2Fe-2S] cluster concentration being only 1.2—1.4 per flavin. The signal enhancement can be clearly observed at 30 K.

The data on the reconstitutively active enzyme are, however, less consistent. Whereas Fig. 1 of Ref. 5 shows a normal Curie behaviour for the line amplitudes of the dithionite induced signal, implicating that the normalized signal

intensity is independent of temperature, Table II of the same paper reports intensity values at $T \leqslant 12$ K that are 1.3—1.6 times those observed at 22 K. Since the change of line amplitudes can be more accurately determined than the change of the direct double integration value of an experimental EPR spectrum, the spin concentration data of Ref. 5 for the reconstitutively active enzyme at $T \leqslant 12$ K must be considered as incorrect. Since the line amplitudes at 33 K of the dithionite induced signal are only slightly greater than those of the succinate induced signal (Fig. 1 of Ref. 5), whereas the shape of both signals is comparable, the signal intensity obtained with both reductants is not much different. These data do not permit the introduction of a second [2Fe-2S] cluster in this type of enzyme. Likewise the presence of cluster 2 in pigeon heart mitochondria and submitochondrial particles of *Saccharomyces cerevisiae* claimed by Ohnishi et al. [5] using the same experimental conditions and criteria can be challenged.

Other investigators [21—23] have carried out more extensive EPR quantitation on the various types of soluble succinate dehydrogenase and Complex II. All enzyme preparations studied showed 1.4—1.5 times more signal with dithionite than with succinate. As the values of cluster 1 scatter around 1 per flavin Beinert et al. [23] clearly state that 'the signal intensity observed in any preparation of succinate dehydrogenase we have examined does not account for the presence of two reduced Fe-S centers'. These authors remark, however, that a definite conclusion about the absolute quantity is not possible since interactions of the kind demonstrated by Ohnishi et al. [5] might diminish the signal intensity.

This last reservation can now be withdrawn, since such spin-spin interactions could be only demonstrated in reconstitutively inactive preparations whereas the saturation characteristics of the succinate- or dithionite-reduced enzyme are independent of the status of the enzyme [23] and independent of the presence of succinate-nonreducible [2Fe-2S] clusters (Fig. 3 of the present paper). It is, therefore, assumed in the rest of this discussion that the quantitations carried out by Beinert et al. [21—23] and those reported by Ohnishi et al. [5] at temperatures above 20 K represent true concentrations of the [2Fe-2S] clusters. It then appears that the total amount of [2Fe-2S] clusters found by both groups agree quite well on the reconstitutively inactive enzymes and Complex II, but that Ohnishi et al. [5], as pointed out above, in fact find practically no cluster 2 in the enzyme active in reconstitution whereas Beinert et al. [23] do find some.

The stoichiometry of the prothetic groups as described in the present paper

It is obvious from Fig. 4 that the normalized intensity of the signal of succinate dehydrogenase as reduced by dithionite is independent of the observation temperature up to 70 K, provided that nonsaturating conditions are used. This confirms the observations of Beinert et al. [23]. From Fig. 2 and Table I but especially from Table II it is clear that the total amount of [2Fe-2S] cluster detectable in succinate dehydrogenase precisely equals the amount of covalently linked flavin, as does the amount of [4Fe-4S] cluster. That the signal obtained with succinate is 88% of the value obtained with dithionite is considered to be insignificant. This may be due to several causes such as some

damage of the succinate dehydrogenase, insufficiently prolonged incubation of the particles with succinate for complete activation of the enzyme or a small systematic error in the procedure used for quantitation, or a combination of these factors.

Under none of the circumstances used to solubilize the enzyme, did the overall concentrations of the FAD, the [2Fe-2S] clusters and the [4Fe-4S] clusters increase (Table III). This excludes the possibility of an EPR-invisible [2Fe-2S] cluster that would only be observable in the soluble enzyme. A high pH in air and in the absence of succinate causes the complete disappearance of the [4Fe-4S] clusters and partial damage of the [2Fe-2S] clusters, sometimes accompanied by a loss of reducibility by succinate/KCN only destroys the [4Fe-4S] clusters.

A rough fractionation of these mixtures (Table V) indicates that under some conditions protein fractions can be obtained where the [2Fe-2S] cluster content is 3 times that of FAD and other fractions where the FAD concentration is 4 times that of the [2Fe-2S] cluster. In these cases less than 30% of the [2Fe-2S] clusters were reducible by succinate. These findings make it likely that the [2Fe-2S] cluster is not attached to the same subunit as the FAD. This is strengthened by the finding that the EPR line shape of the [2Fe-2S] cluster, that very sensitively reflects small changes in the protein environment of the paramagnet, is not noticeably changed in these cases. Such a change would have been expected if the separation of the [2Fe-2S] cluster and the FAD into different fractions was the result of a cleavage, for some reason on another, of one subunit containing both groups. In this context it is noteworthy that the concentration of the [4Fe-4S] cluster in any fraction or preparation of succinate dehydrogenase in this study or in others [11,21—23,25,26] never exceeds that of FAD.

Since all preparations of the soluble enzyme contain only 2 subunits, the following location of the Fe-S clusters is proposed. The 28 000 dalton subunit (further called subunit B) contains the [2Fe-2S] cluster, whereas the [4Fe-4S] cluster is located on the 70 000 dalton subunit (called subunit A) that also binds the FAD [8]. The isolated subunit A is known to contain 4Fe and 4 acid-labile S atoms per FAD molecule [8]. The native enzyme molecule is of the form AB, but the soluble preparations described contain 1.2—1.5 times more subunit B than A. The extra B subunits are probably associated with the AB molecules to form ABB^* molecules, since it is unlikely that a molecule with a molecular weight of 30 000 will copurify with one of 100 000 in an extensive purification procedure. The B^* subunit is not enzymically active and its [2Fe-2S] cluster is only reducible by dithionite. The amount of succinate reducible [2Fe-2S] cluster will then be equal to the amount of FAD as has been experimentally found for all purified soluble preparations [20—23]. The ABB^* type of molecule will originate from an isolation step, such as an alkaline extraction, in which the AB molecule had the chance to dissociate. This proposal is supported by the experimental findings of Coles et al. [42,43] that the stoichiometry of the subunits A and B in soluble forms of succinate dehydrogenase is preparation dependent, the ratio B/A being between 1 and 2.

Summarizing the present paper shows that native succinate dehydrogenase has the same number of each the groups FAD, the [2Fe-2S] cluster and the

[4Fe-4S] cluster. Care should be taken to interpret any experiments carried out with preparations of the enzyme having a different prosthetic group composition.

Effect of a low redox potential on the spin-lattice relaxation of Fe-S cluster 1

As argued above, spin-spin interaction of the succinate-reducible- and non-reducible Fe-S clusters, as demonstrated by Ohnishi et al. [5], cannot be the reason for the relaxation enhancement of cluster 1 at low temperatures, in preparations where the succinate-non-reducible cluster is absent. The only redox group known to be reduced further by dithionite is the flavin. Succinate brings about only a 15—30% decrease of absorption at 460 nm in the optical spectrum of soluble enzymes, whereas with dithionite this decline is more than double this amount [3,44—46]. The intensity of the radical signal induced by succinate suggests that the flavin is reduced to the semiquinone form [22], It is also known that activation of the enzyme by reduction as first reported by Klaasse and Slater [47], takes place at a redox potential in the range where the protein bound histidyl-flavin is expected to become fully reduced [48]. This reductive activation has also been demonstrated in particles [49].

These facts strongly suggest that the only effect of a low redox potential is the complete reduction of the flavin. This causes appreciable changes as is apparent from the drastically lowered affinity of oxaloacetate for the enzyme [48,49]. It is proposed here that these changes in the protein structure are also responsible for the altered temperature dependence of the spin-lattice relaxation time of Fe-S cluster 1 and the small change in EPR line shape of this cluster. The changes in protein structure are quite likely triggered by the geometry of the isoalloxazine ring system of the flavin, which is flat in the oxidized but bent in the reduced form [50]. It must be noted that these changes are probably not physiological since no effect on the spin-lattice relaxation can be observed in particles or beef heart mitochondria reduced with NADH or NAD-linked substrates. This indicates that the semiquinone form of the FAD in the enzyme is flat. The proposal is in line with the conclusions of Gutman and Silman [49] that activation of succinate dehydrogenase by reduced ubiquinone does not proceed via reduction, but does not fit the detailed mechanistic view of Gutman (Scheme IV of Ref. 51) that is based on the assumption that succinate can reduce the histidyl-flavin to a bent conformation.

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