Review Letter

The prosthetic groups of succinate dehydrogenase: 30 years from discovery to identification

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Recent studies using magnetic circular dichroism at cryogenic temperatures, electron paramagnetic resonance (EPR) and linear electric field effect-EPR (LEFE) of succinate dehydrogenase in membranes and in soluble, homogeneous preparations demonstrated the presence of 3 different Fe-S clusters in the mammalian enzyme, as well as in a similar bacterial enzyme, fumarate reductase from *Escherichia coli*. There is one each of the 2Fe, 3Fe, and 4Fe clusters. Thus, succinate dehydrogenase is the first enzyme which has been shown to contain all 3 of these Fe-S clusters. The enzyme also contains 1 mol 8α-[*N*(3)-histidyl]-FAD. It has taken the combined expertise of many laboratories and 15 years of effort to identify the flavin component, and nearly 3 decades to identify the Fe-S clusters. The data from physical methods appear to be internally consistent, in harmony with the results of chemical analysis, and provide a rational explanation for earlier results by the cluster extrusion method. There remains, however, a number of interesting and substantive questions for future investigations. This review traces the tortuous path, the many pitfalls and false leads, which have led us from the discovery of nonheme iron and 'bound' flavin in the enzyme to elucidation of their structures.

1. INTRODUCTION

Some 30 years have passed since the discovery of covalently bound flavin [1], nonheme iron [1], and of labile sulfide [2] in succinate dehydrogenase. The presence of iron and of an unusually tightly bound form of flavin was recognized as a result of analytical studies on the first purified, soluble preparations of the enzyme [3], while the recognition of labile sulfide may be ascribed to serendipity and poor ventilation in our subterranean laboratory.

The central role which succinate dehydrogenase plays in metabolism, coupled with the fascination that bioenergetics held for many biochemists in the ensuing decade attracted many able investigators to the study of the structure of this enzyme, particularly of its prosthetic groups. Yet, the time was not right: the methods which eventually succeeded in unraveling their structure were not yet available to most investigators and apparatus to utilize some of these methods had not even been invented. Thus, many years of well designed and carefully executed experiments succeeded in unraveling only the fact that the FAD moiety was covalently bound to the peptide chain by way of some part of the isoalloxazine ring system, rather than the side chain [4], but the structure eluded identification until ENDOR and EPR techniques and knowledge accrued from their application to model flavins was brought to bear on the problem [5,6]. It took such techniques to pinpoint the site of attachment to the 8α-CH_3 group and eventually modern chemical methods to demonstrate that the substi-

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tuent was histidine via the *N*(3) group of the imidazole [7]. But all this would not have been possible in the 1950's, because the techniques, including those used for the purification of peptides, had not yet been fully developed.

It took much longer to arrive at an understanding of the number of Fe-S clusters in succinate dehydrogenase and their structures. Only now do we seem to be in a position to provide a rational picture of the different clusters which explains the many seemingly irreconcilable observations. As will be apparent, one reason why it took almost 3 decades to arrive at a harmonious description is that the 3 different Fe-S clusters present complicate the identification because of interference in the physical procedures used, so that it took several magnetic resonance methods, some of which have only recently become available, to sort out the data and provide an unambiguous answer. Second, the chemistry of Fe-S cluster interconversions is a very new science. The possible events have been documented only in the past year or two. Lastly, perhaps the very fact that a large number of laboratories have focused their attention on the problem delayed a solution: there were too many conflicting observations and changing interpretations to explain and rationalize before an answer could emerge.

2. PREPARATIONS AND RELEVANT PROPERTIES

It is widely agreed that the properties of succinate dehydrogenase in inner membrane preparations reflect those of the pristine enzyme: it is stable to O_2, shows high Q reductase activity which is inhibited by carboxin and thenoyltrifluoroacetone, and has a high turnover number in the succinate-phenazine methosulfate (PMS) assay. Unfortunately, such preparations do not lend themselves to the application of some of the most useful analytical procedures because of their complexity. Thus, the presence of other Fe-S enzymes complicates the interpretation of EPR data, while cytochromes interfere with the MCD of Fe-S clusters. Hence, purified preparations have been used for the characterization of these clusters. The most useful ones in this regard are Complex II, a membraneous preparation, originally called succinate-Q reductase [8], and the homogeneous, soluble enzyme [9], particularly the fully reconstutively active one [10], i.e., a preparation in which Fe-S center 3 is intact, so that the enzyme can be fully reinserted into membranes, with regain of all of the properties characteristic of the enzyme in the inner membrane [11].

It is important to recall that the properties of the dehydrogenase are quite different in these preparations than in the inner membrane in several regards and that Complex II and the soluble enzyme also show different properties. The dehydrogenase contains 2 subunits of ~70 and ~30 kDa, respectively, the former containing the covalently bound flavin and substrate site [9]. In Complex II 2 small peptides are also present [12], one of which is thought to be cytochrome *b*. These confer O_2 stability and reactivity with Q to the enzyme [13], but the turnover number of the dehydrogenase in the PMS assay is much lower than in the inner membrane, and the hemeprotein renders it unsuitable for MCD studies of the Fe-S clusters. The soluble enzyme which may be extracted from it has a higher turnover number (but not as high as in the inner membrane) [10,11]; it does not reduce Q, and is very labile, so that exposure to O_2 results in partial inactivation and destruction of Fe-S center 3 [14]. It recombines fully with the small peptides, effectively reconstituting Complex II. Pure, soluble preparations are excellently suited for analytical, cluster extrusion, and MCD work, but the oxidative lability of the Fe-S center 3 impedes EPR studies, since an EPR signal can only be detected in oxidized samples. The different turnover numbers of the enzyme in the inner membrane, in Complex II, and in the soluble form have led to the suggestion that the enzyme assumes different conformations in each. This may be relevant in explaining their different behavior in EPR studies.

3. STOICHIOMETRY OF Fe, S, AND FLAVIN

In all of the early preparations extracted aerobically by various methods from beef heart [15,16] or yeast mitochondria [17] the ratio of Fe to covalently bound flavin was 4:1. In later years the fact that Fe is normally associated with S in unique clusters was recognized. Preparations isolated subsequently, including Complex II [18,19], the perchlorate-extracted preparation of Davis and Hatefi [9], and the fully reconstitutive

active butanol enzyme [10] were found to have Fe:S:flavin ratios close to 8:8:1. The average value of many preparations obtained by the butanol procedure [10] in this laboratory is 8.3 ± 0.5 mol Fe per mol flavin [20].

The earlier finding of 4 irons per flavin in the aerobically isolated enzyme in several laboratories remains puzzling, for even though center 3 of the enzyme is destroyed on exposure to air, its iron moieties are not known to be lost from the protein. Thus, the perchlorate-extracted enzyme contains 8 gatom Fe and S [9] but only 15% of center 3 if the preparation is intact [9,10].

4. CLUSTER EXTRUSION

Nonheme iron and labile sulfide in proteins are arranged in clusters. In eukaryotic cells the types of cluster so far identified are [2Fe-2S], [3Fe-xS], and [4Fe-4S]. Among these, the occurrence of trinuclear clusters has only recently been recognized and their structural aspects and acid-labile sulfide content (x = 3 or 4) remain controversial [21,22].

Determination of the type of cluster present used to be based almost exclusively on chemical analysis and EPR data until, in the 1970's, the cluster extrusion (core extrusion) method, introduced by Holm's laboratory, gained prominence [23]. The method seemed straightforward to many of its users, including the authors, since the possibility of cluster conversions under the conditions of protein unfolding used in this procedure was not discovered until several years later [24].

Since EPR data had not yielded an unambiguous answer to the question of the number and type of Fe-S clusters present in succinate dehydrogenase, we turned to the cluster extrusion method for an answer [25]. Two sophisticated variants of the method showed the presence of one [4Fe-4S] and two [2Fe-2S] clusters in homogeneous and fully reconstitutively active preparations. These results were in gratifying agreement with the results of chemical analyses of this type of preparation (8Fe:8S:1 flavin) and seemed to be also in harmony with the interpretations of EPR studies, which suggested the existence of 2 ferredoxin type binuclear clusters and of one 'HiPIP' type tetranuclear cluster in the enzyme [26].

5. EPR SIGNALS AND THEIR INTERPRETATIONS

Much of the past controversy concerning the Fe-S clusters of the enzyme has centered around conflicting observations and interpretations of EPR studies, but they are also the cornerstone of the eventual understanding of the prosthetic groups of the enzyme.

The first demonstration that an asymmetric EPR signal with a major absorption at g = 1.94 develops on the reduction of fragments of beef heart mitochondria by succinate was reported in 1960 [27,28]. These measurements were made at 77 K, where only [2Fe-2S] clusters in the paramagnetic (+1) oxidation state are detected. The signals of [4Fe-4S] clusters in either the +1 or +3 state, or of oxidized [3Fe-xS] clusters, show considerable broadening at temperatures >20 K, so that these clusters were discovered and studied only many years later, when liquid He techniques became available.

These pioneering studies of Beinert and Sands are a milestone in the history of bioenergetics and in the understanding of the structure and function of metalloproteins. Up to that time, measurements of the redox state of the Fe in what was then termed 'nonheme iron proteins' and, hence, conclusions concerning their role in catalysis, had been based on colorimetric measurement of the Fe^{2+} and Fe^{3+} ratio after denaturation, a technique which we pointed out to be fundamentally unsound as far back as 1957 [29].

The next steps were the development of technology for signal measurement at temperatures <20 K, which permitted detection of the EPR signal of an additional Fe-S cluster (center 3) of the enzyme [30,31] and the advent of freeze-quench techniques, which paved the way for the kinetic studies required to show that the oxidation-reduction of a given cluster occurs at rates compatible with the catalytic events.

The second EPR signal of the enzyme, noted by Ohnishi et al. [30] in soluble preparations and by our group [31] in Complex II and other particles, was relatively isotropic, rapidly relaxing and centered at g = 2.01 (g = 2.015, 2.014, 1.990). It was present only in the oxidized form of the enzyme, disappearing on reduction with succinate. Its potential was stated to be 60 mV in sub-

mitochondrial particles and considerably higher in mitochondria. The properties of this new cluster were reminiscent of the HiPIP protein of *Chromatium* [32] and were, consequently, considered to represent a [4Fe-4S] cluster in the +3 state. This assumption seemed to be reinforced by the fact that the aerobically isolated 4Fe preparations lacked this cluster, but it was present in the anaerobically prepared enzyme, which was thought to contain 8 Fe. This second cluster, denoted as 'center 3' or 'HiPIP cluster' in the literature, as well as center 1, were found to be present in amounts nearly equal to that of the flavin in Complex II. In the soluble enzyme center 1 was still present in near stoichiometric amounts in all preparations studied, but center 3 was absent if the enzyme was isolated without succinate and present in well below stoichiometric amounts if isolated with succinate [26].

The explanation became clear when it was found [33] that center 3 was extremely labile to O_2 in the soluble enzyme, so that its EPR signal decayed at the same rate as 'reconstitution activity', i.e., the potential to be reincorporated into the respiratory chain (fig.1). A third property which decayed at the same rate was the 'low K_m' ferricyanide activity, discovered shortly before by Vinogradov et al. [34], which represented the oxidation of succinate by very low concentrations of $Fe(CN_6)^{3-}$, a function of center 3, since it is only seen in pristine soluble preparations, where the center is exposed, but not in membranes, where it is buried and thus

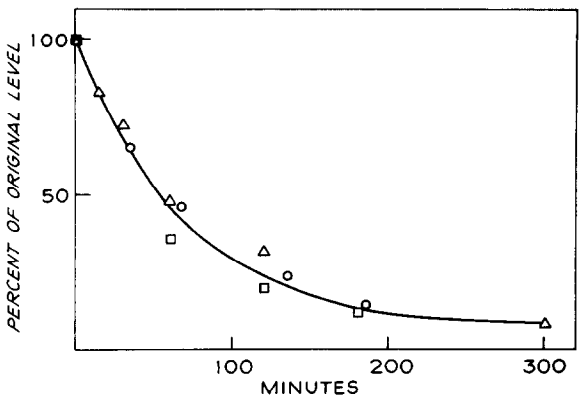

Fig.1. Decay of the reconstitutively active enzyme at 0°C on contact with air. (○) Reconstitution activity, (□) low K_m ferricyanide reductase activity, (△) EPR signal at g = 2.01. From Beinert et al. [14], by permission.

inaccessible to impermeable $Fe(CN_6)^{3-}$. Since the reconstitutively active preparations, i.e., those possessing center 3 preserved during isolation of the enzyme, had to be extracted and processed in anaerobiosis with succinate present, center 3 was in the reduced (EPR-silent) state, but chemical oxidation by agents such as ferricyanide tended to destroy this cluster. A simple means of overcoming this dilemma which has proved useful [35] is the incorporation of the anaerobically isolated (i.e., reduced) enzyme into the inner membrane, followed by exposure to O_2 to oxidize all the Fe-S clusters. This permitted us to quantitate center 3 of the soluble enzyme by EPR and showed that reconstitutively active preparations contain up to 1.0 mol center 3 [35].

A study of the kinetics of the reduction of centers 1 and 3 in Complex II and soluble preparations revealed that not all of the center 1 and 3 population was reduced within the turnover time of the enzyme [26]. The same conclusion was reached on studying the reoxidation of these centers by DPB (2,3-dimethoxy-5-methyl-6-*n*-pentyl-1,4-benzoquinone, a Q_1 analogue, which replaces it quantitatively in steady-state assays) in Complex II [35]. While the reason for these puzzling observations is still not clear, they firmly established that the rate-limiting step in the catalytic cycle is the reduction of these centers by the substrate, not their reoxidation.

The focus of much of the disagreement about succinate dehydrogenase is the third Fe-S cluster, first described by Ohnishi et al. [36] for partially reconstitutively active preparations. They reported that on reduction with dithionite a signal at g ~1.93 appears, with an intensity twice that elicited by reduction with succinate, with a greatly enhanced spin relaxation. The new signal, which became known as center 2, was thought to be detectable only below 20 K and to have a very low mid-point potential (−400 mV). The signal was ascribed to an additional binuclear cluster.

The EPR characteristics of this third cluster were studied in depth by Beinert et al. [26,33], who confirmed the fact that dithionite caused further intensification of the center 1 signal elicited by succinate but found that this was accompanied by a change in line shape and could be seen in Complex II and reconstitutively active, as well as inactive preparations, even at a temperature of 100 K.

They also found that the signal intensity after dithionite was only 20–50% higher than after succinate, rather than double. This behavior was ascribed at the time to spin coupling, a view shared by Ohnishi's group.

The seeming agreement between EPR data, indicating the presence of 2 [2Fe-2S] and of one [4Fe-4S] clusters in the enzyme with the analytical data showing 8 Fe and 8 S, as well as the result of cluster extrusion, was gratifying. There followed a rare period of consensus among workers in the field, terminated by Albracht's report [37] that in his EPR experiments with membranes he could find no sign of center 2 and that, therefore, succinate dehydrogenase may be a 2 cluster enzyme, containing 6, not 8, atoms of Fe and labile S. Significantly, Albracht [37] also could not detect center 2 in the only homogeneous soluble preparation which is 100% active in reconstitution tests [10]. This prompted Albracht to conclude that center 2, when seen, is a preparative artifact. The marked difference in the EPR relaxation properties of center 1 between the succinate and dithionite reduced enzyme, which has been observed with all types of preparations, would still require an explanation, even if center 2 did not exist.

In order to reconcile these views with the analytical data, Albracht [37,38] made several assumptions. One of these was that center 3 must be on the 70 kDa subunit and center 1 on the smaller one. While the location of the various clusters has not been firmly assigned to the 2 subunits, as discussed below, there is suggestive evidence to the contrary. This argument and some internal inconsistencies in the 6Fe hypothesis have been reviewed by one of us [20,39,40]. Another assumption, one which is no longer tenable, is that soluble succinate dehydrogenase contains a 2-fold excess of the 30 kDa subunit over the 70 kDa protein. The basis of this postulate was an earlier report [41,42] that the subunit ratio for various types of soluble preparations based on separation by molecular sieving or gel electrophoresis and various colorimetric procedures was not the expected 1:1 but 1.35 to 1.5 in favor of the smaller subunit. At no time was a ratio of 2 observed, however, as would be required to rationalize the finding of 8Fe and 8S in a postulated 6Fe-6S enzyme.

Only recently has the true explanation emerged for the finding of a subunit ratio greater than 1 (unpublished data of B.A.C. Ackrell, quoted in [40]). It emerged that the behavior of the 70 kDa subunit in Coomassie blue staining is anomalous, deviating from Beer's law at an unexpectedly low concentration, while the staining intensity of the 30 kDa subunit remains proportional to concentration over a much wider range. Thus, only at very low protein concentrations, as had to be used with Complex II, did the staining reaction correctly reflect the weight ratio of the proteins. Coupled with this, the molecular absorbance of the 30 kDa subunit in either the biuret or Lowry reactions is abnormally high, leading also to an apparent excess of the 30 kDa protein. This combination of circumstances, and not overloading of gels in the usual sense, as incorrectly stated [38], explains the unequal subunit ratios reported earlier. These findings remove the experimental basis for the attempt to reconcile the analytical data on the Fe and S content of the enzyme with the assumption that only a binuclear and a tetranuclear cluster are present [37].

6. DISCOVERY OF THE 3Fe CLUSTER

The magnetic field dependence of the linear electric field effect (LEFE) in EPR spectroscopy, particularly in conjunction with measurement of the continuous wave EPR spectra properties, have recently been shown to distinguish 2-, 3- and 4Fe clusters [43]. The LEFE method is particularly useful when several types of clusters are present in a given sample. It was recently applied to air-oxidized Complex II [44]. The curves obtained in LEFE studies closely resembled those previously seen [43] in proteins containing 3Fe clusters (aconitase, glutamate synthase, *Azotobacter* ferredoxin) but differed substantially from results obtained with 2Fe clusters (plant ferredoxin) as well as from the HiPIP type, $[4Fe\text{-}4S]^{2+,3+}$ and bacterial ferredoxin type ($[4Fe\text{-}4S]^{2+,1+}$) clusters.

Peisach et al. [44] suggested, therefore, that center 3 is a [3Fe-*x*S] type, a suggestion later confirmed by MCD. They also proposed that the 3Fe cluster arises by oxidative degradation of a [4Fe-4S] cluster, as in aconitase, and was perhaps very rapidly built up in reducing conditions to a [4Fe-4S] cluster, which would be the catalytically active form. As will be shown, MCD data argue

against such a hypothesis and show that a trinuclear cluster, as such, is present in the catalytically active form. Perhaps the most convincing evidence to date that the 3Fe cluster is not an artifact of the isolation procedure came from the recent demonstration that this cluster is present in vivo in fumarate reductase from *E. coli* [45]. This enzyme is very similar to mammalian succinate dehydrogenase, having the same subunit and Fe-S cluster composition (as shown by EPR and MCD studies [46,47]). Techniques were at hand to amplify the gene expression for this enzyme, so that the signals of the 3Fe clusters of the enzyme were distinctly seen in EPR experiments on whole cells.

7. MCD REVEALS THREE TYPES OF Fe-S CLUSTER IN SUCCINATE DEHYDROGENASE

The identification of center 3 as a 3Fe cluster [44] left us with several major uncertainties. Is there a low-potential center 2 in the enzyme? If so, why is it detectable by EPR in Complex II and some soluble preparations but not in inner membrane or in the most nearly intact soluble enzyme [10]? As mentioned above, the LEFE experiments also posed the question of whether the catalytically active form of the enzyme might not contain a [4Fe-4S] cluster, which breaks down to a [3Fe-*x*S] form during isolation. Definitive answers to these questions called for a new approach. Low-temperature MCD seemed well suited to resolve remaining uncertainties [48], since controversies about the Fe-S cluster composition of the dehydrogenase concerned the existence of paramagnetic centers which were EPR silent because of assumed weak coupling between clusters or zero field splitting. Such electronic ground state perturbations would not be expected to prevent the observation of temperature-dependent MCD spectra.

In deconvoluting the complex MCD spectra, Johnson et al. [48] took advantage of 2 unique properties of the enzyme: (1) that center 2 becomes paramagnetic on reduction with dithionite but not with succinate and (2) that preparations with and without center 3 may be obtained depending on whether or not succinate is present during isolation. Thus, the MCD spectrum of center 3 may be obtained by comparing the MCD spectra of the reconstitutively active enzyme [10] with perchlorate-extracted preparations isolated under argon but without succinate, so that it has very little low K_m ferricyanide or reconstitution activity.

Fig.2 shows the MCD spectra of the 3 clusters obtained as follows. The spectrum of center 1 was obtained by subtracting the spectrum of succinate-reduced perchlorate enzyme (cf. above) from that of an untreated sample of the preparation. The spectrum resembles that of other reduced [2Fe-2S] proteins and confirms that center 1 is binuclear. The spectrum of center 2 is the difference between

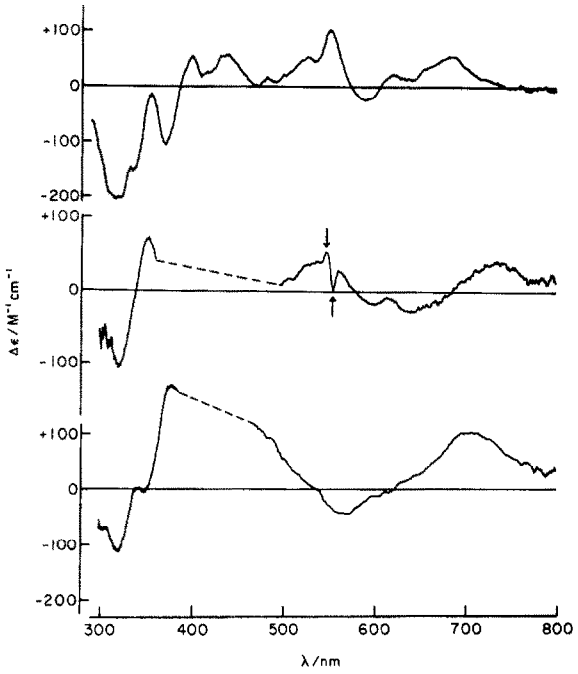

Fig.2. MCD spectra of reduced centers 1, 2, and 3. Conditions: temperature, 4.22 K; magnetic field, 4.5 T. (top) Succinate-reduced minus as isolated perchlorate-extracted enzyme (extracted *without* succinate). Corresponds to reduced center 1. (middle) Dithionite minus succinate-reduced butanol enzyme (reconstitutively fully active). Corresponds to reduced center 2. Similar results were obtained with the perchlorate-extracted enzyme. Arrows indicate derivative due to a trace of cytochrome *b* impurity. (bottom) Succinate-reduced butanol enzyme minus spectrum of reduced center 1. Corresponds to center 3. Dashed lines represent regions where MCD from heme impurity overlaps. From Johnson et al. [48], by permission.

dithionite- and succinate-reduced samples. The characteristic broad positive peaks at ~730 nm and between 560 and 410 nm and the negative trough centered at 630 nm are characteristic of $[4Fe\text{-}4S]^{1+}$ clusters, as seen in bacterial ferredoxins and are clearly distinct from features of reduced [2Fe-2S] clusters [48]. On this basis the authors concluded that center 2 is a $[4Fe\text{-}4S]^{2+,1+}$ cluster. The spectrum of center 3 was obtained as the difference in the MCD spectra of reconstitutively active and nearly inactive preparations. The form of the difference spectrum is reported [48] to be typical of [3Fe-*x*S] clusters. The presence of a 3Fe cluster was also indicated by its unique MCD magnetization properties. Although the number of reduced [3Fe-*x*S] clusters that are responsible for the MCD spectrum cannot be accurately assessed, the results do suggest that in the fully active enzyme bulk conversion to a [4Fe-4S] cluster does not ensue on addition of substrate. Very similar MCD results have recently been reported for the fumarate reductase complex and soluble enzyme from *E. coli* [46].

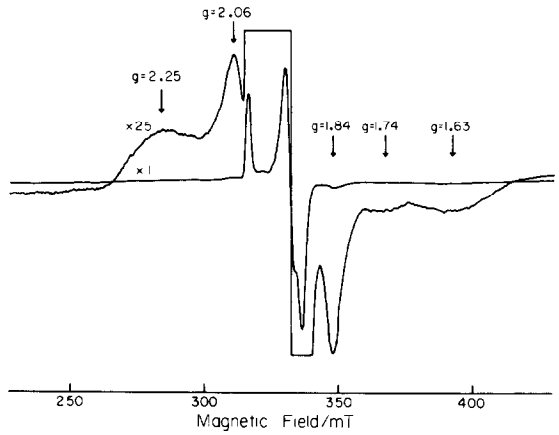

Fig.3. EPR spectrum of dithionite-reduced Complex II. Conditions: 10 K, 0.63 mT modulation amplitude, 5 mW microwave power, 8.99 GHz frequency. Multiplication factor indicates relative gains for the 2 spectra. The conditions of measurement were chosen to highlight the EPR signals from reduced center 2, and consequently the signal from reduced center 1 in the g = 2.03–1.88 region is off-scale. From Johnson et al. [47], by permission.

8. CONFIRMATION THAT A DISTINCT CENTER 2 EXISTS BY EPR

The conclusion from MCD experiments that succinate dehydrogenase contains 3 different types of Fe-S clusters, a [2Fe-2S], a [3Fe-*x*S], and a [4Fe-4S] type, was quite unexpected. This permits rationalization of the cluster extrusion data [25], which showed 2 binuclear clusters to be present, since 3Fe clusters are known to break down to [2Fe-2S] clusters under the conditions of extrusion. While the conclusions regarding centers 1 and 3 confirmed other studies, the demonstration that center 2 is a distinct component of the enzyme and is a tetranuclear rather than a binuclear cluster, as had been believed, was surprising, particularly since, at that time, no EPR signals attributable to a $[4Fe\text{-}4S]^{1+}$ cluster had been reported for dithionite-reduced samples of the enzyme.

However, recent EPR studies of Maguire et al. [49] and Johnson et al. [47] have, for the first time, identified signals characteristic of $[4Fe\text{-}4S]^{1+}$ cluster in dithionite-reduced samples of Complex II and soluble enzyme preparations. Fast relaxing bands attributed to center 2 in dithionite-reduced Complex II are shown by arrows in fig.3 (an additional positive feature with maxima at g = 1.99 is apparent at lower gain settings [49]). Presumably the broadness of this signal has impeded detection by other investigators. It is clear that reduced center 2 does not yield a simple rhombic or axial signal characteristic of a magnetically isolated reduced Fe-S cluster, but rather a broad complex resonance, spanning 150 mT, that is typical of a spin-coupled cluster. Similar complex spectra, albeit spanning a narrower field range, have been reported for spin-interacting $[4Fe\text{-}4S]^{1+}$ clusters in 8Fe ferredoxins [50] and for a $[4Fe\text{-}4S]^{1+}$ cluster spin coupled to a reduced [3Fe-*x*S] cluster (EPR-silent, S = 2 ground state) in the 7Fe ferredoxin from *Thermus thermophilus* [51]. While accurate spin quantitation of the EPR signal from center 2 is hindered by contributions from center 1, the spin concentration has been estimated to be approximately stoichiometric with FAD [47,49]. Broad complex resonances with slightly different band shapes but similar spin quantitations have also been reported for dithionite-reduced samples of fumarate reductase complex and soluble enzyme from *E. coli* [47].

Direct evidence for spin coupling between centers 1 and 2 came from redox titrations monitored by EPR [49]. For Complex II titrations,

with dithionite as reductant and ferricyanide as oxidant in the presence of mediator dyes, were carried out under conditions where center 1 was power saturated. The center 1 EPR signal was monitored at $g = 1.935$, and center 2 at $g = 1.847$. Two inflection points were observed: at 0 mV (E_m of center 1), and at -260 mV (E_m of center 2), both in accord with previous data [52]. Significantly, the appearance of the EPR signal of center 2 paralleled the enhancement of the spin relaxation of center 1. Similar results were obtained with the soluble enzyme [45], except that the midpoint potential of center 2 was -360 mV and its EPR signal is markedly less well resolved. While these experiments provide direct evidence for spin coupling between centers 1 and 2, the possibility of an additional spin interaction between centers 2 and 3 cannot be excluded at this stage.

9. CURRENT STATUS AND PROBLEMS FOR THE FUTURE

The recent studies summarized in this brief review seem to provide, for the first time, an understanding of the nature of the Fe-S clusters of succinate dehydrogenase, since they are in harmony with analytical and cluster extrusion data, as well as with the differences in catalytic properties between preparations having an intact cluster 3 and those which do not. It took 30 years and a plethora of techniques, ranging from sophisticated flavin chemistry through LEFE EPR and low-temperature MCD to gene amplification, to arrive at a coherent picture of the prosthetic groups of this fascinating enzyme. Clearly, no one laboratory could have accomplished this: it took the combined efforts of many laboratories, each with different expertise.

There still remain several unresolved problems. It is not easy to visualize how the early preparations, isolated without succinate, could have given good analytical data in several laboratories for 4Fe/FAD. Had the center 3 Fe atoms been lost from the protein because of exposure to O_2 during isolation by this method [15], the ratio should have been 6, not 4. It is suggestive that preparations isolated by a later modification [42] of this aerobic method, which permitted faster processing, were reported to contain ~6 Fe/flavin, 1 mol center 1, and dithionite reduction showed the presence of center 2, but center 3 was still absent [26]. It seems possible that prolonged exposure of the reconstitutively active enzyme to O_2 may result in progressive destruction of both centers 2 and 3 and eventual loss of their Fe from the protein. The 4Fe enzyme could thus be a mixture of species containing 2 and 6 Fe/flavin.

There is also no completely satisfactory explanation why center 2 gives different EPR spectra in Complex II and soluble preparations [49]. It has been suggested [49] that such differences might reflect changes in the magnitude and/or orientation of the spin coupling between center 2 and one of the other two centers in membranes and different types of soluble preparations. There is evidence from catalytic turnover rates for reversible conformation changes occurring on going from inner membrane to Complex II to the soluble enzyme and on reincorporating the latter in the membrane [11,40,41], which may provide some support for this. Related to this point is the fact that the intercluster magnetic interactions have yet to be fully characterized. Detailed understanding of such interactions will be useful in assessing the spatial disposition of the clusters and possible electron transfer pathways. Now that an EPR signal from cluster 2 has been identified, studies at frequencies other than X-band should prove useful in establishing the clusters involved in magnetic interactions. Also, definitive evidence concerning the subunit location of the Fe-S clusters has yet to emerge.

Perhaps, more important is the still unresolved problem of the biosynthetic machinery involved in the insertion of the 3 Fe-S clusters and of the covalently linked FAD. There also remain questions about the function of the low-potential center 2, which has a redox potential well below that of the fumarate/succinate couple, and why 3 different types of Fe-S clusters and covalently bound FAD are needed by this enzyme.

The picture presented here may not be final; future discoveries may require modification. But it is enough to build on.

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