Biochimica et Biophysica Acta, 462 (1977) 36–48

BBA 47357

THE NUMBER OF Fe ATOMS IN THE IRON-SULPHUR CENTERS OF THE RESPIRATORY CHAIN

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(Received March 10th, 1977)

SUMMARY

1. From the ^{57}Fe hyperfine interaction in EPR spectra of reduced submitochondrial particles from the yeast *Candida utilis*, grown with ^{57}Fe, it is concluded that all Fe-S centers in these particles detectable in spectra at 35–80 K are $[2Fe\text{-}2S]^{2-(2-;3-)}$ centers**. These are the centers 1 of NADH and succinate dehydrogenase, the Rieske Fe-S center and possibly center 2 of succinate dehydrogenase.

2. The signals of the reduced particles detectable only at temperatures below 20 K are $[4Fe\text{-}4S]^{2-(2-;3-)}$ clusters. These are the centers 2, 3 and 4 of NADH dehydrogenase.

3. The EPR spectra of the $[2Fe\text{-}2S]^{3-}$ centers of Complex I and II, but not that of Complex III, display a great inequality of the Fe nuclei in the effective hyperfine interaction in the x-y direction.

INTRODUCTION

Many Fe-S centers are now known to function in the mitochondrial respiratory chain. At least four different Fe-S centers have been characterized in beef-heart Complex I [1]. A more detailed analysis extended this number to 5 [2]. Complex II contains 2 to 3 different Fe-S centers [3, 4] whereas Complex III contains only 1 [5, 6].

Since isolated Complex III contains 2 non-heme Fe atoms per molecule of cytochrome c_1 [7], it is likely that this center, often referred to as the Rieske Fe-S center, is of the cluster type containing 2 Fe and 2 acid-labile S atoms, further indicated as a [2Fe-2S] cluster. Complex II exhibits an EPR signal in the oxidized state [8], whose EPR and redox properties are similar to those of the signal of the high potential iron protein from *Chromatium vinosum*. This signal therefore probably originates from a

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** The formal charge of the cluster in the isolated complex is indicated by 2^-, assuming the acid-labile sulphur to be present as S^{2-} and the cysteine sulphur as RS^-. The charges in brackets refer to the possible charges that the cluster can have in the native protein.

$[4Fe\text{-}4S]^{1-(1-;2-)}$ cluster. For the other Fe-S centers in the respiratory chain the number of Fe atoms per center is not known.

$^{57}Fe(I = \frac{1}{2})$ has been used with putidaredoxin and ferredoxin [9, 10] to show that in the reduced protein the $S = \frac{1}{2}$ system magnetically interacts with the Fe nuclei. Since the hyperfine splitting in the z direction is 1.2–1.4 mT for each of the Fe nuclei, it is possible with a sufficiently small line width to see a typical 1 : 2 : 1 hyperfine structure in X-band EPR spectra. More recently [10–12] ENDOR studies on [2Fe-2S], [4Fe-4S] and 2[4Fe-4S] proteins have also been performed. Hyperfine interactions in the [4Fe-4S] cluster were found to be considerably smaller than in the [2Fe-2S] clusters. The sensitivity of ENDOR is, however, two orders of magnitude less than that of EPR. It is therefore not suitable for determining the number of Fe atoms per cluster in submitochondrial particles or even in the isolated complexes.

Cammack [13] distinguished between [2Fe-2S] and [4Fe-4S] clusters by the effect of 80 % dimethyl sulphoxide on the EPR spectrum of the reduced protein. This method is, however, not applicable when one deals with a mixture of Fe-S proteins as in the respiratory chain.

Since the respiratory chain of the yeast *Candida utilis* is very similar to that of mammalian mitochondria [14–17], growth on a ^{57}Fe-enriched medium provides the possibility of obtaining information on the number of Fe atoms in each of the Fe-S centers of the electron-transfer chain. Part of this work has been reported on a recent congress [18].

MATERIALS AND METHODS

Preparation of cells

The yeast *C. utilis* was grown batchwise in the medium of Galzy and Slonimsky [19] plus 1.3 % ethanol. A 15-times concentrated solution of the bulk salts of this medium (KH_2PO_4, $NH_4H_2PO_4$ and $(NH_4)_2SO_4$) was treated with Chelex 100 (Bio Rad) to remove Fe (determined as described by Ballentine and Durford [20]). The final growth medium contained 0.2 μM Fe^{3+} due to the residual Fe of the bulk salts. No growth of *C. utilis* was observed in this medium in the absence of added iron. To this medium 10 μM Fe^{3+}, either natural Fe (2.2 % ^{57}Fe) or ^{57}Fe (enrichment 93.4 %, Oak Ridge Natl. Lab., Oak Ridge, Tenn.) was added. The ^{57}Fe-containing medium thus had an enrichment of 91.5 %. We will assume this figure to be 90 % in the rest of this paper. The preculture for the ^{57}Fe batch was also grown on the ^{57}Fe-enriched medium.

Preparation of submitochondrial particles

Cells were harvested in the late logarithmic phase and washed twice with water. They were then broken either by vigorous stirring with glass beads for 1 h or by grinding with sand in a mechanical mortar for 15 min, both in 1 M sucrose/20 mM Tris · HCl buffer (pH 8.0)/4 mM EDTA. After a 20-min spin at $3000 \times g$, the material precipitating at $35\,000 \times g$ for 30 min was suspended in 0.25 M sucrose/5 mM Tris · HCl buffer (pH 8.0)/1 mM EDTA. This suspension was sonified for 2–3 min at stand 4 with a Branson sonifier. After centrifuging at $10\,000 \times g$ for 10 min, the material precipitating at $150\,000 \times g$ for 20 min was washed once with 0.25 M sucrose/5 mM Tris · HCl buffer (pH 8.0)/1 mM EDTA. The transparent red-brown pellet was

suspended in a minimal volume of the same buffer and directly used to prepare EPR samples. Submitochondrial particles thus prepared contained about 0.25 nmol cytochrome c_1 per mg protein.

The recording, digitizing and simulation of EPR spectra were performed as described previously [2]. In all simulations it was assumed that the g- and A-tensor have the same principal axes.

Analysis of gaussian curves was performed with a Du Pont 310 curve resolver.

RESULTS

The oxidized state

The submitochondrial particles as isolated were in the oxidized state. The EPR signal of the copper of cytochrome *c* oxidase, as monitored at 50–80 K, was the same in ^{56}Fe and ^{57}Fe preparations, as expected. The g_z line of the low-spin heme *a* showed the presence of the $I = \frac{1}{2}$ nucleus by an increase of its line width at half height from 4.1 mT (^{56}Fe) to 4.6 mT.

The spectrum of the [4Fe-4S]$^{1-}$ center of Complex II was clearly broadened by ^{57}Fe, as shown in Fig. 1. The broadening occurs mainly in the middle and low-field part of the signal. The high-field side contains contributions from the greatly saturated copper signal of cytochrome *c* oxidase. This makes the single integral of the positive part of the spectrum somewhat less than that of the negative part. This is unavoidable. Simulation of the ^{56}Fe signal (best fit with $g_{x,y,z} = 1.997, 2.011$ and 2.020, width (x, y, z) = 2.5, 2.0 and 1.5 mT) indicated slight rhombicity as earlier observed by Ruzicka et al. [21]. We did not succeed in good simulation of the ^{57}Fe signal, corrected for 10 % ^{56}Fe, assuming an equal hyperfine interaction with 1, 2 or 4 nuclei.

The reduced state: shape of the g_z lines

The spectra of the Rieske Fe-S protein, reduced with ascorbate plus tetramethyl-*p*-phenylenediamine, are shown in Fig. 2. No broadening at all is seen in the

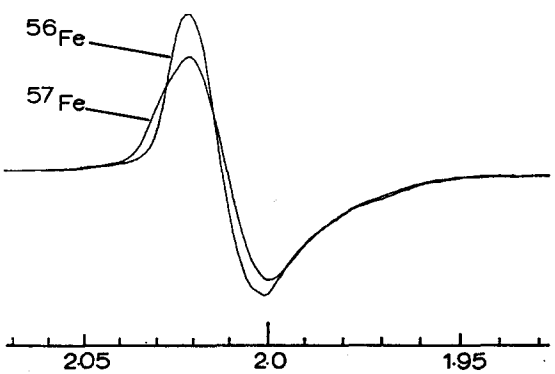

Fig. 1. Effect of ^{57}Fe on the EPR spectrum of center 3 of succinate dehydrogenase. Submitochondrial particles were frozen in liquid nitrogen directly after their preparation. The amplitude of the ^{57}Fe trace was adjusted so that the double integral value was the same as that of the ^{56}Fe trace. The scale refers to g-values. EPR conditions: microwave frequency (F) = 9308 MHz; Temperature (T) = 12 K; microwave power (P) = 20 mW; modulation amplitude (MA) = 0.5 mT; scanning rate (SR) 20 mT/min; modulation frequency = 100 kHz (in expts. of other figures, also).

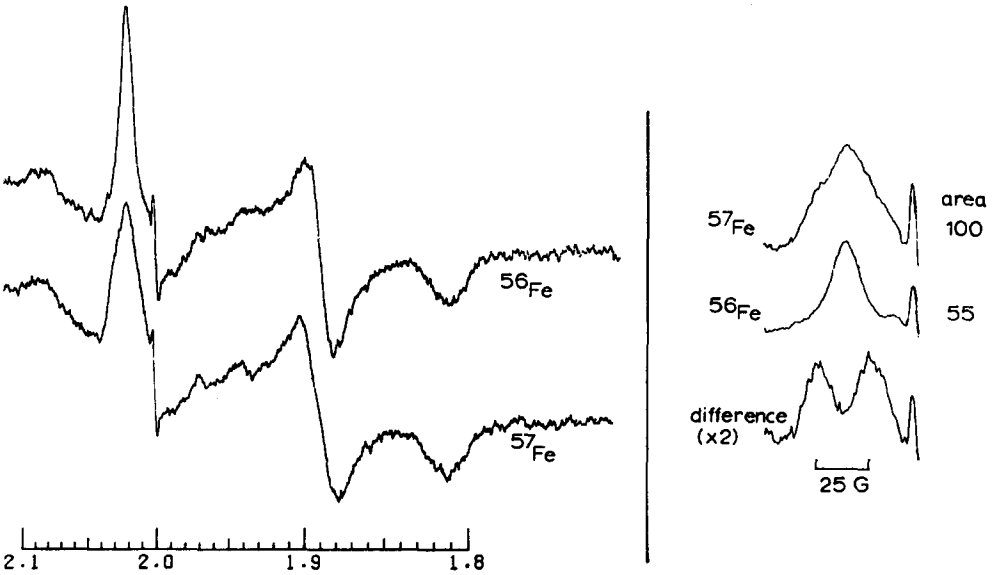

Fig. 2. Effect of ^{57}Fe on the EPR spectrum of the Rieske Fe-S center. Submitochondrial particles were treated for 3 min at 20 °C with 10 mM ascorbate (pH 6.0) plus 60 μM tetramethyl-*p*-phenylenediamine before they were frozen and stored in liquid nitrogen. Left, complete spectra. Right, g_z lines recorded in detail, digitized and replotted on the same *g*-value scale such that the area of the ^{56}Fe line was 55 % of that of the ^{57}Fe line. The lower curve is the 2-times enlarged difference ^{57}Fe minus ^{56}Fe and represents the two outer hyperfine lines. Their distance is 2.5 mT. EPR conditions: *F*, 9310 MHz; *T*, 36 K; *P*, 12 mW for the complete spectra and 3 mW for the detailed g_z lines; *MA*, 0.63 mT; *SR*, 50 mT/min for the complete spectra and 10 mT/min for the detailed g_z lines.

x direction. The *y* direction shows a small broadening, whereas the g_z line is most affected by the presence of ^{57}Fe.

Rieske et al. [7] found 2 non-heme Fe atoms per cytochrome c_1 in isolated Complex III. Since EPR spectra show only a single signal it is generally assumed that this Fe-S cluster is a [2Fe-2S] type. This is confirmed when the g_z is determined in detail (Fig. 2). If we assume that the S = ½ system has an equal effective interaction with 2 ^{57}Fe nuclei, then the ratio of the hyperfine lines would be 1 : 2 : 1. As we have only 90 % ^{57}Fe present, the real peak should consist of three lines in a ratio 22.5 : 55 : 22.5. A subtraction as in Fig. 2 then shows the two outer hyperfine lines as two gaussian peaks. The absence of additional shoulders on the low-field side also suggests that we are indeed dealing with a [2Fe-2S] cluster. The outer hyperfine lines are 2.5 mT apart, so that the effective A_z values are approximately 1.25 mT for both Fe nuclei, a value in good agreement with better characterized [2Fe-2S] ferredoxins [9, 10, 12].

Information about the Fe-S centra in succinate dehydrogenase can be obtained from the spectra in Fig. 3, which are overlapping spectra of the Rieske Fe-S center and the center 1 of succinate dehydrogenase. All the lines of center 1 show broadening in the ^{57}Fe trace. The g_z is examined in Fig. 4. The contribution of the Rieske Fe-S protein has first to be subtracted to obtain the pure g_z of center 1. The result of this subtraction (Fig. 4A) is, however, not a gaussian peak. This is related to the effect [22],

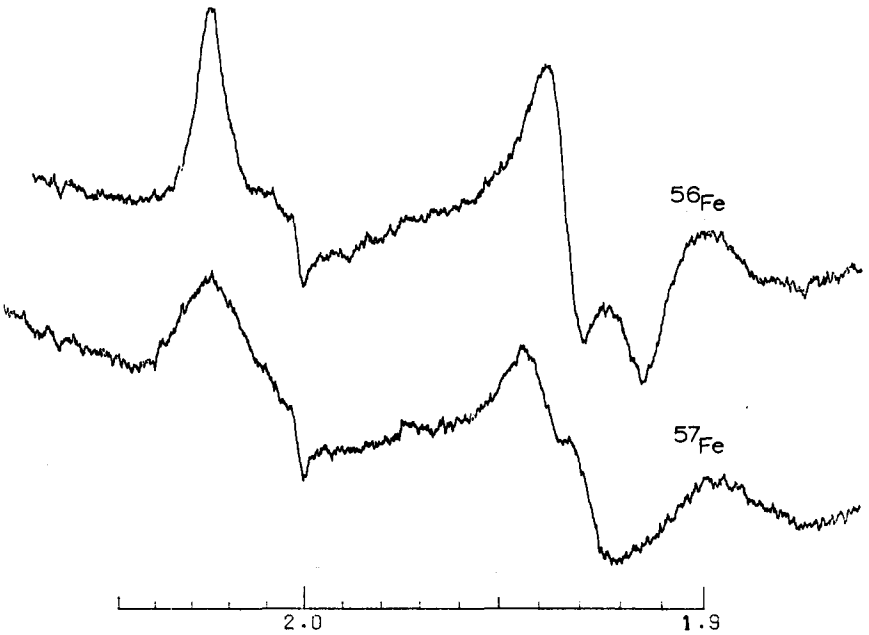

Fig. 3. Effect of ^{57}Fe on the EPR spectrum of center 1 of succinate dehydrogenase. Submitochondrial particles were treated for 3 min at 20 °C with 25 mM succinate before they were frozen. EPR conditions: *F*, 9307 MHz; *T*, 65 K; *P*, 12 mW; *MA*, 0.63 mT; *SR*, 20 mT/min.

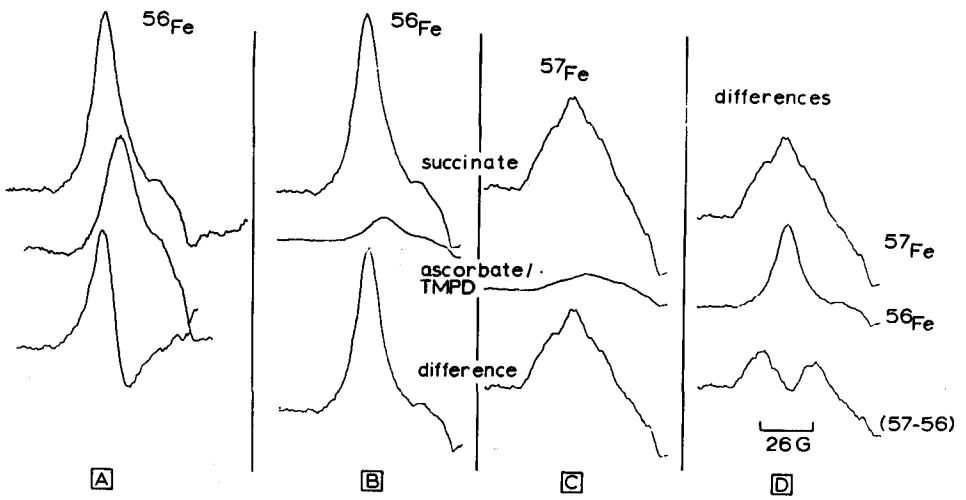

Fig. 4. Effect of ^{57}Fe on the g_z of center 1 of succinate dehydrogenase. The g_z lines of the samples of Figs. 2 and 3 were recorded in detail under identical conditions and digitized. A, subtraction of the g_z obtained with ascorbate (middle trace) from that obtained with succinate (upper trace), both with ^{56}Fe. B, same as A, but the amount of g_z obtained with ascorbate was adjusted so as to obtain a symmetrical difference. C, same as B, but now for ^{57}Fe. The amplification for the g_z with ascorbate was the one determined in B. D, upper trace is the difference spectrum from C (^{57}Fe), middle trace is the difference spectrum from B, plotted on a convenient scale (^{56}Fe). Lower curve is the difference ^{57}Fe$-^{56}$Fe and gives the distance of the two outer hyperfine lines of the g_z of center 1 of succinate dehydrogenase. EPR conditions: *F*, 9308 MHz; *T*, 63 K; *P*, 12 mW; *MA*, 0.63 mT; *SR*, 10 mT/min.

also observable in beef-heart submitochondrial particles, that on reduction of cytochrome *b*-562 the shape of the EPR signal of the Rieske Fe-S center changes. The most prominent change is the shift of the g_x to higher field with a simultaneous broadening. As a consequence the amplitude of the g_y and g_z lines decrease somewhat, assuming no change in line width. Also the *g*-values of the latter lines might shift. It is difficult to observe what happens to the g_z line, as it overlaps with that from center 1. The appoximate shape of the g_z of center 1 was obtained by subtracting less of the g_z of the Rieske Fe-S center, until a symmetrical difference was obtained. The resulting g_z lines of center 1 (Fig. 4, B and C) are compared in Fig. 4D. The area of the two upper curves in Fig. 4D are chosen arbitrarily. From the lower trace the conclusion is drawn that the ^{57}Fe g_z line of center 1 of succinate dehydrogenase displays interaction with only 2 Fe nuclei. This is supported by the fact that the low-field side of the lines has no shoulder. The effective A_z values are approx. 1.3 mT.

The line shape of center 1 of NADH dehydrogenase can be obtained from the difference spectra shown in Fig. 5. NADH alone or NADH plus dithionite both gave a signal of center 1 of succinate dehydrogenase which was 30 % greater than that obtained with succinate alone. The g_z lines were recorded in detail and the results are shown in Fig. 6. A difference ^{57}Fe minus ^{56}Fe in a suitable ratio indicated interaction with 2 Fe nuclei with effective A_z values of approx. 1.2 mT.

The g_z line of center 2 of NADH dehydrogenase also broadens with ^{57}Fe as seen in Fig. 7. The detailed analysis of the g_z line is presented in Fig. 8. Since, especially

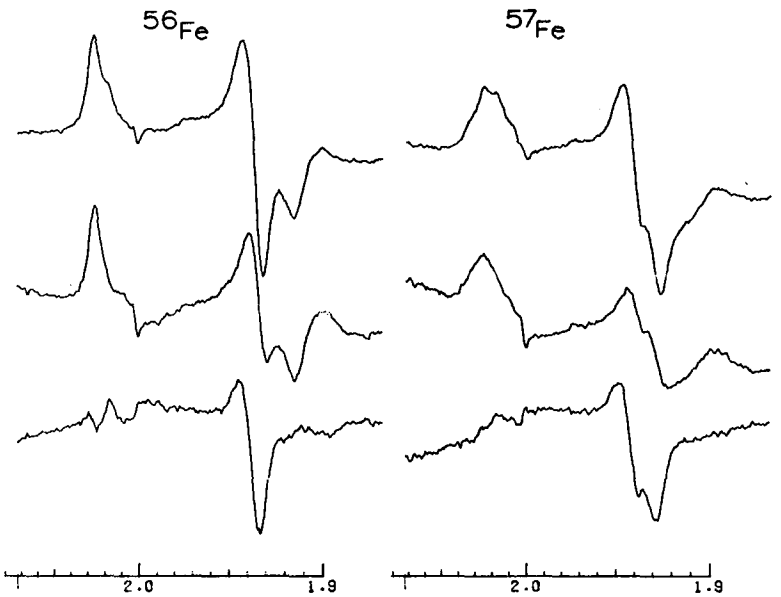

Fig. 5. Effect of ^{57}Fe on the line shape of center 1 of NADH dehydrogenase. Submitochondrial particles were reduced with 10 mM NADH plus a few grains of dithionite. After 3 min at 20 °C they were frozen in liquid nitrogen. Left (^{56}Fe), upper curve, NADH+dithionite; middle curve, succinate (from Fig. 3), enlarged by a factor 1.3, to match the $g = 1.91$ line in the upper trace; lower curve, difference. Right, the same as A, but for ^{57}Fe. All experimental spectra were digitized to the same *g* value scale. EPR conditions as in Fig. 3.

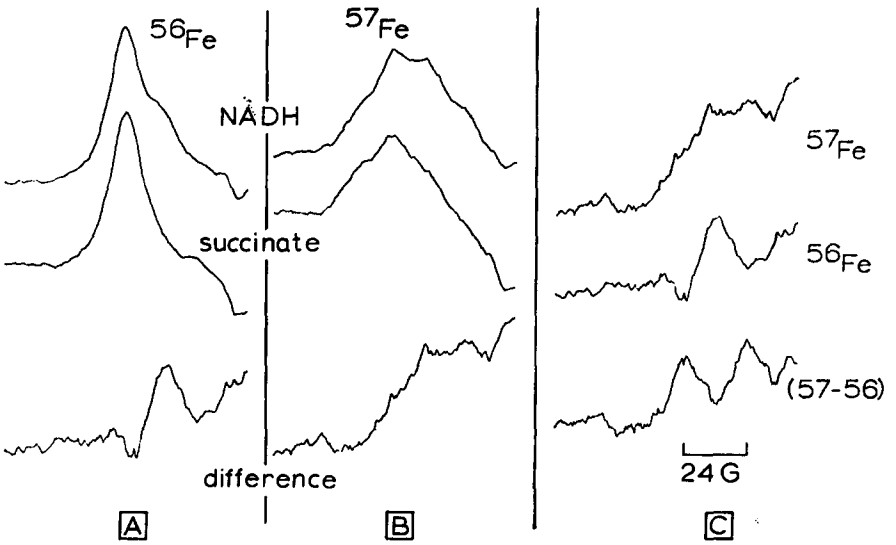

Fig. 6. Effect of ^{57}Fe on the g_z of center 1 of NADH dehydrogenase. The g_z lines of the samples of Fig. 5 were recorded in detail under identical conditions and digitized. A (^{56}Fe), upper curve, g_z obtained with NADH+dithionite; middle curve, g_z obtained with succinate, 1.3 times amplified (see Fig. 5); lower curve, difference. B, same as A, but for the ^{57}Fe traces. C, upper curve, difference curve from B (^{57}Fe); middle curve, difference curve from A (^{56}Fe) plotted on a convenient scale; lower curve, difference $^{57}Fe-^{56}Fe$. This gives the distance of the two outer hyperfine lines. EPR conditions as in Fig. 4.

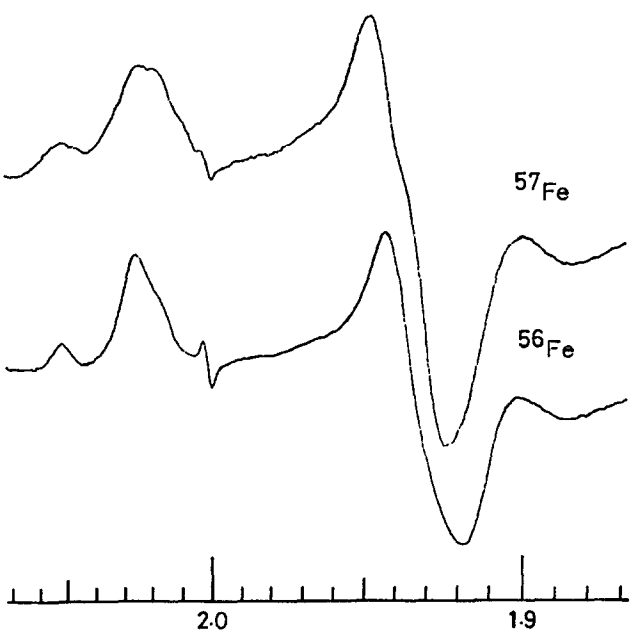

Fig. 7. EPR spectra at 17 K of submitochondrial particles reduced with NADH plus dithionite. Upper curve, ^{57}Fe. Lower curve, ^{56}Fe. EPR conditions; *F*, 9308 MHz; *T*, 17 K; *P*, 12 mW; *MA*, 0.63 mT; *SR*, 20 mT/min.

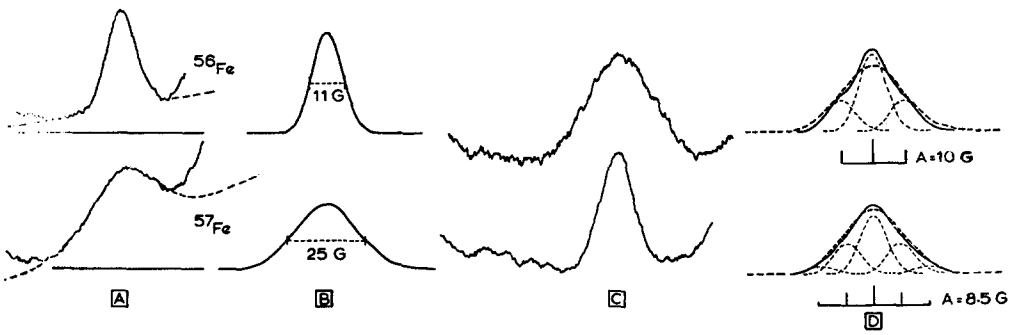

Fig. 8. Effect of ^{57}Fe on the g_z of center 2 of NADH dehydrogenase. A, solid lines, experimental curves; dashed lines, gaussian fits on a tilted base. B, plots of the gaussian fits on a horizontal base. The upper curves of A and B are for ^{56}Fe curves, the lower for ^{57}Fe. C, experimental $g = 2.05$ lines, recorded under saturating conditions for the $g = 2.05$ and $g = 2.02$ lines. The observing phase of the modulation was turned nearly 90° out of phase and adjusted so that the $g = 2.02$ lines were minimal. Upper curve, ^{57}Fe; lower curve, ^{56}Fe. D, analysis of the ^{57}Fe curve in terms of the ^{56}Fe curve. Upper trace, equal interaction of 1 mT with 2 nuclei assumed (ratio 22.5 : 55 : 22.5); lower trace, equal interaction of 0.85 mT with 4 nuclei assumed (ratio 5.6 : 22.5 : 43.8 : 22.5 : 5.6) The thick dashed lines are the gaussian fits to the experimental curve. The thin dashed lines are the individual hyperfine lines. The solid lines are the sum of the hyperfine lines. EPR conditions for A: *F*, 9307 MHz; *T*, 16 K; *P*, 8 mW; *MA*, 0.63 mT; *SR*, 5 mT/min (detection in phase); for C: *F*, 9311 MHz; *T*, 18 K; *P*, 200 mW; *MA*, 0.63 mT, *SR*, 5 mT/min (detection about 90° out of phase).

in the ^{57}Fe trace, the base line is tilted due to overlap with the broadened g_z lines around $g = 2.02$, the curves were fitted with a curve resolver as gaussian lines on a tilted base line (Fig. 8A) and displayed on a flat base line in Fig. 8B. A direct recording of the undisturbed g_z line of center 2 is given in Fig. 8C. In this recording use was made of the fact that the observed phase of the modulation of a signal under saturating conditions can shift somewhat. The results are exactly the same as the gaussian fit of the normal trace. No fine structure can be seen in the ^{57}Fe trace, although the line width (2.5 mT) is more than double that (1.1 mT) of the ^{56}Fe line. Interaction with only two nuclei thus seems less likely. This assumption was strengthened by the result of a gaussian analysis of the ^{57}Fe line in terms of the ^{56}Fe line as shown in Fig. 8D. The best fit was obtained assuming equal interaction with 4 nuclei.

^{57}Fe had very little if any effect on the width of the g_z line at $g = 2.103$ of center 3 of NADH dehydrogenase [2] in 11 K EPR spectra. The g_z line of center 4 [2] cannot be observed separately in these spectra.

The reduced state. shape of g_x and g_y lines

As can be seen in Fig. 9, the line shape of center 1 of NADH dehydrogenase is, in contrast to that of the beef-heart enzyme [2], only composed of a single axial signal and can be readily simulated as such. ^{57}Fe has a marked effect on the $g_\perp$ line (see Figs. 5 and 9). Since the splitting occurs very near to the $g_\perp$ value position, the effective interaction with one nucleus must be much greater than that with the other. Simulations confirmed this. The best fit was obtained assuming a hyperfine interaction of 2 mT with only one nucleus (Fig. 9). Even a 0.1–0.5 mT interaction with the other nucleus, when the sum of $A_{\perp 1}$ and $A_{\perp 2}$ was kept constant, made the simulation worse.

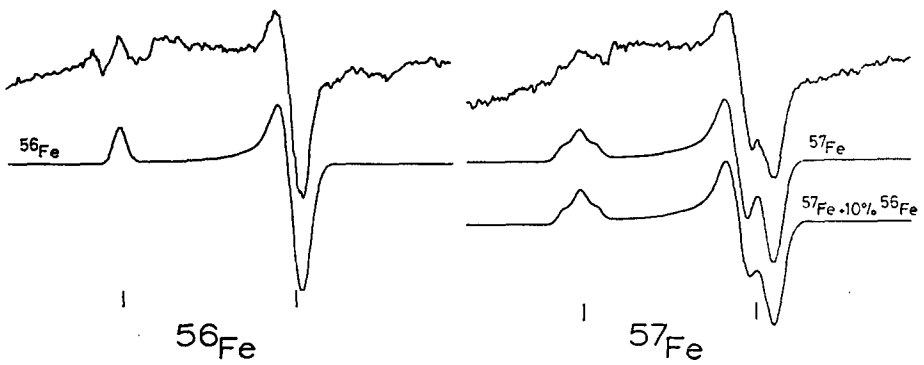

Fig. 9. Simulation of the ^{56}Fe and ^{57}Fe line shape of center 1 of NADH dehydrogenase. Left, upper curve, experimental line shape of center 1 (^{56}Fe, from Fig. 5); lower curve, computer simulation. Parameters, $g_{\|} = 2.0156$, $g_{\perp} = 1.9355$ and widths 1.02 mT and 1.61 mT for the z and x-y direction, respectively. Right, upper curve, experimental line shape of center 1 (^{57}Fe, from Fig. 5); middle curve, simulation. Parameters same as for A, but with $A_{z,1} = A_{z,2} = 1.25$ mT; $A_{\perp,1} = 2.025$ mT and $A_{\perp,2} = 0$ mT. Lower trace, simulation as in the middle trace, but with addition of 10 % line shape of ^{56}Fe (on double integral basis).

A similar asymmetric interaction was seen in the g_y line of center 1 of succinate dehydrogenase (see Figs. 3 and 10). Here too a large hyperfine interaction with one nucleus has to be assumed to obtain good simulations, but the interaction with the other nucleus could not be assumed to be zero, but had to be 0.2–0.3 mT. Best fits for the g_x line could also be obtained only if the interaction with one nucleus was taken to be much greater than that with the other. Simulations are shown in Fig. 10.

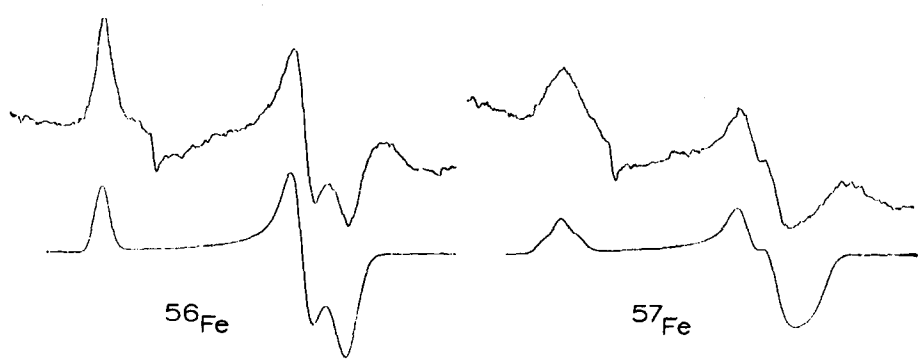

Fig. 10. Simulation of the ^{56}Fe and ^{57}Fe line shape of center 1 of succinate dehydrogenase. Left, upper curve, experimental line shape of center 1 (^{56}Fe, from Fig. 3); lower curve, simulation. Parameters, $g_{z,y,x} = 2.0242$, 1.933, 1.9135 and widths $(z,y,x) = 1.17$ mT, 1.4 mT, 1.7 mT. No effort was made to deal with the overlapping g_z and g_y lines of the Rieske Fe-S center. This means that widths used for the z and x direction are probably not quite correct. Right, upper curve, experimental line shape of center 1 (^{57}Fe, from Fig. 3); lower curve, simulation, consisting of 90 % ^{57}Fe and 10 % ^{56}Fe on double integral basis. Parameters as for A, but with $A_{z,1} = A_{z,2} = 1.3$ mT, $A_{y,1} = 2.1$ mT, $A_{y,2} = 0.3$ mT, $A_{x,1} = 1.6$ mT and $A_{x,2} = 0$ mT for the ^{57}Fe simulation. The shape at the g_y position is very sensitive to small changes in the $A_{y,1}$ and $A_{y,2}$. The shape of the g_x can possibly be refined by introducing a small $A_{x,2}$.

In contrast to these two cases of rather large interactions in the x-y plane, with only one Fe nucleus, no hyperfine interaction was observed with the Rieske Fe-S center in the x direction and only a small interaction in the y direction (see Fig. 2). The peak to peak width of the latter increased from 3.5 mT to 4.6 mT. Since there is no indication that the effective hyperfine interactions in the y direction is different for the two nuclei, the A_y values for both must be about 0.55 mT.

We did not inspect the $g_\perp$ line of center 2 of NADH dehydrogenase in detail. In contrast to beef-heart submitochondrial particles, dithionite reduced not only center 2, but all the other centers of Complex I as well. Reduction of part of center 2 with succinate as occurs in beef-heart particles, was not observed in these particles. From Fig. 7 it can be seen that the $g_\perp$ line of center 2 must be broader than that in beef heart, or its shape must be slightly rhombic, since the overall shape in the $g = 1.91$–1.95 region is quite different from that in spectra of beef-heart particles.

The g_x lines of the centers 3 and 4 of NADH dehydrogenase [2] at $g = 1.86$ and $g = 1.88$, respectively, showed no hyperfine interaction in the ^{57}Fe EPR spectra at 11 K.

DISCUSSION

EPR signals in the reduced particles at 50–80 K

All these signals show hyperfine interaction with 2 Fe nuclei in the z direction. This number 2 is clear for the Rieske Fe-S protein (see Fig. 2) but less straightforward for the other two centers. The change of the signal shape of the Rieske Fe-S center on reduction with succinate compared with reduction by ascorbate makes it difficult to obtain the precise shape of the g_z of center 1 of succinate dehydrogenase. The result from Fig. 4 is only an approximation of the two outer hyperfine lines of this g_z line. A poor signal to noise ratio of the ^{57}Fe g_z line shape of center 1 of NADH dehydrogenase prevented a more convincing analysis. The resulting estimated A_z values, however, are similar to those of known [2Fe-2S] ferredoxins [9, 10, 12]. Moreover, all these g_z lines have a property in common: a g-value very close to 2.02, although the corresponding g_y and g_x values differ very much. This is in accordance with the theoretical g-values of the model of Gibson et al. [23] for [2Fe-2S] clusters.

The behaviour of the g_x and g_y lines of the centers 1 of NADH and succinate dehydrogenase in the ^{57}Fe spectra is quite unique. The effective interaction with the two nuclei is very different. Also the strength of the greatest interaction in the x-y direction is much greater than that in the z direction. The Fe atoms thus are quite unequal with respect to the free electron spin. To our knowledge these are the first examples where this inequality of the Fe atoms shows up directly in the EPR spectra, but it has been demonstrated recently in algal ferredoxin by ENDOR [12]. According to these measurements it is the Fe^{3+} which has a 4 times greater interaction than the Fe^{2+} in the x-y plane. Our estimated A_y values from simulations of the EPR spectra (Figs. 9 and 10), 2.0 mT and 0 mT for center 1 of NADH dehydrogenase and 2.1 mT and 0.3 mT for center 1 of succinate dehydrogenase, agree quite well with the ENDOR-determined values for algal ferredoxin ($A_{x,y} = 1.95$ mT and 1.82 mT for Fe^{3+} and $A_{x,y} = 0.49$ mT and 0.54 mT for Fe^{2+}). Our estimates were made on the assumption that the principle axes of the g- and A-tensors are parallel, which may not be true [12].

TABLE I

PROPERTIES OF THE Fe-S CENTERS IN ^{57}Fe-CONTAINING SUBMITOCHONDRIAL PARTICLES

Center	Complex I				Complex II			Complex III
	1	2	3	4	1	2	3	1
Cluster type	[2Fe-2S]	[4Fe-4S]	[4Fe-4S]	[4Fe-4S]	[2Fe-2S]	[2Fe-2S]	[4Fe-4S]	[2Fe-2S]
Charge	2−(2−; 3−)	2−(2−; 3−)	2−(2−; 3−)	2−(2−; 3−)	2−(2−; 3−)	2−	1−(1−; 2−)	2−(2−; 3−)
$A_{z\,1,2}$ (mT)	1.2	0.85	0	–	1.3	1.3	–	1.25
$A_{y\,1}$ (mT)	2.0	–	–	–	2.1	2.1	–	≈ 0.55
$A_{y\,2}$ (mT)	0	–	–	–	0.3	0.3	–	≈ 0.55
$A_{x\,1}$ (mT)	2.0	–	0	0	≈ 1.6	≈ 1.6	–	0
$A_{x\,2}$ (mT)	0	–	0	0	small	small	–	0

–, not determined

The signal of the Rieske Fe-S center clearly differs from this behaviour, as the hyperfine interaction in the y direction is small and is apparently not asymmetric, whereas no interaction is detected in the x direction.

The above mentioned properties, especially of the g_z lines, lead us to conclude that all these centers are of the $[2Fe\text{-}2S]^{2-(2-;3-)}$ type.

We did not investigate the origin of the observed 30 % extra increase of the signal of center 1 of succinate dehydrogenase, when the reduction was performed with NADH instead of succinate. This extra amount might be related to center 2 of succinate dehydrogenase [3, 4], although Ohnishi et al. [24] state that in pigeon-heart mitochondria this center cannot be reduced by NAD-linked substrates. In any case, its shape must be identical to that of the signal appearing with succinate alone, as difference spectra of NADH minus succinate resulted in an axial signal, which could be easily simulated (Figs. 5 and 9).

EPR signals in reduced particles below 20 K

^{57}Fe more than doubles the width at half height of the g_z line of center 2 of NADH dehydrogenase, without causing any shoulders. Fitting of the ^{57}Fe peak in terms of the ^{56}Fe peak is obviously bad if one assumes equal effective interaction with 2 nuclei (Fig. 8); the total amplitude of the two middle overlapping hyperfine lines is greater than the experimental curve and the flanks do not fit. The overall fit is much better assuming an equal effective interaction with 4 nuclei and would even be improved if the individual interactions were taken to be slightly unequal. The A values are 60–70 % of those found for the [2Fe-2S] clusters. Center 2 is, then, a $[4Fe\text{-}4S]^{2-(2-;3-)}$ cluster, the first example of such a cluster with a relatively high midpoint potential.

^{57}Fe has no effect on the g_z and g_x of center 3 of NADH dehydrogenase nor on the g_x of center 4. It has been demonstrated [15] that these signals are not present in *C. utilis* cultures lacking Fe or S in the medium, so they are quite likely due to Fe-S clusters. The hyperfine values observed with ENDOR in ^{57}Fe-substituted [4Fe-4S] proteins [11] are smaller than for [2Fe-2S] centers. This makes it likely that the centers 3 and 4 are not [2Fe-2S] but probably [4Fe-4S]. Since EPR spectra at 35 GHz [25] have the same line shape and g values as 9 GHz spectra, we see no reason to assume the presence of triplet states from two interacting $[4Fe\text{-}4S]^{3-}$ centers as is the case in some $2[4Fe\text{-}4S]^{3-}$ ferredoxins [26, 27]. Table I summarizes our conclusions.

EPR spectra of yeast particles at 4.2 K and high power showed no extra lines, in contrast to beef-heart particles. This supports the conclusion that these extra lines are not due to NADH dehydrogenase as pointed out previously [2].

Making use of the stoichiometry for the Fe-S center of Complex I determined by Albracht et al. [2] and extrapolating the results of this paper to beef-heart mitochondria, we can now account for 13 of the 16 chemically determined Fe atoms [28, 29] per FMN in NADH dehydrogenase. For Complex II, using the stoichiometry determined by Beinert et al. [4] 6.2–7 of the 8 Fe atoms [30, 31] per FAD can now be accounted for in EPR spectra.

ACKNOWLEDGEMENTS

The authors thank Professor E. C. Slater for his valuable criticism and his

continuous interest. Part of this work has been supported by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.). One of the authors (J.S.) is grateful to the Gesellschaft für Biotechnologische Forschung (G.B.F.) for granting leave to enable him to participate in this project and to Professor Dr. J.-H. Fuhrhop for his kind encouragement.

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