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EFFECTS OF DIETARY IRON DEFICIENCY ON IRON-SULFUR PROTEINS AND BIOENERGETIC FUNCTIONS OF SKELETAL MUSCLE MITOCHONDRIA

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Decreases in the mitochondrial content of Fe-S proteins appear to cause a loss of bioenergetic functions in skeletal muscle mitochondria during dietary iron deficiency. Following 21 days of an iron-deficient diet (2 mg iron/kg), significant decreases in muscle mitochondrial iron protein content, dehydrogenase and oxidase activities, and rates of ATP synthesis were observed relative to rats fed a control diet (50 mg iron/kg). Fe-S proteins were studied by EPR spectroscopy at low temperatures, following incubation with substrates, and/or redox titrations. Submitochondrial preparations were utilized in order to overcome membrane barriers to electron donors and to concentrate inner mitochondrial membrane Fe-S proteins. The Fe-S proteins of rat muscle mitochondria (not previously studied) appear remarkably similar to those of other tissues and species. Although the concentration of most Fe-S proteins studied was decreased by iron deficiency, the Rieske cluster of Complex III ($Fe\text{-}S_{bc-1}$) was only minimally affected. In Complex II, Fe-S cluster S-1 was decreased 38% and cluster S-3 was decreased 59%. Complex I Fe-S clusters were substantially decreased by iron deficiency. The Fe-S cluster of electron-transferring flavoprotein dehydrogenase was 55% lower in the iron-deficient submitochondrial preparations. Succinate and NADH dehydrogenase activities, and maximal rates of reversed electron transport were about 80% lower in iron-deficient submitochondrial preparations than in controls, and oxidase activities were decreased about 70%. Decreases in cytochrome content were too small to explain loss of oxidase activity, and clearly could not affect dehydrogenases or reversed electron transport. Despite reductions in maximal rates of ATP formation, the efficiency of phosphorylative coupling was not affected. Our studies suggest that the loss of Fe-S clusters causes decreases in the dehydrogenase activities and that the iron depletion from the complexes is not a random process.

Introduction

The mitochondrial transfer of electrons from substrates to oxygen involves a number of iron-containing proteins. Fe-S proteins are the principle (in number) electron carriers. The diversity of membrane bound Fe-S proteins, and the fact that they can only be readily detected by EPR spectroscopy at low temperatures, have made functional studies difficult to perform. Nevertheless, significant understanding of Fe-S protein function has been gained from studies of submitochondrial preparations and isolated electron-transport chain complexes [1,2].

The experimental intervention of iron deficiency affords an opportunity to alter the iron content of mitochondrial proteins and to investigate associated bioenergetic effects. Such an approach was employed by Ohnishi et al. [3,4] who demonstrated that low

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; RCR, respiratory control ratio; TMPD, *N,N,N',N'*-tetramethylphenylenediamine.

iron content in the growing medium of yeast cultures significantly decreased the mitochondrial content of many EPR-detectable Fe-S clusters. Dietary iron deprivation of growing rats induces large decreases in mitochondrial oxidase activities, without affecting phosphorylative efficiency [5]. In rats, skeletal muscle appears to be the tissue most strongly affected by iron deficiency [6]. The effects of dietary iron deficiency on Fe-S proteins are unknown in rat skeletal muscle mitochondria. This report deals with the possible relationships between mitochondrial Fe-S protein content and bioenergetic functions in rat skeletal muscle during dietary iron deficiency.

Methods

Male Wistar rats were obtained at 21 days of age (Simonsen Labs, Gilroy, CA). The rats were provided an iron-deficient diet containing 2 mg iron/kg, (Teklad, Madison, WI) for 21 days. Control rats were fed an identical diet which was iron supplemented to 50 mg/kg. Hematocrits were measured after centrifugation of blood in heparinized capillary tubes, and hemoglobin concentration was estimated by the cyanomethemoglobin method (Data Medical Associates, Inc.).

Muscle mitochondria were prepared from both hindlimbs, both forelimbs, and the abdominal muscles of each rat, according to the methods of Bullock et al. [7], with modifications described by Davies et al. [8]. Six to ten iron-deficient and control animals were used for each mitochondrial isolation. Submitochondrial preparations were obtained by sonication of mitochondria suspended in 30 mM sodium phosphate, pH 7.0, as described by Kielly and Bronk [9].

Fe-S proteins

Fe-S proteins were studied by low-temperature EPR spectroscopy of submitochondrial preparations. Samples were reduced or oxidized in quartz tubes of known internal diameter with either 5 mM dithionite, NADH, succinate or ferricyanide, according to the method of Beinert [10]. Redox titrations of selected samples were performed as described by Dutton [11] using calomel and platinum electrodes. Mediator dyes were chosen to scan a wide range of potentials. The mediators used were: 2,5-cyclohexanediene-1,4-dione compounded to 1,4-benzenediol (1 : 1) (quinhydrone); 2,3,5,6,-tetramethyl-2,5-cyclohexadiene-1,4-dione (duroquinone); 9,10-anthracenedione-1,5-disulfonate (anthroquinone-1,5-disulfonate); *N*-ethylphenazonium ethosulfate (phenazine ethosulfate); safronin-T (see Ref. 12); 1,1'-dibenzyl-4,4'-bipyridilium dichloride (benzyl viologen); 6,7-dihydrodipyrido-(1,2-*a* : 2',1'-*c*)pyrozinediium dibromide (diquat); and 7,8-dihydro-6-H-dipurido-(1,2-*a* : 2'1'-*c*)diazepinediium dibromide (triquat); each at 8 μM. All samples were rapidly frozen in liquid N_2-cooled 2-methylbutane and methylcyclohexane (1 : 1) and stored at 77 K until needed. EPR spectroscopy, using a Varian E109-E instrument, was performed at various temperatures using either a liquid helium cooling system (Air Products, Heli-tran) or a Varian liquid N_2 EPR cooling accessory. Liquid helium was used for temperatures below 77 K. The spectrometer was coupled to a Digital Equipment Corp. PDP 11-34 minicomputer and spectra were stored for subsequent baseline subtraction and spectral analysis.

Cytochromes and flavoproteins

The cytochrome and flavoprotein content of mitochondria was measured according to the methods of Schollemeyer and Klingenberg [13], with minor modifications. Concentrations were obtained from oxidized minus reduced difference spectra (DW-2-UV-VIS spectrophotometer, American Instrument Co.) at 25°C. The following wavelength pairs and extinction coefficients were employed: cytochrome *a*, 605–630 nm, $\epsilon_{mM} = 24.0$ [14]; cytochrome b_{total}, 562–575 nm, $\epsilon_{mM} = 20.0$ [15]; cytochrome $c(+c_1)$, 550–540 nm, $\epsilon_{mM} = 19.1$ [16]; flavoprotein content was estimated at 465–510 nm, $\epsilon_{mM} = 11.0$ [16].

Electron transport

Succinate and NADH dehydrogenase activities were measured in submitochondrial preparations according to the method of Singer [17]. ATPase activity in submitochondrial preparations was assayed as described by Melnick et al. [18]. Reversed electron transport (ATP-energized succinate reduction of NAD^+) in the preparation was measured as described by Danielson and Ernster [19]. Protein was determined by a biuret procedure [20].

Mitochondrial and submitochondrial oxidase activities were measured polarographically at 37°C in a medium adapted [8] from that of Dow [21] contain-

ing 15 mM KCl, 0.4 mM NAD^+, 45 mM sucrose, 12 mM mannitol, 5 mM $MgCl_2$, 7 mM EDTA, 20 mM glucose, 0.2% bovine serum albumin (essentially fatty acid free), 30 mM potassium phosphate and 25 mM Tris, pH 7.4. Substrates used were 10 mM pyruvate + 2.5 mM malate, 10 mM succinate + 4 μM rotenone, or 5 mM ascorbate + 0.5 mM TMPD + 2 nM cytochrome *c*. Mitochondria were maximally stimulated with either 0.2 mM ADP + 1 μM FCCP. Respiratory control ratios and ADP/O ratios were determined according to the method of Chance and Williams [22].

Results

A dietary deficiency that causes dramatic changes in the molecular composition of mitochondria might be expected to result in overt physiological manifestations. After 42 days of age, the iron-deficient rats were 20% lower in weight than control animals. Iron-deficient rats' eyes and ears were pale; their hematocrits were down to about 18%, from 42%, and hemoglobin concentrations were down to 3.5, from 14.0 g/dl. If the iron-deficient rats were refed a normal diet, they rapidly gained weight and had no apparent lasting effects. The yield of mitochondria (mg/g muscle) from the iron-deficient animals was 20–30% lower than from controls. Mitochondria from the iron-deficient rats had a pale green-brown color rather than the usual red-brown.

The trypsin-isolated mitochondria did not metabolize free fatty acids but carnitine derivatives of fatty acids were readily oxidized. It appears that the specific activities of other enzymes were not affected by the trypsin in the isolation procedure, in agreement with the observations of Bullock et al. [7]. Mitochondrial ATPase, which is a not an iron-containing enzyme, had slightly higher specific activity in the iron-deficient submitochondrial preparations compared to the controls (control 1.0 μmol/min per mg, iron-deficient 1.2 μmol/min per mg) and was inhibited by oligomycin.

TABLE I

OXIDASE ACTIVITIES IN MITOCHONDRIA AND SUBMITOCHONDRIAL PREPARATION

Activities expressed as nmol O_2/min per mg protein.

	Controls	Iron deficient	% change
Mitochondrial oxidases			
Pyruvate-malate	416 ± 17	99 ± 5	−76
Glutamate	415 ± 20	157 ± 9	−62
Succinate	349 ± 17	98 ± 7	−72
Ascorbate + TMPD	1 567 ± 149	1 036 ± 54	−34
Submitochondrial oxidases			
NADH	648 ± 56	196 ± 27	−70
Succinate	204 ± 13	55 ± 5	−73
Ascorbate + TMPD	1 850 ± 132	1 133 ± 98	−39

Effects of iron deficiency on oxidase and dehydrogenase enzymes, energy coupling, and cytochrome and flavin content

All iron-containing enzymes studied exhibited decreased specific activities in both submitochondrial preparations and mitochondria from the iron-deficient animals. The data in Table I show that there were distinct differential changes in various iron-containing respiratory components of iron-deficient mitochondria. The least affected was cytochrome oxidase, and the most affected were the NADH- and succinate-oxidizing systems. Table II shows that respiratory control and ADP/O ratios were not

TABLE II

RESPIRATORY CONTROL (RCR) AND ADP/O RATIOS IN MITOCHONDRIA

Substrate	Control mitochondria		Iron-deficient mitochondria	
	RCR	ADP/O	RCR	ADP/O
Pyruvate-malate	6.00 ± 0.38	2.79 ± 0.17	6.61 ± 0.45	2.86 ± 0.21
Succinate	2.34 ± 0.21	1.97 ± 0.16	2.06 ± 0.27	1.90 ± 0.19
Ascorbate-TMPD	1.21 ± 0.09	1.10 ± 0.08	1.24 ± 0.08	1.11 ± 0.09

TABLE III

NADH AND SUCCINATE DEHYDROGENASE ACTIVITIES IN SUBMITOCHONDRIAL PREPARATIONS

	NADH dehydrogenase		Succinate dehydrogenase	
	V' [a]	K'_m [b]	V' [c]	K'_m [d]
Submitochondrial preparation				
Control	11.9 ± 0.7	3.13 ± 0.2	1.8 ± 0.2	0.48 ± 0.06
Iron deficient	2.0 ± 0.1	0.78 ± 0.1	0.4 ± 0.1	0.08 ± 0.01
% decrease	83		78	

[a] Apparent V, μmol $Fe(CN)_6^-$ reduced/min per mg protein.
[b] Apparent K_m (mM) with respect to $Fe(CN)_6^-$.
[c] Apparent V, μmol dichlorophenolindophenol reduced/min per mg protein.
[d] Apparent K_m (mM) with respect to phenazine methosulfate.

affected by iron deficiency. It is interesting to note that even though maximal rates of oxidation were suppressed by as much as 60–70%, there was no loss in the efficiency of conversion of ADP to ATP. It is clear that these iron-deficient mitochondria could readily perform their major function, ATP synthesis, albeit at a much lower rate than normal mitochondria.

The apparent V (V') value for NADH dehydrogenase was decreased 83% in submitochondrial preparations from iron-deficient animals, and V for succinate dehydrogenase was decreased 78% (Table III). Apparent K_m (K'_m) values for these enzymes were dramatically altered, possibly indicating changes at the acceptor dye-binding sites. Rates of reversed electron transport were decreased from 24.1 nmol/min per mg in control to 3.9 nmol/min per mg in iron-deficient submitochondrial preparations (83% decrease).

Mitochondrial cytochrome and flavoprotein concentrations were decreased 25–50% by iron deficiency (Table IV). Cytochrome aa_3 was the least affected cytochrome.

Fe-S clusters of Complex II, Complex III, and electron-transferring flavoprotein dehydrogenase

The absolute concentration of membrane-bound Fe-S clusters in submitochondrial preparations cannot readily be determined by EPR alone, however, differences in concentration of specific Fe-S clusters in control compared to iron-deficient submitochondrial preparations can be measured.

EPR spectroscopy following succinate reduction demonstrated a marked decrease in the g 1.93–1.94 feature of iron-deficient skeletal muscle submitochondrial preparations (Fig. 1). The g 2.00–2.02 region was much less affected by iron deficiency in both NADH- and succinate-reduced samples. In mitochondria from other tissues, succinate can reduce Fe–S components of Complex II, III and IV, with the exception of cluster 2 of Complex II, (designated S-2) *, apparently because its redox midpoint potential is too low to be reduced by succinate [23]. As there was complete reduction of cluster S-3 (indicated by the complete absence of a temperature-sensitive EPR feature at g 2.01), it is clear that cluster S-3 was not a component of the succinate-reduced spectra (S-3 is an Fe-S cluster that is paramagnetic only when oxidized [24] (Fig. 2). The main components that contributed to the succinate-reduced spectra were cluster S-1 of Complex II [23], electron-transferring flavoprotein dehydrogenase [25], the Rieske Fe-S cluster [26], and possibly a component of the outer membrane. In submitochondrial preparations, the outer membrane component would represent a very small contribution to the spectra at g 1.93 [27], which leaves electron-transferring flavoprotein dehydrogenase (g_z 2.086, g_y 2.083 and g_x 1.89) and cluster S-1 (g_z 2.03, g_y 1.93 and g_x 1.91) [28] as the principle clusters that contributed to resonances at g 1.93. The decrease in the g 2.03 signal, indicative of cluster S-1, was 38% in the iron-deficient submitochondrial preparations. As the g 1.93 derivative peak was decreased 60% by iron deficiency, and the g 1.89 peak was decreased 55%, electron-transferring flavoprotein dehydrogenase seems to be the cluster that was most decreased in the spectra of suc-

* Fe-S clusters from the NADH-ubiquinone reductase part of the respiratory chain are referred to as N-1, N-2, N-3, etc. Clusters from the succinate-ubiquinone reductase are called S-1, S-2 and S-3.

TABLE IV

MITOCHONDRIAL FLAVOPROTEIN AND CYTOCHROMES

Content expressed as nmol/mg mitochondrial protein.

Mitochondria	Flavoprotein	Cytochrome c ($+c_1$)	Cytochrome b	Cytochrome a
Control	1.22 ± 0.07	0.84 ± 0.04	0.42 ± 0.03	0.43 ± 0.02
Iron deficient	0.82 ± 0.04	0.43 ± 0.01	0.25 ± 0.01	0.32 ± 0.02
% decrease	32.7	48.8	40.4	25.5

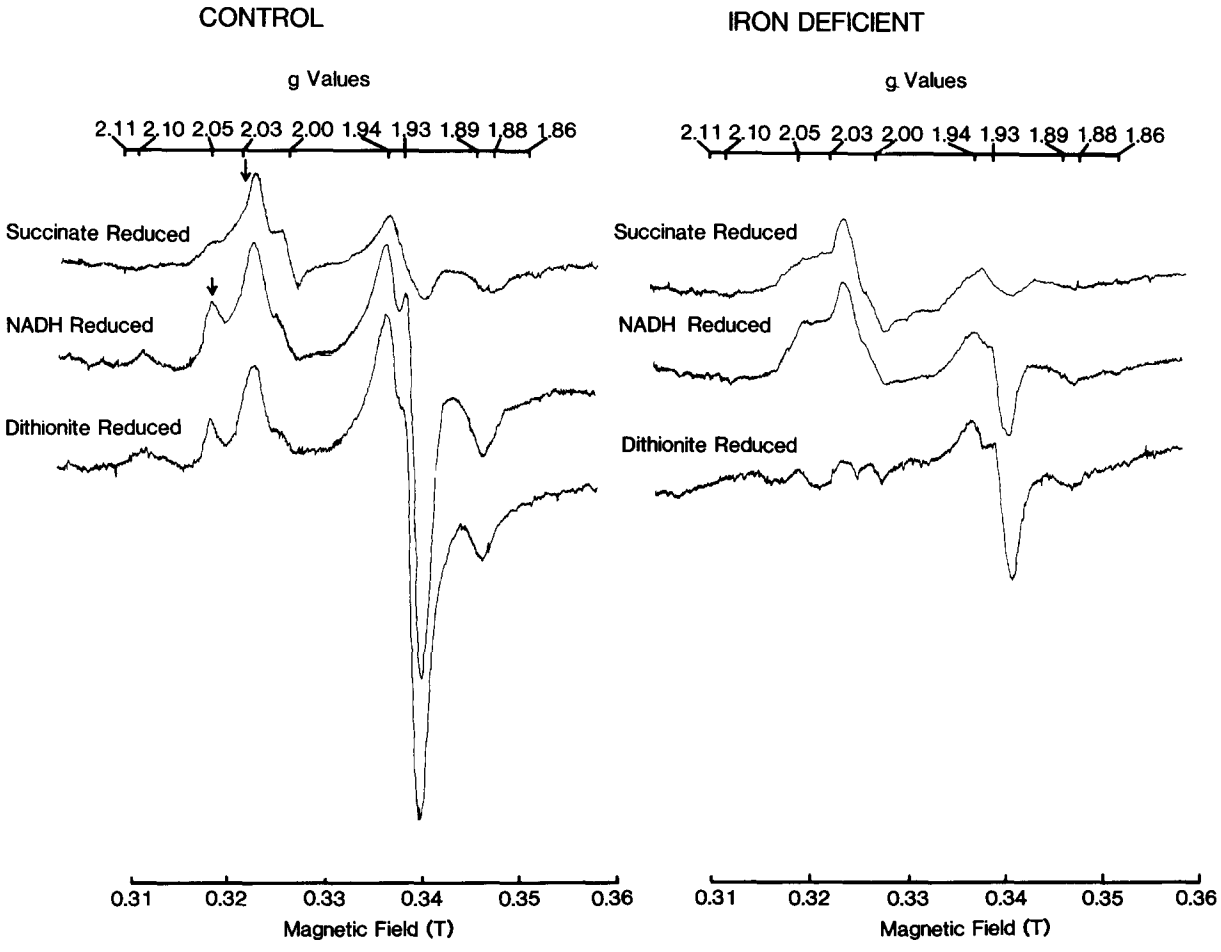

Fig. 1. Comparison of EPR spectra from control and iron-deficient submitochondrial preparations incubated anaerobically with various indicated substrates (5 mM) prior to freezing. The spectra were recorded at 20 K and the spectrometer settings were as follows: microwave frequency 9.14 GHz, modulation frequency 100 kHz and amplitude 1.0 mT, microwave power 10 mW, scan rate 25 mT/min, protein concentration was 10 mg/ml.

cinate-reduced iron-deficient submitochondrial preparations. Succinate might be expected to partially reduce cluster N-2 of Complex I, however, in these samples succinate did not produce a clear signal with a g value of 2.05 (indicative of cluster N-2) in either control or iron-deficient preparations [32].

The concentration of cluster S-3 was 59% lower in skeletal muscle submitochondrial preparations from iron-deficient animals than from controls. During redox titration of the preparations, a spin-spin interaction was readily observed. While the exact nature of this interaction is not clear, it appears to involve ubisemiquinone (QH·) and may be a QH·-QH· interaction, perhaps influenced by oxidized center S-3 [29, 30]. The hyperfine splitting of the g 2.01 feature was seen clearly with the potential poised at 149 mV. Redox titration of the g 2.01 oxidized component (cluster S-3) showed a midpoint potential (E_m) of +150 mV, which is also similar to that reported for another tissue (Fig. 3) [28].

Reduction of the control and iron-deficient submitochondrial preparations with ascorbate-TMPD, measured at 60 K, showed spectral features at g 2.03 and 1.89, suggestive of the Rieske Fe-S cluster [26]. Ascorbate-TMPD would not reduce the components of Complex I or II, and, in fact, center S-3 of Complex II was detectable at temperatures below 20 K indicating that it was still oxidized. There was no noticeable decrease in concentration of the Rieske cluster on comparing control and iron-deficient submitochondrial preparations at g 1.89. There was a small decrease at g 2.03 (about 20%). Resolution of the Rieske cluster in skeletal muscle submitochondrial preparations was very poor in comparison to Rieske centers from other tissues [25,31].

Fe-S clusters of mitochondrial Complex I

NADH and dithionite reduced the Fe-S proteins of Complex I and cluster S-2 of Complex II, in addition to those reduced by succinate. NADH reduction revealed decreases in the components at g 1.89, 1.93, 1.94, 205 and 2.10 in the iron-deficient submitochondrial preparations (Fig. 1). Probably the most unambiguously resolved component in these spectra was the $g_{\parallel}$ component of cluster N-2 [32] with a derivative peak at g 2.05. This peak was decreased 72% in the iron-deficient preparations. Dithionite reduction of the control submitochondrial prepara-

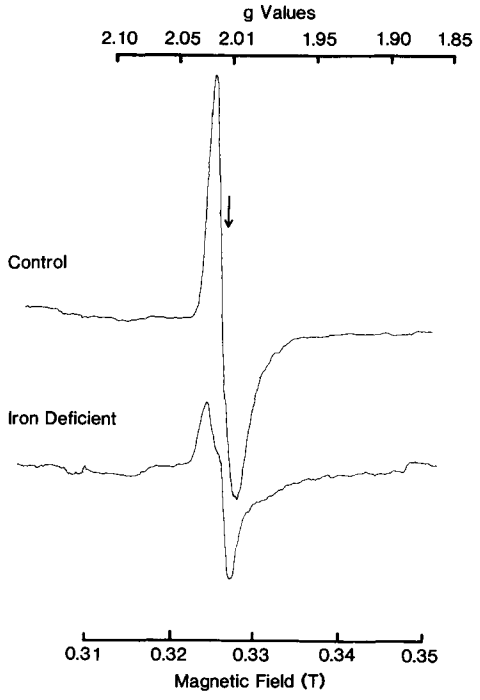

Fig. 2. Comparison of control and iron-deficient submitochondrial preparations at 10 mg/ml incubated with 5 mM $K_3Fe(CN)_6$ prior to freezing. EPR spectra were recorded at 10 K, and the spectrometer settings were the same as in Fig. 1, except that a lower gain was used.

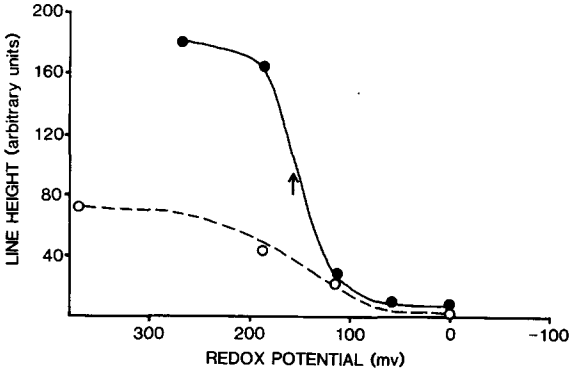

Fig. 3. Redox titration of the paramagnetic oxidized Fe-S cluster in control (•———•) and iron-deficient (o- - - - -o) submitochondrial preparations, at 10 mg/ml. The signal height is from the g 2.01 feature seen in Fig. 2. The voltage is expressed relative to a standard hydrogen electrode. The dashed line indicates a tentative fit for a one-electron carrier. $Na_2S_2O_4$ was the reductant and $K_3Fe(CN)_6$ the oxidant.

tions did not appear to resolve any components not visible by NADH reduction, however, an ambiguous spectrum was obtained with dithionite reduction of the iron-deficient preparations. In iron-deficient submitochondrial preparations, in which succinate or NADH reduction showed prominent g 2.01–2.02 derivative peaks, dithionite incubation resulted in a significantly altered EPR signal: dithionite or NADH reduction, however, produced equivalent signals at g 1.89, 1.93, 1.94, 2.05 and 2.10.

In order to determine the midpoint potential of some identified clusters, and to resolve which components contributed to the decreased g 1.93–1.94 signal in the iron-deficient submitochondrial preparations redox titrations were carried out in the presence of various mediator dyes. The only low-potential mediator dyes available are one-electron carriers which are themselves paramagnetic when reduced. Accordingly, spectral features at g 2.0–2.01, at potentials below about 100 mV, are due in part to the dyes used in the redox system.

Fig. 4 shows the redox titrations of iron-deficient and control submitochondrial preparations at 20 K.

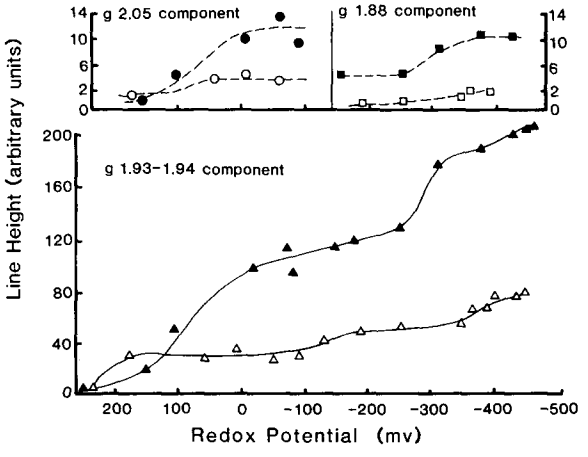

Fig. 4. Redox titration of the g 1.93–1.94 feature of control (▲———▲) and iron-deficient (△———△) submitochondrial preparations (prominent in Fig. 1) at 20 K. Protein concentration was 20 mg/ml. The insets show the simultaneous changes at g 2.05 (●, control; ○, iron-deficient) and g 1.88 (■, control; □, iron deficient). The dashed lines in the insets are tentative titration curves for determining the midpoint potential. The voltage is expressed relative to a standard hydrogen electrode. $Na_2S_2O_4$ was the reductant and $K_3Fe(CN)_6$ the oxidant.

In this study the overlap of the g 1.93 and 1.94 components did not afford differentiation and they have, therefore, been analyzed together. The $g_\perp$ and $g_\parallel$ components of center N-2 have been reported to exhibit g values of 1.93 and 2.05, respectively, in pigeon heart [32]. In this study, a g 1.93 component with an $E_{m7.5}$ value of approx. +55 mV titrated simultaneously with a g 2.05 derivative peak, in the control preparations. Because of the size of the g 2.05 peak, and because the amount of submitochondrial preparation available for the redox titrations was quite limited, $E_{m7.5}$ of the iron-deficient preparations could not be accurately measured. The $E_{m7.5}$ value of cluster N-2 determined seems to be higher than that reported in other tissues [1,32]. The remaining principal component of the g 1.93–1.94 signal at 20 K had an $E_{m7.5}$ value of about –300 mV, and also titrated in conjunction with another signal at g 1.88 (Fig. 4, inset). A recent study on rat liver submitochondrial preparations [34] suggests that center N-4, with features at g 1.94 and 1.88, might be the cluster detectable in our redox titrations at these g values, however, overlapping EPR signals and differences between different tissues make exact identification difficult. In the iron-deficient preparations, spectra taken at 20 K indicate that cluster N-2, and the

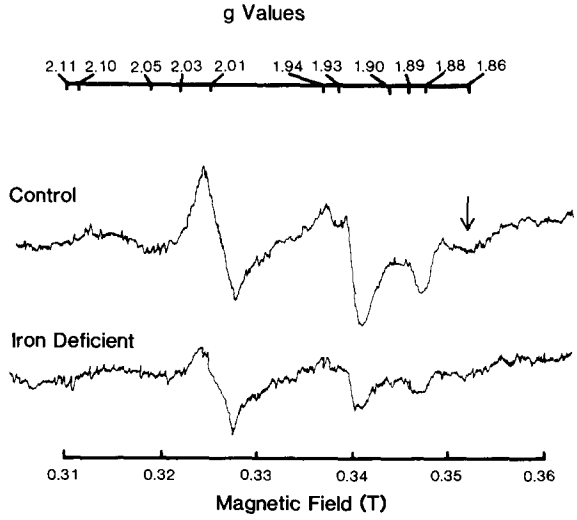

Fig. 5. EPR spectra of control and iron-deficient submitochondrial preparations incubated with 5 mM NADH prior to freezing, recorded at 10 K. Spectrometer settings were the same as in Fig. 1. Protein concentration was 10 mg/ml.

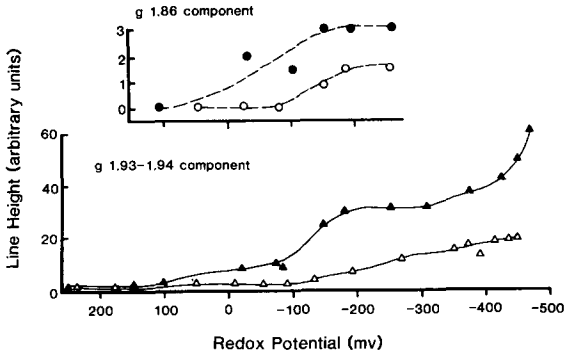

Fig. 6. Redox titration of the g 1.93–1.94 feature of control (▲——▲) and iron-deficient (△——△) submitochondrial preparations, at 10 K, see in Fig. 5, protein concentration was 20 mg/ml. The inset displays the titration of the g 1.86 feature (•, control; ○, iron deficient). The dashed titration curve of the inset is a tentative measure of the midpoint potential. The voltage is expressed relative to a standard hydrogen electrode. $Na_2S_2O_4$ was the reductant and $K_3Fe(CN)_6$ the oxidant.

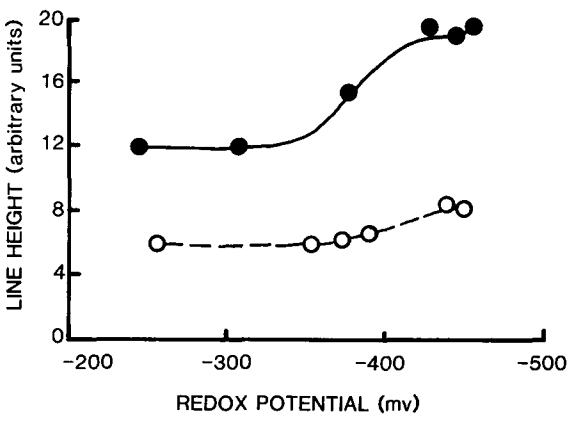

Fig. 7. Redox titration of the g 1.93–1.94 feature (N-1) at 90 K, at low poised potentials. The voltage is expressed relative to a hydrogen electrode. The sample is the same as in Figs. 4 and 6.

$E_{m7.5}$ −300 mV component (which is perhaps cluster N-4) were dramatically decreased in concentration.

Spectra recorded at 10 K of samples reduced by NADH are shown in Fig. 5. A feature at g 1.86 can be identified in these spectra. Redox titrations at 10 K revealed a cluster with an $E_{m7.5}$ of −130 mV that was almost completely absent in the iron-deficient submitochondrial preparations (Fig. 6). The concmitant appearance of a g 1.86 peak suggests that this was in part cluster N-3 (Fig. 6, inset) [34]. Cluster N-1b might also be expected to contribute to the g 1.93–1.94 signal, however, the identification of center N-1B was not clear at this temperature [32]. The g 1.86 feature of Fig. 5 was poorly resolved and estimation of the midpoint redox potential could not be made accurately. The redox titration at 10 K demonstrated another feature with an $E_{m7.5}$ of less than −450 mV that was not apparent at 20 K. This feature was not fully resolved but it is apparent that in the iron-deficient submitochondrial preparations it was significantly decreased. Based on previously reported g values and midpoint potentials, of cluster N-1a

TABLE V

SUMMARY OF Fe-S PROTEIN RESULTS

Fe-S cluster	Principal g values used for identification	How determined	$E_{m,7.5}$	% decrease in iron deficient submitochondrial preparation
S-1	2.03	Succinate reduction	–	38
S-3	2.01	$Fe(CN)_6$ oxidation	+150	59
N-1	1.93–1.94	Redox titration	−380	61
N-2	2.05, 1.93	NADH reduction and redox titration	+55	73
N-4 [a]	1.93–1.94, 1.88	Redox titration	−280	65
N-3 [a]	1.93–1.94, 1.86	Redox titration	−120	46
Electron-transferring flavoprotein dehydrogenase	1.89	Succinate reduction	–	66
Rieske (Fe-S_{bc-1})	1.89	Ascorbate-TMPD reduction	–	20

[a] Assignment of g values and $E_{m7.5}$ should be considered tentative because of low resolution of g_x components in these experiments and the likelihood of overlapping features in the g_y (1.93–1.94) area of the spectrum.

[32], it seems possible that the low-potential feature detected at 10 K is this cluster.

To measure cluster N-1 more quantitatively, the redox titration of submitochondrial preparations was measured at 90 K (Fig. 7). At this temperature, contributions to the EPR spectra of clusters N-2, N-3 and N-4 are insignificant. The titration represented a composite of cluster N1a and N1b [2] and the $E_{m7.5}$ obtained (−380 mV) was also a composite of these two features. The concentration of cluster N-1 was 62% lower in iron-deficient submitochondrial preparations than controls. Table V summarizes the results of Fe-S measurement by EPR..

Discussion

The studies of normal and iron-deficient mitochondrial Fe-S proteins and bioenergetics suggest the following conclusions which are subsequently discussed. (1) The Fe-S proteins of normal muscle mitochondria are similar to those of other tissues and species. (2) Severe dietary iron deficiency decreases the Fe-S cluster content of skeletal muscle mitochondria. Fe-S proteins would appear to be integral components of mitochondrial electron transport and decreases in mitochondrial Fe-S protein content limit dehydrogenase activity, and ultimately, oxidase activity. (3) The decrease in mitochondrial Fe-S protein content, induced by severe dietary iron deficiency, is probably a nonrandom process resulting in more complete Fe-S depletion from some mitochondrial complexes, but no depletion from the remaining functional complexes.

Fe-S proteins of normal muscle mitochondria

The midpoint potentials of Fe-S clusters N-2, N-3 and N-4 were all slightly higher than those reported for various other tissues (beef heart, pigeon heart, pigeon breast, and rat liver) [1]. Cluster N-1 exhibited a slightly lower $E_{m7.5}$ than previously reported [33], whereas cluster S-3 was similar [24]. The fact that the dipolar coupling of ubisemiquinone (g 2.04 and 1.96) was clearly visbible at a redox potential poised at 149 mV, as previously reported [2], may be taken as evidence of the accuracy of potentiometric measurements. The differences in midpoint potential between rat skeletal muscle Fe-S clusters and those of other tissues may be reflectice of slightly different protein environments. Midpoint potential measurements from rat skeletal muscle were not, however, sufficiently different to suggest any unique arrangement of Fe-S proteins. The overall EPR spectral characteristics of muscle submitochondrial preparations Fe-S clusters were also remarkably similar to those from other tissues [1,2]. Temperature dependence, and the relative contribution of each Fe-S cluster to the EPR spectra, were also not significantly different.

Fe-S proteins and mitochondrial bioenergetics

The relatively simple technique of dietary iron deficiency would appear to provide an excellent mechanism with which to probe further the energy-transducing systems of mitochondria. Dietary iron deficiency induced dramatic decreases in the Fe-S cluster content of mitochondria. These changes were most pronounced in skeletal muscle, but were also evident in mitochondria from liver (data not shown). Mitochondria isolated from iron-deficient rats' skeletal muscle were comparable to those isolated from control rats in respect to ATPase activity, a non-iron-containing component.

The concentrations of all Fe-S proteins studied were decreased by iron deficiency. Both degradation of previously assembled clusters, and the inability to continue normal rates of Fe-S protein synthesis may have been contributing factors. Whether the protein components of iron-containing enzymes continued to be synthesized is not known. The protein 'shells' of iron-containing enzymes, however, constitute a large number of mitochondrial proteins and their loss would be expected to increase the specific activities of non-iron-containing enzymes; however, this did not occur to any significant degree. Resolution of the g 1.93–1.94 feature by redox titration helped quantitate some specific changes that occurred in the Fe-S clusters of submitochondrial preparations.

Why dithionite did not cause the appearance of a signal at g 2.0–2.02 in the iron-deficient mitochondria is not understood. Succinate and NADH reduction demonstrated the existence of this spectral feature, and also showed that the pathway of reduction via ubiquinone was operative. Cluster S-3 (oxidized paramagnetic) from succinate dehydrogenase was reduced by dithionite. Whatever dithionite did to this cluster, it was the exception, as the only significant

alterations to other features of the EPR spectra of iron-deficient submitochondrial preparations were decreases in concentration.

Decreases in yeast mitochondrial content of NADH dehydrogenase Fe-S clusters reduce the overall rate of Complex I activity, when cells are grown in media of low iron content [4]. Similarly, chronic ethanol consumption decreases both the liver mitochondrial Fe-S protein content of Complex I, and the activity of NADH dehydrogenase [34]. Trumpower et al. [35] have also demonstrated that extraction of the Rieske Fe-S protein from Complex III results in loss of ubiquinol-cytochrome *c* reductase activity, and that reconstitution restores activity. Additionally, studies on isolated Complex II have correlated loss of cluster S-3 with loss of enzymatic activity [28], and have shown that cluster S-1 is integrally involved in succinate dehydrogenase activity [1]. These studies provide evidence of the essential roles of Fe-S clusters in Complex I, II and III activities. In the present study, the similarities between decreases in Fe-S cluster content and dehydrogenase activities strongly implicate Fe-S clusters as essential components of electron transport. Decreases in K'_m values of the acceptor dyes, $K_3Fe(CN)_6$ and phenazine methosulfate (in NADH and succinate dehydrogenase, respectively) were also observed. It appears that the electron acceptors may have been binding at altered active sites.

Succinate and NADH oxidase activities appear to have been limited by the suppressed dehydrogenase activities. Since ubiquinol-cytochrome *c* reductase and cytochrome oxidase activities have much higher rates of activity than NADH and succinate oxidase, it seems unlikely that they limit O_2 consumption [36]. In the present study, cytochrome concentration was decreased less than were dehydrogenase activities or rates of reversed electron transport. It therefore appears that electron flow through the Fe-S clusters limits dehydrogenase activities, which, in turn, limits maximal rates of substrate oxidation.

In this context, it is important to note that normal mitochondria rarely, if ever, perform at their *V* activity, which is the condition under which most biochemical studies are performed. It has also been shown that normal muscle maximal O_2 consumption is greatly in excess of whole animal maximal O_2 consumption [8]. Thus, it is not particularly surprising that iron-deficient mitochondria, while 70 and 73% decreased in NADH and succinate oxidase activities, respectively, were adequate for physiological maintenance at rest. Although maximal oxidase activities decreased enormously, the efficiency of ATP synthesis was not decreased at all. This indicates that the working mitochondrial electron-transport complexes all operated in a fully functional manner. It follows that the sequence of electron flow essential for energy coupling was not altered, as this would presumably decrease the efficiency of ATP synthesis, but instead there was a reduced number of functional electron-carrying components. The remaining functional components in the iron-deficient mitochondria were able to maintain an adequate electrical and proton gradient to facilitate ATP synthesis.

This study has focused on the Fe-S clusters that can be identified and are likely to be integral components of the respiratory chain. It should be noted that cytochromes and Fe-S clusters account for only about 66% of the total iron in mitochondria. The nonheme, non-Fe-S iron has recently been measured by Tangerås et al. [37], who speculated that it might be an iron pool available for heme synthesis. As this iron has not been shown to have a role in electron transport or energy coupling, it was not evaluated in this study.

Mechanism of Fe-S protein depletion

From comparisons of Complex I activities and EPR changes in the iron-deficient submitochondrial preparations, it seems reasonable to assume that any molecule of Complex I cannot readily exchange electrons with other molecules of Complex I, and that each identified Fe-S cluster is an integral part of the electron-transport chain. In the present study, only four of the Complex I Fe-S proteins were identified so that estimates of Fe-S cluster loss are probably low. If iron were removed randomly during iron deficiency, there would necessarily be a much larger decrease in NADH dehydrogenase activity than was actually observed. A random removal of clusters would have resulted in less than 2% of complexes having all four clusters, and NADH dehydrogenase activity would have decreased more than 98%. In actuality, the activity of NADH dehydrogenase was decreased by 83% and NADH oxidase by 70%. This suggests that there was sequential removal of Fe-S

protein iron from mitochondrial complexes, and that the removal was not random.

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