THE PATTERN OF IRON–SULFUR CENTERS IN BROWN ADIPOSE TISSUE MITOCHONDRIA: PREPONDERANCE OF ETF DEHYDROGENASE AND INVARIANCE WITH THE THERMOGENIC STATE

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1. Introduction

Most of the resonances observed in heart mitochondria by e.p.r. spectroscopy at presently attainable sensitivities have been recently identified (for review, see [1]) inculding the so-called iron–sulfur (Fe–S) 'center 5' [2], recognized by its low field resonance (g_z = 2.086) in the reduced state of mitochondria from a variety of species and tissues [1]. The assignment of this e.p.r. line was based on the purification of a thus far unknown iron–sulfur (Fe–S) flavoprotein from beef heart mitochondria [3]. Since the purified Fe–S flavoprotein was most readily reduced by fatty acyl-CoA in the presence of acyl-CoA dehydrogenase and electron-transferring flavoprotein (ETF), it was suggested [3] that this Fe–S flavoprotein may function as an electron transfer protein between the fatty acyl-CoA dehydrogenase system [4] and the terminal part of the respiratory chain. In support of this conclusion, we would like to present further experimental evidence based on e.p.r. spectra of mitochondria from brown adipose tissue, a tissue highly specialized for fatty acid oxidation and non-shivering thermogenesis (for review, see [5]). Furthermore, we want to present results of e.p.r. measurements on whole brown adipose tissue during the entire period of adaptation to a cold environment, which establishes the suitability of e.p.r. spectroscopy as a non-destructive tool for studying aspects of cold-acclimatization related to a change in the rate of mitochondrial biogenesis in this tissue.

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2. Methods

2.1. *Animals*

Weaned guinea pigs of an inbred strain from Marvin O'Brien, Rt. 3, Syene Road, Madison, WI were used. Approximately 4 weeks old, the animals were transferred from an environment of 22°C to 5–6°C in which they remained for different lengths of time [6].

2.2. *Isolation of mitochondria*

Brown adipose tissue mitochondria were isolated essentially as described [7,8]. Heart mitochondria were prepared by the nagarse method [9]; the final mitochondrial pellet was washed twice. Protein was determined by the biuret method after precipitation with trichloroacetic acid and dissolution of the pellet in the presence of 2% deoxycholate.

2.3. *E.p.r. spectroscopy*

E.p.r. spectroscopy and anaerobic experiments were carried out as described [10,11]; complete reduction was achieved by addition of various substrates and dithionite. The samples were frozen following a reaction period of one minute at 25°C. Samples of whole heart or brown adipose tissue were frozen 3–4 min after decapitation of the animal. Because of the relatively long time required for this procedure, the respiratory chain components in these tissues were largely reduced [11].

3. Results and discussion

3.1. *E.p.r. spectra of isolated mitochondria and whole tissue*

Fig.1A-B shows typical e.p.r. spectra at 13.3°K of isolated mitochondria from brown adipose tissue and heart of cold-acclimated guinea pigs in the completely reduced state. The same principal resonances are seen in both spectra indicating that there are no detectable qualitative differences between the Fe–S centers of these two types of mitochondria. The same conclusion is valid also for the spectra obtained in the oxidized state (not shown). Since a detailed interpretation of the e.p.r. signals from heart mitochondria has been given previously (for review, see [1]), only the most significant features of those from brown adipose tissue mitochondria are considered here. The most prominent difference between the spectra from the two tissues is observed in the region arround $g = 2.1$, notably in the resonances centered at $g = 2.10$ and $g = 2.086$. Thus, an exceptionally strong line at $g = 2.086$, relative to that at $g = 2.10$, is seen in the brown adipose tissue mitochondria.

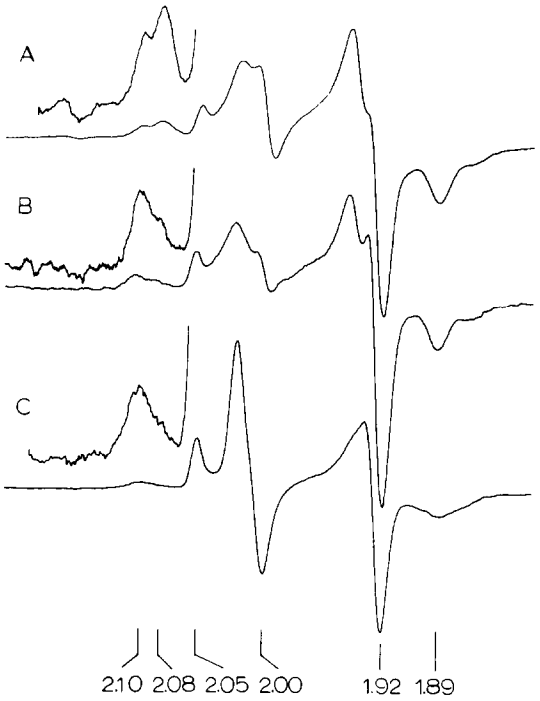

Fig.1. E.p.r. spectra of mitochondria. (A) Brown adipose tissue mitochondria of guinea pigs (pooled sample of 8 animals) were suspended in 0.25 M sucrose, 1 mM HEPES, pH 7.3 at 66.2 mg per ml and reduced anaerobically (see Methods). (B) Heart mitochondria of the same animals as in (A) were suspended in the same buffer at 19.5 mg per ml and reduced anaerobically. (C) Blowfly flight muscle mitochondria, approx. 40 mg per ml, of a solution containing 0.15 M KCl, 10 mM Tris, 1 mM EDTA, 0.5% bovine serum albumin, pH 7.3 and reduced anaerobically for 5 min at 30°C with 0.1 M *sn* -glycerol 3-phosphate. The conditions of e.p.r. spectroscopy were: microwave power, 2.7 mW; modulation amplitude and frequency, 800 G and 100 KHz, respectively; scanning rate, 400 G per min; time constant, 0.5 sec; and temperature, 13.3°K. The amplification ratios with respect to the main spectrum of (A) were: (A, inset) 6.4; (B, main curve) 2.5; (inset) 16; (C, main curve) 1; (inset) 10.

Fig.2A-C shows e.p.r. spectra recorded on samples

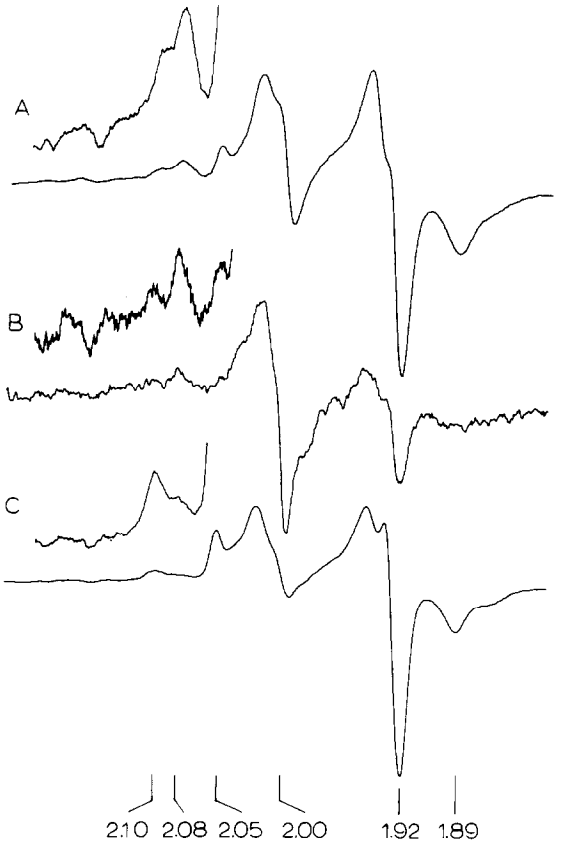

Fig.2. E.p.r. spectra of whole brown adipose tissue of cold-acclimated (A) and warm-acclimated guinea pigs (B). (C) Whole heart tissue of cold-acclimated guinea pig. The conditions of e.p.r. spectroscopy were as in fig.1. The amplification ratios with respect to the main spectrum of (A) were: (A, inset) 6.4; (B, main curve) 5; (inset) 18.6; (C, main curve) 0.64; (inset) 4. Because of the poor signal-to-noise ratio the enlarged spectrum of the low field peak of fig.2B was obtained through a signal averager (8X; Nicolet 1020 A).

of whole heart and brown adipose tissue of warm- and cold-acclimated guinea pigs. No significant difference was observed in the heart preparations from animals in either thermogenic state and the relative intensities of the e.p.r. lines (fig.2C) are similar to those previously reported for rat heart [12]. On the other hand, from a comparison of fig.2A and B, it is seen that the content of e.p.r.-detectable components is almost 8 times higher in brown adipose tissue of cold-acclimated animals (fig.2A) as compared to the warm-acclimated animals (fig.2B). Except for this difference in total concentration, however, the spectra are very similar, and particularly the relatively strong line at $g = 2.086$ is seen in both thermogenic states.

3.2. *Assignment of the* g = *2.086 line to ETF dehydrogenase*

From the comparison of the e.p.r. spectrum of brown adipose tissue mitochondria (fig.1) with that of heart mitochondria it is evident that the line at $g = 2.086$ belongs to a component most abundant, and therefore probable specifically required, in the metabolism of brown adipose tissue mitochondria. Characteristic features of these mitochondria are their high capacity for oxidation of fatty acids and *sn*-glycerol 3-phosphate as well as their high content of flavoproteins related to the oxidation of these substrates [5].

That the newly discovered Fe–S flavoprotein with $g_z = 2.086$ [3] is unrelated to the *sn*-glycerol 3-phosphate dehydrogenase is supported by two experimental facts. First, purified preparations of the Fe–S flavoprotein have revealed only minimal or no *sn*-glycerol 3-phosphate dehydrogenase activity [3]. Secondly, the flight muscle of a number of insects is probably the tissue richest in *sn*-glycerol 3-phosphate dehydrogenase activity. From fig.1C it is seen, however, that the e.p.r. spectrum of flight muscle mitochondria from blowflies shows only a weak line at $g = 2.08$, when these mitochondria are reduced anaerobically by *sn*-glycerol 3-phosphate. Thus, the high level of the Fe–S flavoprotein in brown adipose tissue and the low level in insect flight muscle are strong evidence that the Fe–S flavoprotein represented in the line at $g = 2.086$ is indeed linked to the acyl-CoA dehydrogenase system.

Whereas the fatty acyl-CoA dehydrogenases and ETF are only loosely, if at all, bound to the mitochondrial inner membrane (they are readily recovered in the soluble fraction on sonication [13]), the Fe–S flavoprotein is tightly bound to the inner membrane. It would then appear that transfer of reducing equivalents to the terminal electron transfer system is only possible via a membrane-bound electron carrier as is also the case in the succinate and the NADH dehydrogenase systems. In view of the accumulating evidence concerning its function, we propose the tentative term 'ETF dehydrogenase' for the new Fe–S flavoprotein.

3.3. *Estimation of the ETF dehydrogenase concentration*

The availability of purified ETF dehydrogenase [3] and of well resolved e.p.r. spectra from tissues and mitochondria containing this protein, make it possible to estimate its concentration in various tissues. Since ETF dehydrogenase has a single Fe–S center and thus an uncomplicated and easily interpretable e.p.r. signal and can also be obtained in high purity [3], it is to date probably the respiratory carrier of this type most suited for quantitative considerations. We estimate that mitochondria from brown adipose tissue of cold-acclimated guinea pigs contain 60 nmol of ETF dehydrogenase per g of protein (Biuret) and in whole tissue we calculate 2.5 to 3.0 nmol per g of fresh weight; the concentration in whole tissue from warm-acclimated guinea pigs was 1/5 to 1/10 of this value. The concentration of ETF dehydrogenase in guinea pig heart mitochondria was, on a protein basis, equal to that in brown adipose tissue mitochondria, but the concentration of Fe–S centers belonging to other parts of the electron transfer system is obviously higher in heart. From a comparison of the peaks of center 2 of NADH dehydrogenase ($g = 2.054$) and ETF dehydrogenase ($g = 2.086$) and the double integrals of spectra of the pure species [3,10,12] we find that the NADH dehydrogenase concentration in brown adipose tissue is approx. 0.5 and in heart 2 times that of ETF dehydrogenase. These estimates are in good agreement with estimates arrived at independently from the concentration of FMN and acid extractable FAD in heart mitochondria [1]. In comparison to other tissues, in which the fatty acid oxidation pathway is of lesser importance, the quantitative relationship of the Fe–S centers of ETF dehydro-

genase to those of NADH dehydrogenase observed in brown adipose tissue more closely reflects the balance of these enzymes required in the oxidation of fatty acids.

If the e.p.r. signal from the high potential type of Fe–S proteins (g = 2.01, in the oxidized state) as observed at 13.5 mW of microwave power and 7.1°K is taken as a measure of the concentration of succinate dehydrogenase, then the concentration of this enzyme in brown adipose tissue is about 1.3 times that in heart.

3.4. *Time course of the increase in mitochondrial Fe–S centers (proteins) of brown adipose tissue during cold exposure*

Although it is well established that there is a considerable increase in the mitochondrial mass of the brown adipocytes during the initial period of cold-acclimation (for review, see [5]), it has been found difficult to provide accurate estimates of the rate of this increase based on electron microscopy or recovery of mitochondria on differential centrifugation of homogenates. We have, therefore, approached this problem by measuring the increase in mitochondrial electron carriers in whole tissue using e.p.r. as a non-destructive technique of quantitative assay.

Since at early stages of acclimation the signal to noise ratio of whole adipose tissue is poor, we chose the amplitude of the strong line centered at g = 1.93 (fig.2) as a measure of the concentration of Fe–S centers present. We are aware that this line has contributions from a number of Fe–S centers belonging to different dehydrogenases [1], but since we did not observe any differences in the pattern of development of the various signals at different stages of acclimation, we consider this as a valid relative measure of total Fe–S centers. The time course of the increase of the line at g = 1.93 in whole tissue with acclimation is shown in fig.3. Although there is obviously a great individual variation in the rate at which the guinea pigs adapt to the cold environment, the transition from the warm- to the cold-acclimated state appears on an average to take approximately 14 days of cold exposure (5°C). The new steady state is characterized by a 5- to 10-fold increase in mitochondrial Fe–S centers (proteins) per wet weight of tissue, and this increase is largely due to an increased concentration of mitochondria [5]. When compared to previous

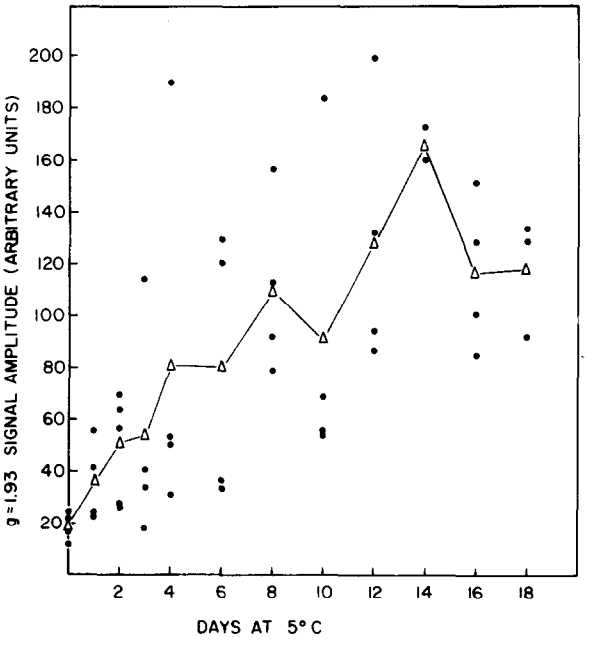

Fig.3. Time course of the increase in the signal intensity of the composite high field line (centered at g = 1.93) of various Fe–S centers in whole brown adipose tissue of guinea pigs during exposure to 5°C. The conditions of e.p.r. spectroscopy were as indicated in the legend to fig.1. Each point represents a single experimental animal; the triangles the arithmetic average.

studies (for review, see [5]) on the induction of loose-coupling of these mitochondria, being fully manifest after 3–4 days under similar experimental conditions, the process leading to stimulation of mitochondrial biogenesis appears to be a slower process. The mechanism of this process, however, remains to be elucidated.

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