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Two modes of irreversible inactivation of the mitochondrial electron-transfer system by tetradecanoic acid

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Bovine heart submitochondrial particles were incubated for 2–6 h at 37°C with various concentrations of tetradecanoic acid, and the effects on the activities, the total acid-labile sulphide content and EPR spectra of the electron transfer system were studied. Two distinct time-dependent processes of the slow irreversible inactivation of the electron-transfer system were found. They differ in the concentration of tetradecanoic acid required. The more specific effect, induced by 100–400 nmol tetradecanoic acid per mg protein, consists of a selective blockage of electron transfer between the Fe-S clusters of the NADH dehydrogenase and ubiquinone, without damage to any of the Fe-S clusters. Higher concentrations of tetradecanoic acid caused gradual destruction of all Fe-S clusters of NADH dehydrogenase and of the 3-Fe cluster of succinate dehydrogenase, leading to complete inactivation of both NADH and succinate oxidation.

Introduction

A slow irreversible inactivation of the respiratory chain in submitochondrial particles by free fatty acids, differing from the well-known instantly reversible inhibition [1], was discovered by Ludwig and co-workers [2,3] Tetradecanoic acid (myristic acid) was shown to be the most effective among the even-numbered saturated fatty acids. The inactivation is localized in the NADH:Q oxidoreductase segment of the electron transfer system and is strongly dependent on temperature. Inactivation of the respiratory chain in submitochondrial particles caused by heating to 40–45°C is due to the action of endogenous free fatty acids [4]. The present communication deals with the characterization of the sites of the inactivations.

Materials and Methods

Submitochondrial particles from bovine heart were prepared essentially as described by Crane et al. [5]. Disintegration of the mitochondria was achieved, however, by sonification. The particles were stored in liquid nitrogen until used. Activity measurements of the several individual enzyme systems of the respiratory chain were performed spectrophotometrically or with an oxygen electrode at 37°C [3,6,7]. Acid-labile sulphur was assayed according to King and Morris [8]. EPR spectra were recorded on a Varian E-9 EPR spectrometer. Cooling of the samples and processing of the spectra were described elsewhere [9,10].

Results

Activities of a suspension of submitochondrial particles treated aerobically with varying amounts

of tetradecanoic acid for 2 h at 37°C are shown in Fig. 1. The contents of acid-labile sulphur of the washed particles are also given in this figure. In the range between 60 and 400 nmol tetradecanoic acid per mg protein, inhibition of the NADH oxidase activity occurred with little effect on the NADH-ferricyanide activity or acid-labile sulphur content. The succinate-cytochrome *c* activity was markedly enhanced. The selective inactivation of the NADH oxidase activity was identical to that reported earlier [2,3]. An increase in the concentration of tetradecanoic acid up to 1.8 μmol per mg protein, however, caused complete inhibition of the NADH-ferricyanide and succinate-cytochrome *c* oxidoreductase activities, as well as a two-thirds loss of acid-labile sulphur. Identical results were obtained after treatment with oleic acid under anaerobic conditions (not shown), excluding the involvement of oxidative processes.

In order to check whether the effects at high concentrations of tetradecanoic acid might be due to solubilization of components of the electron transfer system, the incubated suspensions were centrifuged and the enzymic activities were determined separately in the supernatant fluids and in the resuspended particles. Both the incubation with tetradecanoic acid and the centrifugations were done under anaerobic conditions. The supernatant fluids contained only about 8% of the NADH-ferricyanide oxidoreductase and about 3% of the succinate-phenazine methosulphate oxidoreductase activity, whether tetradecanoic acid was present or not (not shown). There was also no increase in the total protein content of the supernatant fluids, even after high concentrations of tetradecanoic acid had been used. The enzymic activities of the resuspended particles (Fig. 2) were

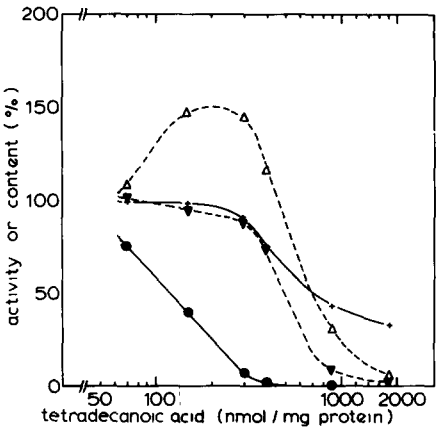

Fig. 1. Acid-labile sulphur content and enzyme activities of submitochondrial particles treated with various concentrations of myristic acid Submitochondrial particles (1.68 mg protein per ml in 0.25 M sucrose and 10 mM phosphate buffer (pH 7 4) were incubated aerobically for 2 h at 37°C in portions of 5 ml each with amounts of tetradecanoic acid (added as methanolic solutions) as indicated in the figure. The final methanol concentration was 1% (v/v) in all cases. Enzyme activities, expressed in percentages of that of a control incubated with methanol, were assayed directly in the incubation mixtures. Acid-labile sulphur in the particles was determined after centrifugation and resuspension in 0.5 ml buffered sucrose. +——+, acid-labile sulphur content (control· 5.95 nmol S^{2-} per mg protein); ●——●, NADH oxidase activity (control 10.0 nkat/mg protein; ▼------▼ NADH-ferricyanide oxidoreductase activity (control 66.3 nkat/mg protein), △- - - - -△ succinate-cytochrome *c* oxidoreductase activity (control 3 12 nkat/mg protein). The symbols indicate mean values of double or triplicate experiments

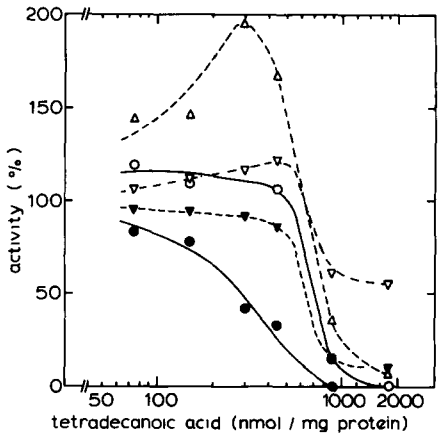

Fig. 2 Enzyme activities of washed submitochondrial particles after treatment with tetradecanoic acid. Samples of 1.68 mg protein of freshly prepared submitochondrial particles (different batch from that in Fig. 1 and Table I) in 1 ml 0.25 M sucrose and 10 mM phosphate buffer (pH 7.4) were incubated for 2 h at 37°C in a nitrogen atmosphere with amounts of the fatty acid as indicated. Thereafter the suspensions were centrifuged anaerobically and the particles were resuspended in the original volume of buffered sucrose for enzyme assay. O——O succinate oxidase activity; ▽·-·-·▽ succinate-phenazine methosulphate-dichlorophenolindophenol oxidoreductase activity; other symbols as in Fig 1. Activities are expressed in percentages of that of a control incubated with methanol. Control activities (nkat/mg protein): succinate oxidase, 3.35; succinate-cytochrome *c* oxidoreductase, 3.9; succinate-phenazine methosulphate-dichlorophenolindophenol oxidoreductase, 27.9; NADH oxidase, 4.4; NADH-ferricyanide oxidoreductase, 21.8.

similar to those in the experiment in Fig. 1. Control incubations with methanol, used as the solvent of tetradecanoic acid, did not affect the activities except for a decrease up to 50% in the succinate-cytochrome *c* oxidoreductase activity. This inactivation was presented by low concentrations of tetradecanoic acid (Figs. 1 and 2). Moreover, low concentrations of tetradecanoic acid caused a slight stimulation of the other succinate dehydrogenase activities (Fig. 2). The loss of sulphide as depicted in Fig. 1 is due to real destruction of this form of sulphur, rather than to solubilization, since identical results were obtained upon analysis of the total suspension of submitochondrial particles (not shown).

The effects of low and high concentrations of tetradecanoic acid on submitochondrial particles were also studied by means of EPR spectroscopy. Spectra from particles after treatment with a low concentration of tetradecanoic acid, which strongly inhibited the NADH oxidase activity but had little effect on the NADH-ferricyanide oxidoreductase activity (Table I), are shown in Fig. 3. Trace A is the spectrum at 52 K of the treated and washed particles without any additions. The broad trough around $g = 1.99$ is due to the $g_{x,y}$ lines of oxidized Cu_A in cytochrome *c* oxidase [11]. Trace B was obtained when these particles were mixed with an excess of NADH for 10 s at 0°C. The difference in amplitude between the spectrum at $g = 2.07$ and $g = 1.99$ is a measure of the amount of oxidized Cu_A. As seen from a comparison between the traces A and B, there was no apparent reduction in Cu_A after addition of NADH. The additional lines in trace B at $g = 2.025$ and around $g = 1.93$ are characteristic for reduced cluster 1 of NADH dehydrogenase [12]. Trace C is the spectrum of control particles to which 518 nmol tetradecanoic acid per mg protein was added under conditions identical to those in trace A except for omission of the 6-h incubation at 37°C. No inhibition of NADH oxidase was noticed after washing with serum albumin. Reduction with NADH was performed as for the samples in trace B. The spectrum is quite different from that in trace B: Cu_A is more reduced and now also the characteristic lines were observed of the reduced [2Fe-2S] cluster [12] of succinate dehydrogenase ($g_{x,y,z} = 1.91$, 1.93, 2.03). The trough around $g = 1.89$ indicates that

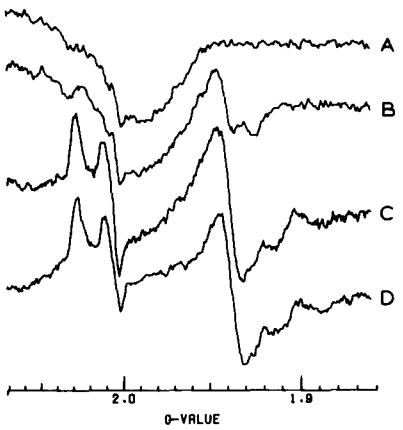

Fig 3 Effect of the incubation of submitochondrial particles with a low concentration of tetradecanoic acid on their EPR spectra at 52 K Particles were treated with 259 nmol of tetradecanoic acid as described in Table I. (A) Particles in the oxidized state. (B) As A but after mixing with 7 mM NADH for 10 s at 0°C before freezing in liquid nitrogen. (C) Control particles that were only shortly (5 min at 37°C) incubated with 518 nmol of tetradecanoic acid per mg protein and subsequently washed as described in Table I. Particles were then mixed with 7 mM NADH for 10 s at 0°C and frozen in liquid nitrogen. (D) Particles as in B, but with subsequent mixing with a few grains of $Na_2S_2O_4$ for 1 min at 0°C before freezing. EPR conditions. microwave frequency, 9333.2 MHz, temperature, 52 K; incident microwave power, 2.2 mW, modulation amplitude, 0.63 mT. The modulation frequency for this and the other EPR spectra in this paper was 100 kHz

also the Fe-S clusters in cytochrome *c* reductase [13] were reduced. Thus, in the control particles (trace C) NADH had reduced not only NADH dehydrogenase, but also succinate dehydrogenase, cytochrome *c* reductase, and part of cytochrome *c* oxidase. In the particles treated with tetradecanoic acid (trace B), however, only NADH dehydrogenase was reduced and the electron transfer to other components was inhibited. The latter components were not destroyed and could all be reduced with dithionite (trace D). In this case also complete reduction of Cu_A was achieved.

Fig. 4 shows the behaviour of the clusters 2, 3 and 4 of NADH-dehydrogenase and of the 3-Fe cluster in succinate dehydrogenase [14] which can be observed only at lower temperatures. In trace A, oxidized particles treated with tetradecanoic acid, the characteristic signal of the oxidized succinate:Q oxidoreductase around $g = 2.02$ can be observed superimposed on the signal of Cu_A. In

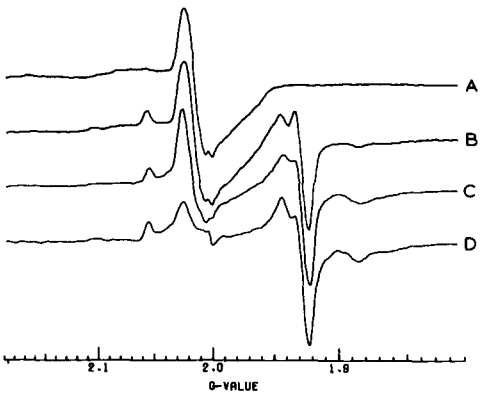

Fig. 4. Effect of the incubation of submitochondrial particles with a low concentration of tetradecanoic acid on their EPR spectra at 12 K The traces A–D refer to the same treatments as described in Fig. 3. The EPR conditions were also the same, except for the temperature.

trace B, in addition to the signal of cluster 1 of NADH dehydrogenase that was already detected at 52 K (Fig. 1, trace B), also the lines of the reduced cluster 2 ($g_{\|} = 2.056$, $g_{\perp} = 1.922$) can be clearly observed. Some of the lines of the reduced clusters 3 and 4 can be seen: at $g = 2.10$, 1.88 and 1.86. Due to greater anisotropy of these signals, the latter lines are relatively small in amplitude and can best be observed if one looks along the baseline. Dithionite did not increase the degree of reduction of NADH dehydrogenase (trace D). The apparent greater amplitude of the peak at $g = 1.945$ in trace D versus that in trace C reflects the fact that the 2-Fe cluster of succinate dehydrogenase is less saturated under these EPR conditions, when reduction is performed with dithionite [15]. Although it is of no further concern for the present study, it can be noted that in the control samples the 2-Fe cluster of succinate dehydrogenase was fully reduced by NADH (Fig. 3, trace C) whereas the 3-Fe cluster in the same enzyme was not (Fig. 4, trace C). The Figs. 3 and 4 demonstrate that the treatment with a low concentration of myristic acid leads to a blockage of electron transfer between the NADH dehydrogenase and the ubiquinone pool. There is no apparent destruction of any of the Fe-S clusters in the respiratory chain.

A high concentration of tetradecanoic acid, which causes strong inhibition of both the NADH oxidase and the NADH-ferricyanide oxidoreductase activity, resulted in a severe destruction of Fe-S clusters of NADH and succinate dehydrogenase. An example is given in Fig. 5. Trace A is from particles treated with a high concentration of tetradecanoic acid and trace B is from particles treated with a low concentration. In both cases reduction was performed with excess of NADH for 10 s at 0°C. It can be clearly seen that the amount of reduced cluster 2 of NADH dehydrogenase ($g_{\|} = 2.056$, $g_{\perp} = 1.922$) is much less in trace A. Addition of dithionite did not increase this signal, so the cluster has been destroyed by the treatment. The same holds for the 3-Fe cluster of succinate dehydrogenase, the signal of which is absent in trace A. The effect on other clusters was investigated under conditions for optimal detection of these clusters and is summarized in Table I. The resistance of the 2-Fe cluster of succinate dehydrogenase and the lability of the 3-Fe cluster in the same enzyme towards high concentrations of tetradecanoic acid, is compatible with the irreversible destruction of half the succinate-phenazine methosulphate activity and the complete inactivation of the succinate oxidase activity (Fig. 2). The destruction of several Fe-S clusters of the electron transfer system as detected by EPR spectroscopy (Fig. 5 and Table I) is consistent with the loss of labile sulphide observed in Fig. 1.

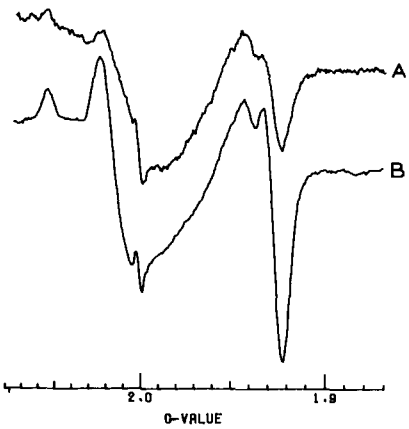

Fig. 5. Comparison of the effects of the incubation of submitochondrial particles with a high and a low concentration of tetradecanoic acid on their EPR spectra at 17 K. Particles were incubated with 518 nmol (A) or 259 nmol (B) of tetradecanoic acid per mg protein as described in Table I. The suspensions were then mixed with 7 mM NADH for 10 s at 0°C and frozen in liquid nitrogen. The EPR conditions were as in Fig. 3, except for the temperature.

TABLE I

Effect of the treatment with tetradecanoic acid on the Fe-S clusters and the activity of submitochondrial particles

Submitochondrial particles (of the same batch as in Fig 1; 1.45 mg protein/ml in 0.25 M sucrose and 10 mM phosphate buffer, pH 7.4) were incubated with tetradecanoic acid for 6 h at 37°C and subsequently washed, first with the original volume of 2% bovine serum albumin in 0.25 M buffered sucrose and thereafter in 0.25 mM sucrose. For EPR spectroscopy the concentration of the particles was increased 40-fold. The control values for the NADH oxidase and the NADH-$K_3Fe(CN)_6$ oxidoreductase activities were 8.76 and 32.1 nkat/mg protein, respectively

	Residual activity or concentration (%) after treatment with tetradecanoic acid (nmol/mg protein)	
	259	518
NADH oxidase activity	22	1
NADH-$K_3Fe(CN)_6$ oxidoreductase activity	70	48
Clusters in NADH dehydrogenase:		
1	100	57
2	100	42
3	100	23
4	100	0
Clusters in succinate dehydrogenase·		
2-Fe	100	100
3-Fe	100	0

Discussion

The results in this paper demonstrate two distinct and irreversible modes of action of tetradecanoic acid on the electron transfer system in submitochondrial particles. One effect occurs at low concentrations of tetradecanoic acid and is very specific with respect to its site of action. The NADH oxidase activity is severely inhibited, with hardly any effect on the succinate oxidation (Figs. 1 and 2). The EPR spectra (Figs. 3 and 4) indicate that none of the Fe-S clusters in the respiratory chain is affected. Instead, they show a specific interruption of electron transfer from NADH dehydrogenase to the ubiquinone pool. This effect is irreversible as was demonstrated in a former study [3]; human serum albumin which is known to bind free fatty acids tightly in a reversible manner [20], protected the submitochondrial particles completely from the slow inhibition by tetradecanoic acid, whereas subsequent addition of the same or even higher concentrations of human serum albumin failed to reverse the inhibition. The participation of the known reversible inhibition in the effects observed in the present study can be excluded, since all measurements were performed after gentle washing of the particles with serum albumin, which does not exclude, however, the possibility that the slow irreversible inactivation may be preceded by the instant reversible inhibition. The reason for the irreversibility and the slowness of the inactivation deserve further investigation. An effect on cluster 2 of NADH dehydrogenase is improbable owing to the lack of any change in its EPR lineshape. The activation of an endogenous phospholipase action in the particles was excluded earlier [3]. Extraction of Q-10 by this low concentration of tetradecanoic acid is also unlikely, because the succinate oxidation activity is not affected. It is therefore proposed that tetradecanoic acid acts on the site of interaction of Q-10 with NADH dehydrogenase. A structural property common to both free fatty acids and Q-10 is the presence of a long unpolar side-chain. Tetradecanoic acid thus may compete for the Q-binding site, thereby lowering the thermodynamic stability of this component, which may lead to its slow irreversible denaturation. The high activation energy of the inactivation of the NADH oxidase system as reported earlier [3] would be in line with this hypothesis. The fatty acid-sensitive component may be a Q-binding protein [16] or a protein conferring Q-reactivity, the existence of which has also been established for the succinate:Q oxidoreductase system [17]. The second effect, occurring at higher concentrations of tetradecanoic acid, involves the destruction of several Fe-S clusters of the electron transfer system. This may be due to a detergent-like action of fatty acids by which hydrophobic interactions are weakened and may render the Fe-S clusters accessible to the nucleophilic attack of water leading to the hydrolysis of the clusters. A loss of EPR signals of Fe-S clusters was also reported after prolonged treatment with high concentrations of detergents [18]. We failed to detect H_2S in the gas phase of the incubation

sample, but the same was the case after addition of a known amount of sodium sulphide to the particle suspension; apparently H_2S reacted with components of the submitochondrial particles so that it escaped detection. A severe loss of acid-labile sulphur was also observed after heating the particles to 60°C (not shown) conforming that the Fe-S clusters are generally sensitive towards denaturing conditions. From Figs. 1 and 2 it may be seen that the various parameters, except for NADH oxidase, strongly decrease simultaneously only above a critical concentration of tetradecanoic acid (over 500 nmol per mg protein). Under these conditions the molar ratio of tetradecanoic acid to phospholipid approaches 1. The partition of the fatty acid molecules within the phospholipid bilayer may induce phase transitions followed by a destabilization of the membrane structure and exposition of the Fe-S clusters to hydrolytic breakdown. A slow irreversible selective denaturation by free fatty acids has also been reported for several yeast enzymes [19]. Here again tetradecanoic acid was the most effective among saturated fatty acids, indicating that such effects of tetradecanoic acid are not restricted to the mitochondrial respiratory chain.

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