Reversible Inhibition of the Mitochondrial Ubiquinol–Cytochrome *c* Oxidoreductase Complex (Complex III) by Ethoxyformic Anhydride†

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ABSTRACT: The mitochondrial ubiquinol–cytochrome *c* oxidoreductase (complex III) is inhibited by ethoxyformic anhydride (EFA). The inhibition is readily reversed by hydroxylamine, suggesting the involvement of essential histidyl or possibly tyrosyl residues. The spectrum of ethoxyformylated complex III in the UV region showed a peak at 238 nm, indicative of *N*-(ethoxyformyl)histidine. Addition of hydroxylamine caused a large decrease of the 238-nm peak, which amounted to 16 mol of (ethoxyformyl)histidine/mol of cytochrome c_1. Hydroxylamine addition to ethoxyformylated complex III also caused a small change at about 280 nm, which could be due to reversal of 1.6 O-ethoxyformylated tyrosyl residues/mol of cytochrome c_1. Among many inhibitors of the cytochrome bc_1 region of the respiratory chain, EFA is the only reagent known to cause reversible inhibition by covalent modification of amino acid residues. The inhibition site of EFA was determined to be between cytochromes *b*-562 and c_1. However, unlike antimycin, which also inhibits in the same region, EFA did not promote the reduction of cytochrome *b*-566 in particles treated with substrates. In addition, it was found that EFA inhibits proton translocation in the cytochrome bc_1 region and is a more effective electron transport inhibitor when added to reduced particles as compared to oxidized particles. These results together with the strong possibility that the EFA target is a histidyl or possibly a tyrosyl residue have been discussed in relation to the mechanism of proton translocation by complex III.

The mitochondrial uniquinol–cytochrome *c* oxidoreductase (complex III) contains as electron carriers two species of cytochrome *b* (*b*-562 and *b*-566), a cytochrome *b* like chromophore (chromophore 558) first recognized in beef heart mitochondria in 1973 (Davis et al., 1973) and recently shown to exist also in yeast (Briquet et al., 1981), a high potential iron–sulfur protein containing a binuclear iron–sulfur cluster (Trumpower, 1981), and cytochrome c_1. In addition, two forms of ubisemiquinone are considered to be involved in electron transfer to and from the *b* cytochromes (de Vries et al., 1981; Trumpower, 1981; Van Ark et al., 1981). The sequence of electron carriers in complex III is still unknown, although recent investigations have provided considerable useful information on the mechanism of electron transfer and proton translocation in the cytochrome bc_1 region of the respiratory chain (Trumpower, 1981; Van Ark et al., 1981; Guierrieri et al., 1981; Alexandre et al., 1980).

Among the probes used for studying the mechanism of electron transfer in complex III are various site-specific inhibitors. Early studies with mitochondria and submitochondrial particles showed that antimycin A, 2-alkyl-4-hydroxyquinoline *N*-oxide, 3-alkyl-2-hydroxynaphthoquinone (SN-5949), and BAL[1] inhibited electron transfer in the bc_1 region of the respiratory chain (Rieske, 1967; Slater, 1949). The first three compounds inhibited electron transfer between the *b*- and *c*-type cytochromes, and BAL has been shown to inhibit at the level of the iron–sulfur protein of complex III (Slater & de Vries, 1980). In addition, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone and 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) have also been shown recently to inhibit at the level of the Rieske-type iron–sulfur proteins in chloroplasts and mitochondria, respectively [Malkin, 1981; Trumpower, 1981; see also Trumpower & Haggerty (1980)]. Other inhibitors acting in the bc_1 region of beef heart and yeast mitochondria are myxothiazol, oudemansin, and strobilurins A and B, all of which contain a β-methoxyacrylate system in common (Becker et al., 1981), and mucidin (Subik et al., 1974), funiculosin (Moser & Walter, 1975; Nelson et al., 1977), and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Briquet et al., 1981).

In the present paper, we report on the effect of ethoxyformic anhydride (EFA) on complex III. This compound, which ethoxyformylates various protein residues (e.g., histidyl, tyrosyl, lysyl, cysteinyl, and arginyl), inhibits electron transfer in complex III but in a manner different from antimycin. Thus EFA inhibits the oxidation of cytochrome *b*-562 and the reduction of cytochrome c_1 + *c*, but unlike antimycin it does not promote the reduction of cytochrome *b*-566. The inhibition by EFA is readily reversed by addition of hydroxylamine, suggesting modification of a histidyl or possibly a tyrosyl residue. Identification of specific protein residues involved in catalysis should be of value in understanding the mechanisms of electron transfer and proton translocation in complex III.

Materials and Methods

EFA, sodium succinate, sodium fumarate, cytochrome *c* (type III), Tris, PMS, and DCIP were obtained from Sigma; antimycin A and sodium ascorbate were from Calbiochem; TMPD was from Eastman; hydroxylamine was from Mallinkrodt. Ubiquinone 10 (Q_2) was a gift from Dr. S. Osono, Eisai Co. Ltd., Tokyo, Japan. All other chemicals were reagent grade or of the highest quality available.

Complex III (Hatefi, 1978) and submitochondrial particles (SMP) (Hanstein et al., 1974) were prepared as described. Protein was estimated by the biuret method (Gornall et al.,

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[1] Abbreviations: BAL, British antilewisite (2,3-dimercaptopropanol); HOQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; EFA, ethoxyformic anhydride; Q_2 and Q_2H_2, respectively, oxidized and reduced coenzyme Q_2 (ubiquinone 10); PMS, phenazine methosulfate; DCIP, 2,6-dichlorophenolindophenol; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; Ant A, antimycin A; SMP, submitochondrial particles; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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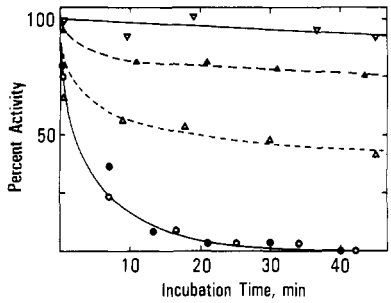

FIGURE 1: Effect of EFA on activities of segments of respiratory chain from succinate to oxygen. SMP at 8.6 mg/mL was incubated at 0 °C in a mixture containing 0.25 M mannitol, 50 mM potassium phosphate, pH 7.0, 17 mM fumarate except in (△), and 6.7 mM EFA and sampled at the intervals shown for activity assay. In the polarographic assays for succinate → O_2 (○), Q_2H_2 → O_2 (●), and ascorbate + TMPD → O_2 (▽) the SMP concentration was 0.23–0.24 mg/mL; and in the spectrophotometric assay for succinate → PMS/DCIP, in the absence (△) and presence (▲) of fumarate, the SMP concentration was 43 μg/mL. In addition the latter assay mixture contained 1.75 mM NaN_3. Unless otherwise specified, in this and all subsequent experiments EFA was added from a freshly made ethanolic solution at 690 mM. Controls in the absence of EFA received an equivalent volume of ethanol.

1949) in the presence of 1 mg of sodium deoxycholate/mL. Ubiquinone 10 (coenzyme Q_2) was reduced with sodium dithionite (Hatefi, 1978), and Q_2H_2–cytochrome *c* reductase activity of complex III was assayed as described elsewhere (Hatefi, 1978). Assays for succinate oxidase and succinate–PMS/DCIP reductase activities of SMP were conducted at 30 °C as before (Hatefi & Stiggall, 1978), except that 0.25 M mannitol was used instead of 0.25 M sucrose and no cytochrome *c* was added to the reaction mixture in the succinate oxidase assay. Ascorbate + TMPD oxidase activity was assayed in a reaction mixture containing 0.25 M mannitol, 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 11.3 mM sodium ascorbate, 180 μM TMPD, 8.5 μg of antimycin/mL, and 0.19 mg of SMP/mL. Ubiquinol oxidase activity was assayed in a similar reaction mixture, except that antimycin was omitted, and ascorbate and TMPD were replaced with 5.6 μL of Q_2H_2 (28 mM in ethanol)/mL of the reaction mixture. Oxygen consumption was measured at 30 °C, with a Clark-type electrode. Substrate-induced reduction of *b* and $c + c_1$ cytochromes of SMP was studied in the Aminco-Chance dual-wavelength spectrophotometer, and their spectra were recorded by the Aminco DW-2a spectrophotometer in the split-beam mode. Details of reaction conditions are given with the appropriate figures.

Results

Inhibition of Complex III by EFA. Figure 1 shows the effect of preincubation time of SMP with 6.7 mM EFA at 0 °C on several electron transport activities of the particles. It is seen that EFA treatment of SMP resulted in complete inhibition of succinate oxidase and ubiquinol oxidase activities, while succinate–PMS/DCIP reductase activity was partially inhibited at the EFA concentration used, and cytochrome *c* oxidase activity as measured in the presence of ascorbate + TMPD as electron donors to cytochrome *c* was only slightly affected. As shown elsewhere (Vik & Hatefi, 1981), higher concentrations of EFA can result in complete inhibition of succinate dehydrogenase activity, and succinate, fumarate, malonate, and oxaloacetate protect this enzyme against inhibition by EFA. Figure 1 also demonstrates the protection afforded by 17 mM fumarate in the SMP incubation mixture on the inhibition of succinate–PMS/DCIP reductase activity of SMP by 6.7 mM EFA. Thus, the results of Figure 1 show

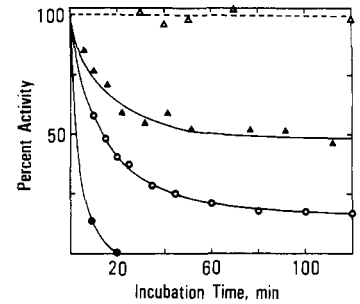

FIGURE 2: Effect of EFA on Q_2H_2–cytochrome *c* reductase activity of complex III. Complex III at 1.6 mg/mL in 100 mM potassium phosphate, pH 6.0, was incubated at 0 °C with 0.0 (△), 0.28 (▲), 1.07 (○), or 5.3 (●) mM EFA and sampled at the intervals shown for activity assay. The concentration of complex III in the assay was 16 μg/mL. The specific activity of complex III at 30 °C in the absence of EFA (△) was 73 μmol min^{-1} mg^{-1}.

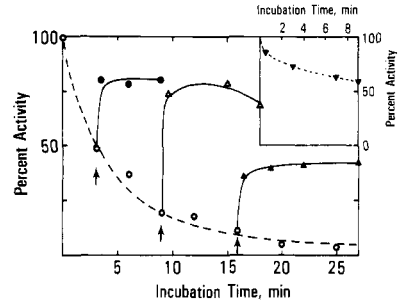

FIGURE 3: Reversal of EFA inhibition of Q_2H_2–cytochrome *c* reductase activity of complex III by hydroxylamine. Complex III at 0.26 mg/mL in 100 mM potassium phosphate, pH 7.0, was incubated at 0 °C with 0.69 mM EFA. At the intervals shown by arrows 360 mM hydroxylamine (pH 7.0) was added, the incubation continued, and the mixture sampled for activity measurement as shown. The inset shows the effect of 360 mM hydroxylamine added to the complex III incubation mixture in the absence of EFA. Complex III activity in the absence of inhibitors was 120 μmol min^{-1} mg^{-1}.

that EFA inhibits the succinate oxidase activity of SMP primarily in the region between ubiquinol and cytochrome *c*, i.e., at the level of complex III. Figure 2 shows that ubiquinol–cytochrome *c* reductase activity as catalyzed by purified complex III is inhibited by EFA and that both the rate and extent of inhibition are functions of EFA concentration in the complex III incubation mixture. As shown in Figure 3, the EFA inhibition of Q_2H_2–cytochrome *c* reductase activity could be reversed to a considerable extent upon subsequent addition of hydroxylamine. The inset shows the effect of hydroxylamine alone on complex III activity. Although EFA can modify various protein residues as indicated above, the reversal by hydroxylamine is considered to be diagnostic for modification of histidyl residues (Burstein et al., 1974). However, a much slower hydroxylamine reversal of EFA-modified tyrosyl residues has also been reported (Melchior & Fahrney, 1970; Miles, 1977). It has been demonstrated that EFA shows greater specificity for histidyl residues at pH ~6.0, but reaction with histidyl residues at higher pH values also occurs (Miles, 1977; Tudball et al., 1972). Our results showed that the inhibitory effect of EFA on complex III increased considerably as the pH of the incubation medium was changed gradually from pH 6.5 to pH 8.0. However, pH studies as applied to detergent-solubilized complex III are probably complicated by the effect of pH on complex III solubility.

While the above experiments do not allow a distinction between essential histidyl and tyrosyl residues as EFA targets in complex III, the spectral change of EFA-treated enzyme in the UV region shown in Figure 4 might be more in favor

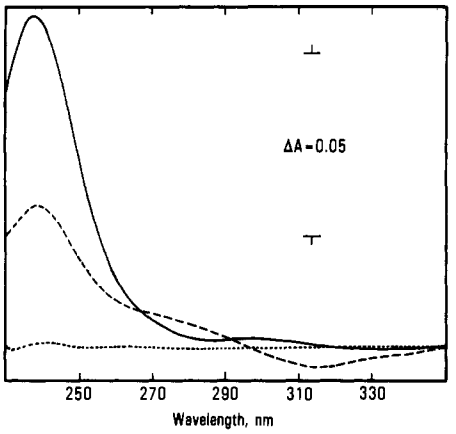

FIGURE 4: Effects of EFA and hydroxylamine on absorbance of complex III in UV region. Complex III at 0.26 mg/mL was dissolved in 100 mM potassium phosphate, pH 7.0, and placed in the sample and reference cuvettes (2.7 mL each) and the base-line difference spectrum (dotted trace) recorded. Then 27 μL of 69 mM EFA in ethanol was added to the sample cuvette and an equal volume of ethanol to the reference cuvette. After 25 min, the difference spectrum shown by the solid trace was recorded. At this point, 270 μL of 4 M hydroxylamine (neutralized) was added to each cuvette, and the difference spectrum shown by the dashed trace was recorded immediately. The spectral studies were carried out at 5 °C.

of histidyl residues. The experimental conditions were the same as in Figure 3. Complex III at 0.26 mg/mL was treated at 0 °C with 0.68 mM EFA for 25 min; then its spectrum was recorded against a complex III solution treated only with ethanol (EFA solvent). The difference spectrum (Figure 4, solid trace) showed a peak at 238 nm, indicative of *N*-(ethoxyformyl)histidine (Burstein et al., 1974; Miles, 1977). The absence of a trough at about 278 nm suggested that under the conditions used little or no tyrosyl residues were modified by EFA. When hydroxylamine was added, the absorbance at 238 nm diminished by 60% (Figure 4, dashed trace), which is consistent with the reversal of activity by hydroxylamine shown in Figure 3. This suppression of the 238-nm absorbance by hydroxylamine amounted to 16 mol of histidine/mol of cytochrome c_1 [$\Delta\epsilon$ for *N*-(ethoxyformyl)histidine was taken to be 3200 L mol^{-1} cm^{-1}]. As seen in Figure 4, hydroxylamine addition also caused a small absorbance increase at about 280 nm. If we assume that this is due to reversal of tyrosine modification, then the number of tyrosyl residues involved would be 1.6 mol/mol of cytochrome c_1 [$\Delta\epsilon$ for *O*-(ethoxyformyl)tyrosine was taken to be 1310 L mol^{-1} cm^{-1}; Burstein et al., 1974]. Thus, while the above results might be in favor of modification of essential histidyl residues by EFA, the possibility that complex III contains per mole one to two essential tyrosyl residues that are reversibly modified by EFA cannot be ruled out on the basis of the available data.

Site of EFA Inhibition in Complex III. The experiments described below were done with SMP rather than complex III so that the site of EFA block could be (a) delineated under conditions that both substrate and oxygen were present to result in oxidation–reduction "crossover" on the substrate and oxygen sides of the block and (b) compared with the site of the antimycin block. The substrate used in these experiments was succinate, since extensive inhibition of succinate dehydrogenase by EFA could be prevented in the preincubation medium by the addition of fumarate. NADH could not be used as substrate, because EFA also inhibited NADH–ubiquinone reductase activity, and this inhibition could not be prevented by the addition of any protecting agent.

Figure 5 shows the effect of antimycin on succinate-induced reduction of the *b* cytochromes at 565 nm minus 575 nm and

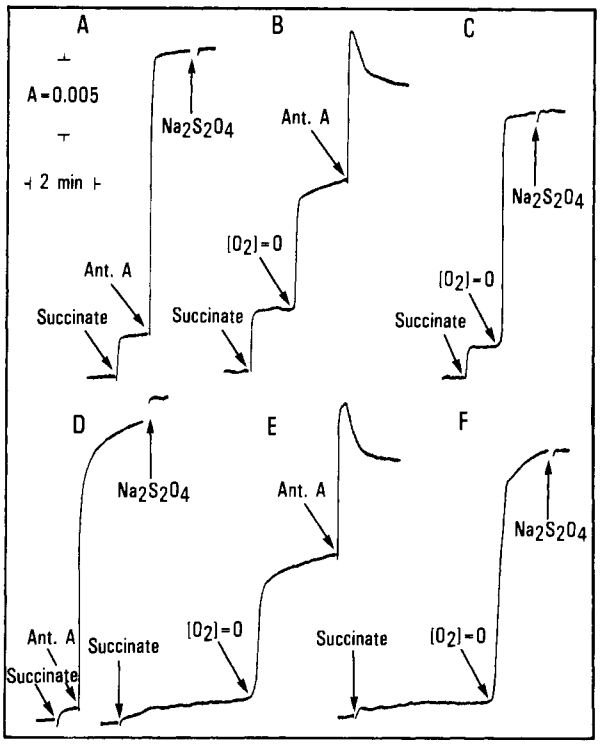

FIGURE 5: Effects of antimycin and anaerobiosis on succinate-induced reduction of *b* and $c + c_1$ cytochromes of SMP in the absence and presence of fumarate. SMP at 20 mg/mL in 0.25 M mannitol containing 50 mM potassium phosphate, pH 7.0, was incubated at 0 °C in the absence (traces A, B, and C) and presence (traces D, E, and F) of 5 mM fumarate and then diluted to 2 mg/mL into an assay cuvette containing 0.25 M mannitol, 50 mM Tris-HCl, pH 7.5, and 0.5 mM EDTA. Where indicated, 6.7 mM sodium succinate, 10 μg/mL antimycin, and a few grains of solid $Na_2S_2O_4$ were added. The assay temperature was 30 °C. Traces A, B, D, and E were recorded at 565 nm minus 575 nm to monitor cytochrome *b* reduction, and traces C and F were recorded at 550 nm minus 540 nm to monitor cytochrome $c + c_1$ reduction. In traces D, E, and F, the fumarate concentration in the assay mixture was 0.5 mM.

of the *c* cytochromes at 550 nm minus 540 nm in SMP (traces A, B, and C) and in SMP pretreated with 4.72 mM fumarate (traces D, E, and F). Fumarate concentration in the assay medium was 0.5 mM. It is seen that addition of 6.7 mM succinate resulted in partial reduction of the *b* (traces A and B) and *c* cytochromes (trace C). Addition of antimycin in trace A caused further reduction of the *b* cytochromes, while subsequent addition of $Na_2S_2O_4$ caused no further reduction. Traces B and C show the effect of anaerobiosis. In trace B anaerobiosis caused *b* reduction to the extent of 55% of the maximum reduction, which was achieved transiently by subsequent addition of antimycin. The partial reversal after the reduction caused by antimycin appeared from difference spectra to be due to reoxidation of cytochrome *b*-566. Trace C shows the effect of anaerobiosis on reduction of the *c* cytochromes. Traces D, E, and F show similar experiments with SMP pretreated with fumarate, i.e., in the presence of 0.5 mM fumarate in the assay mixtures. The results were essentially the same as in traces A, B, and C, respectively, except that fumarate had caused partial inhibition of succinate oxidation with the consequence that steady-state reduction of the cytochromes was less pronounced and it took longer for the reaction mixtures to become anaerobic. The experiments shown in traces D, E, and F of Figure 5 are, therefore, the controls for those carried out with EFA-treated SMP. Thus, as seen in Figure 6, trace A, pretreatment of SMP for 25 min at 0 °C with 6.7 mM EFA resulted in a block in the respiratory chain such that steady-state reduction of the *b* cyto-

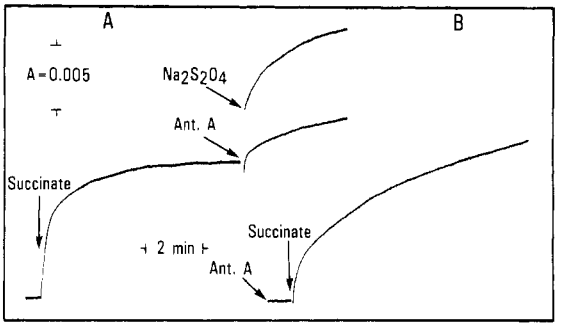

FIGURE 6: Effects of succinate and antimycin on reduction of *b* cytochromes of SMP treated with EFA. Experimental conditions were the same as in Figure 5, except that the preincubation mixture contained 6.7 mM EFA and 5 mM fumarate, and SMP was incubated at 0 °C with EFA for 25 min. Reduction of the *b* cytochromes was monitored at 565 nm minus 575 nm.

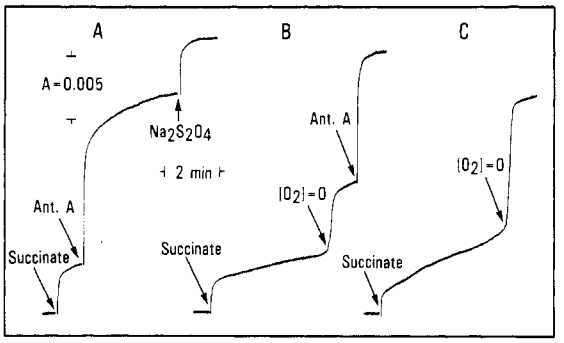

FIGURE 7: Reversal by hydroxylamine of effect of EFA on succinate-induced reduction of *b* and $c + c_1$ cytochromes of SMP. Experimental conditions were the same as in Figure 5, except that after 25-min incubation of SMP with EFA and fumarate, 150 mM hydroxylamine was adde to the mixture and the incubation continued for an additional 30 min at 0 °C before assay. Traces A and B show the reduction of cytochromes *b* at 565 nm minus 575 nm, and trace C shows the reduction of cytochromes $c + c_1$ at 550 nm minus 540 nm.

chromes upon addition of succinate had increased considerably (compare with Figure 5, trace D). Subsequent addition of antimycin caused a slow increase in absorbance at 565 nm minus 575 nm, while dithionite addition doubled the extent of reduction. When antimycin was added to the reaction mixture before addition of succinate, a biphasic reduction was observed (trace B), with the first phase being smaller than that shown in trace A. It should be noted, however, that in the presence of antimycin, the extent of *b* reduction increased beyond that shown in trace A prior to antimycin addition, suggesting a slow reduction of *b*-566 in addition to *b*-562. In EFA-treated SMP there was no detectable steady-state reduction of the *c* cytochromes (not shown), but the EFA concentration used did not completely abolish electron transfer through the respiratory chain since after 13 min the reaction mixture became anaerobic and the *c* cytochromes were reduced. Thus, by comparison to Figure 5, the above results suggested that, similar to antimycin, EFA inhibits at a site on the substrate side of the *c* cytochromes but that the effect of EFA on substrate-induced reduction of the *b* cytochromes is different from that of antimycin. Figure 7 shows the reversal by hydroxylamine of EFA inhibition. It is seen that traces A, B, and C of Figure 7 are respectively similar to traces D, E, and F of Figure 5, which were from SMP pretreated with fumarate but not with EFA. The slow reduction of the *c* cytochromes in trace C of Figure 7 after addition of succinate and prior to anaerobiosis is because the reaction mixtures in the experiments of Figure 7 contained 16 mM hydroxylamine,

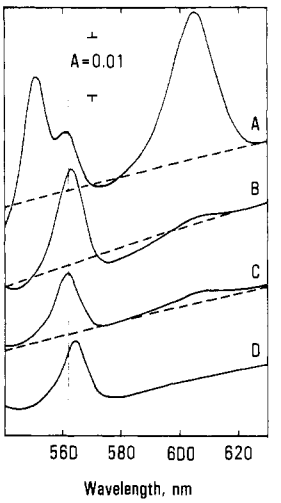

FIGURE 8: Effects of antimycin and EFA on cytochrome spectra of succinate-reduced SMP. 0.3 mL of SMP at 10 mg/mL in 0.25 M mannitol and 50 mM potassium phosphate, pH 7.0, was treated for 25 min at 0 °C with 10 mM fumarate in traces A, B, and C; 18 μg of antimycin in trace B; and 13 mM EFA in trace C. It was then diluted to 1.1 mg/mL in a medium containing 0.25 M mannitol, 50 mM Tris-HCl, pH 7.5, and 0.5 mM EDTA at room temperature. 1 mL of the mixture was placed in each of a pair of cuvettes, and their difference spectra (dashed lines) were recorded. Then 10 μL of 0.5 M sodium succinate was added to the sample cuvette and 10 μL of water to the reference cuvette, and their difference spectra were recorded again. In trace A, the cuvettes were allowed to stand for several minutes after succinate addition to achieve anaerobiosis; then the difference spectrum shown was recorded. Trace D is the spectrum of the antimycin- and succinate-treated SMP (the experimental cuvette of trace B) minus the spectra of EFA and succinate-treated SMP (the experimental cuvette of trace C).

which caused partial inhibition of cytochrome oxidase.

Figure 8 shows the absorption spectra of SMP treated with EFA or antimycin and then reduced with succinate. Trace A shows in untreated SMP the succinate-induced reduction after anaerobiosis of cytochromes $a + a_3$ at about 605 nm and $c + c_1$ at 551 nm and partial reduction of the *b* cytochromes at about 562 nm. As expected, the *b* cytochrome reduced under these conditions was *b*-562. Trace B is antimycin-treated SMP reduced with succinate. It shows inhibition of reduction of cytochromes $c + c_1$ and $a + a_3$ and increased reduction of the *b* cytochromes with a peak at about 563.5 nm. This peak is due to the sum of reduced *b*-526 and *b*-566. Trace C shows the spectrum of EFA-treated SMP reduced with succinate. This spectrum shows the reduction of *b*-562 only.[2] Subtraction of trace C from trace A (result not shown) deleted the *b* peak from trace A, indicating (a) that in the presence of EFA cytochrome *b*-562 was almost completely reduced and (b) that EFA treatment did not alter the spectrum of reduced *b*-562. Subtraction of trace C (reduced *b*-562) from trace B (reduced *b*-562 + *b*-566) gave trace D, which is reduced *b*-566. The peak as recorded was, however, at 564.5 nm, which could be the result of distortions by the sloping base line and subtraction of the spectrum of EFA-treated SMP from that of antimycin-treated SMP. At any rate, the above results indicate that in the complex III segment of the respiratory chain EFA

[2] The shoulder on the short-wavelength side of this peak might be due to reduction of the cytochrome *b* like component, chromophore 558, which we discovered in complex III in 1973 (Davis et al., 1973). Recently Briquet et al. (1981) have also seen this component in yeast mitochondria and have designated it cytochrome *b*-558. However, since a complete spectrum of this component with α, β, and Soret peaks has not yet been obtained, we prefer not to designate it a cytochrome on the basis of only a single peak at 558 nm.

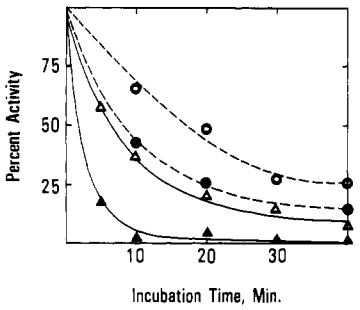

FIGURE 9: Effect of EFA on Q_2H_2–cytochrome *c* reductase activity of oxidized and reduced SMP. SMP at 10.75 mg/mL in 0.25 M mannitol and 50 mM potassium phosphate, pH 7.0, was treated at 0 °C with 0.96 mM KCN, 19 mM succinate (▲, ●) or 19 mM fumarate (△, ○), and 3.6 (○, ●) or 7.2 mM (△, ▲) EFA. At the intervals shown, 100 μL of each mixture was withdrawn and treated with 4.5 μL of 10% sodium deoxycholate and 100 μL of 2 M KCl. Additions of deoxycholate and KCl resulted in solubilization of SMP, which was then diluted by further addition of 800 μL of the mannitol–phosphate buffer and assayed for Q_2H_2–cytochrome *c* reductase activity in the presence of 20 mM NaN_3 and 4 mM malonate.

inhibits electron transport between cytochromes *b*-562 and c_1 + *c*. While to this extent the effect of EFA is similar to that of antimycin, there are some important differences, however. (i) Unlike antimycin, EFA treatment of SMP does not bring about substrate-induced reduction of *b*-566. (ii) Prereduction of cytochromes c_1 + *c* (presumably also the iron–sulfur protein of complex III) with ascorbate + TMPD in cyanide and antimycin-treated particles inhibits the substrate-induced reduction of *b*-566 (Rieske, 1971; Erecinska et al., 1972) and *b*-562 (Davis et al., 1973). Addition of ascorbate + TMPD to EFA- and cyanide-treated SMP had little or no effect on the substrate-induced reduction rate of *b*-562. (iii) EFA is a protein residue modifier, and the ready reversal of EFA inhibition of complex III by hydroxylamine suggests the ethoxyformylation of an essential histidyl or possibly tyrosyl residue. As far as is known, antimycin and most other inhibitors of complex III appear to inhibit through noncovalent binding and not by way of protein modification.

Another interesting feature of EFA is that it is a more effective inhibitor against substrate-reduced particles. As shown in Figure 9, the QH_2-cytochrome *c* reductase activity of SMP was more readily inhibited when EFA was added to particles pretreated with succinate as compared to fumarate. These results suggest a possible effect of oxidation–reduction on target conformation and/or the p*K* of the residue modified by EFA. The former possibility agrees with the results of Ball et al. (1977), who found that trypsin inhibited reduced complex III faster than the oxidized complex. The latter possibility has interesting implications regarding the mechanism of proton translocation and will be discussed below.

Discussion

It has been shown that treatment of SMP or complex III with EFA inhibits electron transport. In SMP, this compound inhibits at three different levels, namely, (a) at the level of succinate dehydrogenase where the inhibition appears to be at the active site (Vik & Hatefi, 1981), (b) between NADH dehydrogenase and ubiquinone (unpublished results), and (c) at the level of the bc_1 cytochromes in complex III. The latter site is particularly sensitive to treatment of SMP with EFA. Reversibility of EFA inhibition by hydroxylamine, as well as the spectral change of EFA-treated complex III in the UV region, suggests modification most likely of an essential histidyl or possibly a tyrosyl residue. The heme groups of the cytochromes do not appear to be modified by EFA as judged by their unaltered absorption bands in the α region.

Comparative studies of the effects of EFA and antimycin on the cytochrome region of the respiratory chain have indicated the following. Both EFA and antimycin inhibit electron transfer from substrates to cytochromes *c* + c_1 and *a* + a_3, and both result in accumulation of reduced cytochrome *b*-562. Thus, although these observations seem to suggest that EFA and antimycin interrupt electron transfer between cytochromes b_{562} and c_1, their sites of action may be very different, especially if the Q-cycle hypothesis of Mitchell (1976) and the recent Q-cycle-type electron transfer schemes of Trumpower (1981) and Van Ark et al. (1981) are taken into consideration. In addition, it should be kept in mind that antimycin treatment makes cytochrome *b*-566 reducible, while EFA treatment does not. Furthermore, addition of antimycin to EFA-treated SMP inhibits the reduction of *b*-562 by succinate (Figure 6, trace B), which suggests different inhibition sites for EFA and antimycin.[3] The dual effects of these two inhibitors are reminiscent of the combined effects of antimycin and UHDBT. Bowyer & Trumpower (1980) have shown that oxidant-induced reduction of cytochrome *b* in antimycin-treated preparations was inhibited by UHDBT. Indeed, in this and other respects EFA seems to share phenomenological inhibitory features with UHDBT and the inhibitors of Becker et al. (1981), which contain in common a β-methoxyacrylate group.

Whether these latter compounds inhibit by noncovalent binding or through covalent modification is not known. However, EFA most likely inhibits QH_2–cytochrome *c* reductase activity by modification of an essential histidyl or tyrosyl residue, thus allowing for two important lines of investigation. First, should the structure modified by EFA be an imidazole group, then it might be possible to use other histidyl residue modifiers, which might result in a stable product allowing analysis and target identification (Roberts et al., 1977). Second, we have shown that EFA inhibits proton translocation coupled to ubiquinol–ferricyanide reductase activity in mitochondria (unpublished results). Therefore, the putative histidyl or tyrosyl residue modified by EFA might be involved in proton translocation through a Bohr-type mechanism, which has been proposed by Guerrieri et al. (1981) for proton translocation in the bc_1 region of the respiratory chain. In this regard, the finding (Figure 9) that EFA more rapidly inhibits the QH_2–cytochrome *c* reductase activity of reduced rather than oxidized particles is interesting. By analogy to the hemoglobin Bohr effect, it is possible that proton translocation results from the p*K* changes of protein residues whose environment is altered by enzyme conformation changes coupled to electron transfer or substrate binding. Such protein residues could be the EFA targets, and modulation of their p*K* by the redox state of complex III could be the basis of the results shown in Figure 9.

[3] In agreement with this conclusion we have shown that HOQNO, which apparently acts at the same site as antimycin (Van Ark et al., 1981), does not protect complex III against EFA inhibition. The experiment was done as follows. Complex III at 250 μg/mL was treated with 43 μM HOQNO, then incubated at 0 °C with 1.45 mM EFA, and sampled for activity measurement after 15 s and every 3 min thereafter up to 15 min. In the assay, addition of 43 μM HOQNO completely inhibited QH_2–cytochrome *c* reductase activity, but when the above complex III incubation mixture was sampled for assay, HOQNO concentration in the assay was lowered to 0.15 μM, which by itself resulted in only about 20–25% inhibition. Thus, it was found that pretreatment of complex III with 43 μM HOQNO afforded no protection against EFA inhibition, which after 15 s of incubation was ~50% and after 10 min ~95%, regardless of the absence or presence of HOQNO in the preincubation medium.

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Proline Transport by Synaptosomal Membrane Vesicles Isolated from Rat Brain: Energetics and Inhibition by Free Fatty Acids†

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ABSTRACT: Synaptosomal membrane vesicles have been employed to study the energetics of proline transport and the inhibition of proline transport by unsaturated free fatty acids. Active uptake of proline into synaptosomal membrane vesicles requires extravesicular Na^+ and is primarily driven by a Na^+ gradient created by diluting K^+-loaded vesicles into Na^+-containing buffers. Uptake of proline under these conditions is enhanced up to 2-fold by a valinomycin-induced diffusion potential (interior negative). Proline transport is reduced in the absence of external Cl^- or internal K^+. Strong (40–90%) inhibition of proline uptake occurs upon collapse of the Na^+ gradient by ionophores such as gramicidin D or activation of the action potential Na^+ channel by veratridine or *Tityus serrulatus* venom. Less (15–25%) inhibition is obtained with the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone, which also prevents the stimulation of proline uptake by the valinomycin-induced diffusion potential. Unsaturated free fatty acids inhibit proline uptake. The inhibition is greatest for arachidonic acid and was somewhat less for oleic acid. The saturated fatty acids palmitic and stearic have little or no inhibitory capacity. Endogenous unsaturated free fatty acids may exert similar inhibitory effects on the reuptake systems for neuroactive amino acids and thus modulate their action in the central nervous system.

High-affinity, sodium-dependent uptake systems for proline and other putative neurotransmitters have been described in isolated nerve ending (synaptosome) preparations (Peterson & Raghupathy, 1972; Bennett et al., 1973; Snyder et al., 1973). It has been suggested that such transport systems function as reuptake mechanisms in the termination of neurotransmitter actions (Iversen, 1971). Alternatively, since amino acid neurotransmitters are localized primarily in the cytoplasm rather than in synaptic vesicles, these transport systems may operate in the reverse direction under depolarizing conditions and release neurotransmitters from the nerve ending (O'Fallon et al., 1981). The inhibition of proline uptake by

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