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Action of bovine serum albumin on cytochrome *c* oxidase activity and proton pumping: a role for fatty acids in enzyme function?

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Abstract Bovine serum albumin (BSA) at micromolar concentrations causes a red shift of the Soret band of bovine cytochrome *c* oxidase with a slow biphasic time course. It also inhibits the turnover of detergent-isolated enzyme in a similarly slow manner; the progress of this inhibition is halted by palmitate and other fatty acids. The inhibitory bovine serum albumin effect may involve fatty acid depletion from the enzyme. Respiration by cytochrome *c* oxidase vesicles (proteoliposomes) in the presence of ionophores (uncontrolled) shows only a small inhibition by BSA but preincubation of such vesicles with BSA induces a loss of proton pumping activity. After incubation of BSA-depleted proteoliposomes in the presence of reductant with combinations of fatty acids, pumping activity can be fully restored, suggesting a supportive or even essential role of endogenous fatty acids in H^+ translocation by this membranous enzyme.

Key words: Cytochrome *c* oxidase; Fatty acids; Palmitate; Oleate; Bovine serum albumin; Spectral change; Proteoliposomes; Respiratory control; Proton pumping

1. Introduction

Free fatty acids (FFA) are always present in biomembranes [1]. They catalyse cation exchange [2], act as electrophoretic protonophores, and are weak acid uncouplers in planar bilayers [1] and in liposomes [3,4]. They may also act directly on membrane enzymes and carriers; thus they catalyse downhill proton transport (H^+ leak) mediated by thermogenin [5] and by the ATP/ADP-antiporter [6,7]. Labonia et al. [8] reported that palmitate and myristate cause significant decreases in respiratory control in cytochrome oxidase-containing proteoliposomes, and concluded that fatty acids react with the oxidase. Although Thiel and Kadenbach [9] found no effect upon respiratory control measured polarographically, they identified a FFA-induced loss of control when cytochrome *c* oxidation was measured spectrophotometrically, and suggested that fatty acids increase ferricytochrome *c* dissociation from some oxidases. Subsequently Kohnke et al. [10] proposed that fatty acids can induce a membrane potential-dependent electrophoretic proton movement and a loss of respiratory control in COV without direct interaction with the oxidase protein.

As both detergent-isolated and proteoliposomal enzymes not only respond to added FFA but may contain intrinsic FFA or related low molecular weight species, it is important to examine the effects of removal of such fatty acids from the assays. We have followed up studies on fatty acid effects in liposomes [3,4] and proteoliposomes [2,11,12] by examining the action of bovine serum albumin (BSA) on cytochrome *c* oxidase. BSA binds FFA tightly and can therefore deplete enzyme systems of fatty acids and related compounds. Inhibitory effects of BSA on both isolated enzyme and proteoliposomes have been observed. With proteoliposomes we report for the first time a way of blocking and then restoring proton pumping activity. Some of these results have been communicated previously in abstract form [13,14].

2. Materials and methods

Bovine heart cytochrome *c* oxidase was purified according to Kuboyama et al. [15], with Tween-80 substituting for Emasol in the final stages. The enzyme was stored at −80°C in 100 mM sodium phosphate buffer containing 0.25% Tween-80. Protein concentration was determined by the biuret method of Gornall et al. [16], and cytochrome *c* oxidase concentration using an extinction coefficient of 27 mM^{-1} cm^{-1} for reduced minus oxidized enzyme (cytochrome aa_3) at 605–630 nm [17].

Reagents used included sodium ascorbate, cytochrome *c* (horse heart), MES (2-[*N*-morpholino]-ethanesulfonic acid), TMPD (N,N,N′,N′-tetramethyl-*p*-phenylenediamine), and BSA (type 7511, < 0.05% fatty acids) from Sigma Chemical Co. (St. Louis, MO). All fatty acids used were obtained as the free acids from Sigma. HEPES (4-[2-hydroxyethyl]-1-piperazine-ethane-sulphonic acid) buffer was from Research Organics Inc. (Cleveland, OH).

Spectra were obtained using a Beckman DU-7HS spectrophotometer interfaced to a Apple IIGS microcomputer. The data files were then exported to an Apple Macintosh LC 630 and manipulated using MS Excel. Cytochrome *c* and *a* steady states were monitored using an Aminco DW-2 dual wavelength spectrophotometer, from which the spectral files were exported to a Compaq 286 computer using a Olis (Olis Instruments Ltd., Bogart, GA) data acquisition package. Steady-state levels of cytochrome *c* were monitored at 550–540 nm, with dithionite used to attain full reduction. Cytochrome *a* was monitored at 605–630 nm; it was assumed that cytochrome a_3 makes no contribution at this wavelength pair in the aerobic steady state [18], but contributes 15% after dithionite reduction.

Oxygen consumption was measured with a Clark-type oxygen electrode (Yellow Springs Instruments) and the signal recorded with a Cole-Parmer Versagraph recorder. The 4-ml reaction chamber was magnetically stirred and thermostatically controlled by a water jacket. The buffering system used was usually 100 mM K^+-HEPES, 30 μM EDTA, 0.1% lauryl maltoside at pH 7.0 and 30°C.

Proton pumping was assayed using a Radiometer Copenhagen

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Abbreviations: FFA, free fatty acids; BSA, bovine serum albumin; COV, cytochrome *c* oxidase containing vesicles; HEPES, 4-[2-hydroxyethyl]-1-piperazine-ethane sulphonic acid; MES, 2-[*N*-Morpholino]-ethanesulfonic acid; TMPD, N,N,N′,N′-tetramethyl-*p*-phenylenediamine; EDTA, ethylene diamine tetraacetic acid; LM, lauryl maltoside; C12:0, lauric acid; C13:0, tridecanoic acid; C14:0, myristic acid; C15:0, pentadecanoic acid; C16:0, palmitic acid; C17:0, heptadecanoic acid; C18:1 oleic acid; C18:2, linoleic acid; C20:4, arachidonic acid

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GK2321C pH electrode attached to a Radiometer Copenhagen PHM64 Research pH meter. pH changes were recorded on a Radiometer Copenhagen REC61 Servograph connected to the pH meter via a REA100 interface. The pH electrode was placed in a sealed 4.4 ml reaction chamber, with a syringe access port. The vessel was magnetically stirred and thermostatically controlled by a water jacket. The reaction mixture contained 64 mM K_2SO_4, DOPC/DOPE COV with 0.2 μM outwardly facing aa_3, 400 nM valinomycin, 10 nM FCCP, pH 7.0, at 30°C. Reductant driven pulses were initiated by the addition of 30 nmol ferrocytochrome *c*. The pH of the cytochrome *c* was adjusted to pH 7.0, just prior to injection into the vessel. After equilibrium was reached the pH was readjusted to 7.0. Three more pulses were then recorded in the same fashion. Following the last cytochrome *c* pulse, 10 mM ascorbate and 150 μM TMPD were added. After anaerobiosis the pH was readjusted to pH 7.0 and the COV were pulsed four times with 50 μl of air-saturated 64 mM K_2SO_4. After the fourth pulse 1 μM nigericin was added and the COV were pulsed twice more. The electrode response was then calibrated with aliquots of standard H_2SO_4 and KOH solutions (the presence of cytochrome *c* increases buffering capacity).

To deplete them of fatty acids COV were incubated with 150 μM BSA overnight (~16 h) at 4°C. They were then passed through a Sepharose 6B column equilibrated with 64 mM K_2SO_4, pH 7.0, at 25°C, to separate COV from BSA. H^+/e^- ratios were then measured as described above.

Reinfusion of FFA into BSA-treated COV was achieved by incubating the latter with 10 mM ascorbate, 10 μM cytochrome *c*, and appropriate quantities of the fatty acid or mixture being tested for 1 h at 4°C. The COV were then passed again through a Sephadex 6B column equilibrated with 64 mM K_2SO_4, pH 7.0. H^+/e^- ratios were measured as before in the absence of FCCP.

3. Results

As the enzyme may be exposed to fatty acids in vivo, it was examined after the removal of any intrinsic fatty acids by incubation with BSA (Fig. 1). Fig. 1A shows the spectroscopic effect of bovine serum albumin (BSA) addition on the oxidase. The difference spectra clearly indicate a progressive red shift of the Soret peak. This change is biphasic, as shown by the time course plotted in Fig. 1B. At least two populations of fatty acids may be trapped by BSA, with rapid and slow dissociation rates. The fatty acid released more slowly has the greater effect on the binuclear centre. Preincubation of enzyme with BSA and laurate (C12:0), both of which induce a red shift of the oxidized Soret spectrum, also slows the binding of formate to the enzyme (results not shown).

Removal of intrinsic FFA from cytochrome *c* oxidase by incubation with BSA inhibits the enzyme. Where in the electron transfer sequence does BSA exert its effect? To answer this question we examined the steady-state reduction of cytochromes *c* and *a* during ascorbate oxidation. Fig. 2 shows a pattern of competition between BSA and fatty acids for the enzyme. BSA addition increases the steady-state reduction of cytochrome *c* during ascorbate oxidation in a concentration and time dependent manner (Fig. 2A). This implies an inhibition of turnover. The inhibition of the enzyme by BSA is blocked by fatty acids, as shown in Fig. 2B. In this case 30 μM palmitate (C16:0) arrested the increase in reduction of cytochrome *c* (and hence oxidase inhibition) induced by BSA.

Fig. 2C shows the corresponding changes in steady-state redox level of cytochrome *a*. After addition of ascorbate plus TMPD cytochrome *a* is ~45% reduced in the control enzyme. Pre-incubation for 30 min with BSA caused an elevated reduction level of cytochrome *a* and also inhibited turnover (as shown by the longer anaerobiosis time). Pre-incubation with palmitate (C16:0) produced an effect opposite to

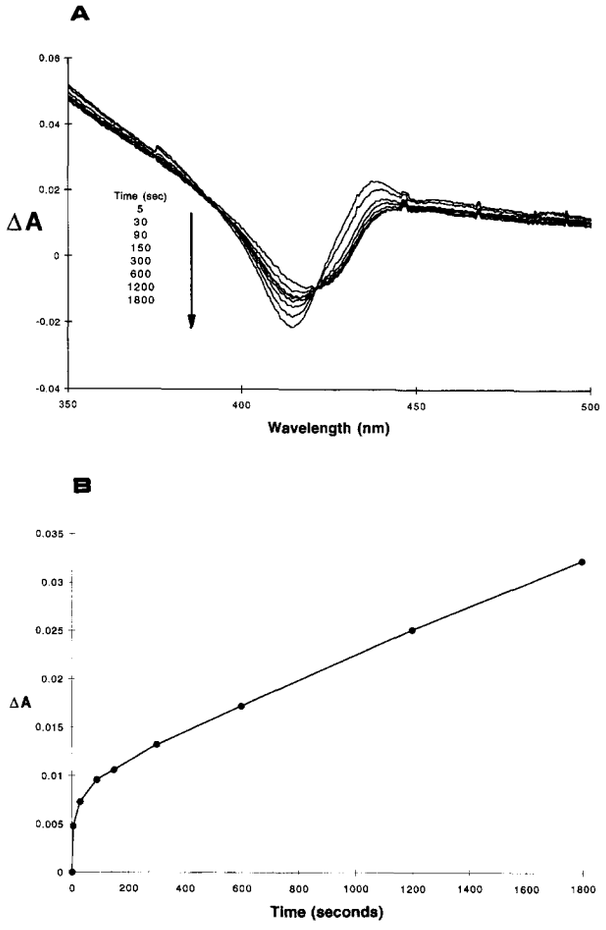
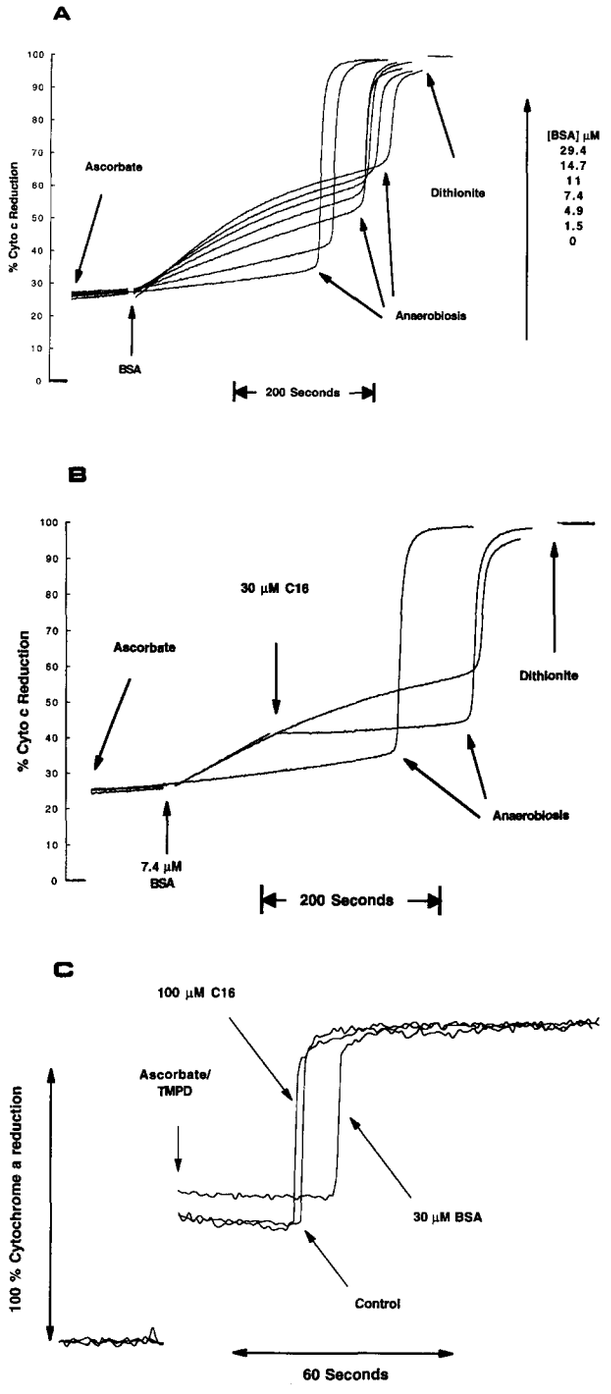


Fig. 1. The spectroscopic effect of serum albumin on cytochrome *c* oxidase. A: Difference spectra induced by serum albumin in the Soret region. 'Oxidized enzyme+BSA' minus 'oxidized enzyme'. 5 μM cytochrome *c* oxidase, 100 mM K^+-HEPES, 30 μM EDTA, 0.1% LM, pH 7.0, at 30°C. BSA (Sigma type 7511) was present in the experimental cuvette at an approximate concentration of 150 μM (10 mg/ml). B: Time course of the bovine serum albumin-induced spectral effects. The plot shows the increase in 430–413 nm absorbance differences with respect to time. Other conditions as in A.

that seen with BSA. In its presence the steady-state reduction level of cytochrome *a* is slightly lower and the onset of anaerobiosis occurs sooner. We conclude that the inhibition by BSA (or the activation by fatty acids) occurs between cytochrome *a* and the oxygen sensitive site, probably at the reduction step(s) involved in the transfer of electrons from cytochrome *a* to the binuclear centre.

Similar treatment of COV with BSA gave only a small inhibition of enzyme turnover and did not markedly affect respiratory control ratio (results not shown). A substantial effect can, however, be seen upon proton pumping (Fig. 3). In control COV the H^+/e^- ratio was 0.9 ± 0.1 (see summary in Table 1) for ferrocytochrome *c* pulses, remaining close to the theoretical 1.0 for each initial pulse and declining slightly with subsequent pulses [19]. After reduction of the proteoliposomal enzyme by ascorbate plus TMPD, the COV were pulsed with aliquots of O_2-containing medium. The resulting H^+/e^- ratios approach 0.8 ± 0.1 (Fig. 3A). Following incubation with BSA and separation of the latter on a Sepharose B column (Section 2 the COV lost cytochrome *c*-dependent H^+-pumping and

Fig. 2. Effects of serum albumin and palmitate on the steady state reduction levels of cytochromes *c* and *a* during oxidase turnover. A: The effect of BSA alone. Redox level of cytochrome *c* was monitored at 550–540 nm. 150 nM cytochrome *c* oxidase and 15 μM cytochrome c were preincubated in 50 mM potassium phosphate, 0.1% LM, pH 7.4, 30°C. The reaction was started with the addition of 5 mM ascorbate. After approximately 1 min BSA was added. After the onset of anaerobiosis dithionite was added to reduce cytochrome *c* fully. B: Effect of palmitate (C16:0) on BSA-treated oxidase. Conditions are as in A. One minute after the addition of ascorbate, 7.4 μM BSA was added to two samples. To the second BSA-treated sample 30 μM palmitate (C16:0) was added 2 min after addition of BSA. C: Effect of BSA and palmitate (C16:0) on cytochrome *a* steady-state reduction level. 50 mM potassium phosphate, pH 7.4, 0.1% LM, 30°C. Cytochrome aa_3 ~1 μM, cytochrome *c* ~1 μM. The reaction was initiated with addition of 10 mM ascorbate plus 75 μM TMPD. The 605–630 nm absorbance was followed. 100 μM palmitate (C16:0) and 30 μM BSA were preincubated with enzyme for 30 min at 30°C. After anaerobiosis, dithionite was added to secure 100% reduction.



showed very low H^+:O ratios following pulses of O_2-containing medium (Fig. 3B).

This result suggested that FFA may be implicated in proton pumping. Several attempts were therefore made to restore proton pumping in the BSA-treated COV fractions. Additions of oleate or palmitate, alone or in combination, were ineffective, whether the COV were incubated with these fatty acids or they were simply added to the test system (results not shown). However certain fatty acid combinations were more effective in securing a restoration of proton pumping. Fig. 3C shows the results obtained when the BSA-treated COV were incubated with a mixture of longer and shorter chain fatty acids (C13, C15, and C17) together with ascorbate and cytochrome *c* at 4°C for 1 h. Following removal of the incubation mixture components by passing the COV through a second Sepharose 6B column, the vesicles were tested for the ability to pump protons in response to pulses of ferrocytochrome *c* or oxygen-containing buffer. As shown in Fig. 3C substantial proton ejection reappeared in this system, both in the reductant and the oxidant pulse tests. Indeed, the early reductant pulses gave an estimated H^+/e^- ratio close to 1.0, almost completely restoring the stoichiometry seen prior to BSA treatment.

4. Discussion

BSA is a protein with six high affinity fatty acid binding sites. Its action on cytochrome *c* oxidase – a red shift of the Soret band – indicates that 'resting' enzyme may contain intrinsic bound fatty acids, probably of a long chain variety. The kinetics of the spectroscopic change induced by BSA

Fig. 3. Abolition of proton pumping in COV by serum albumin incubation and its restoration. A: Proton pumping in control COV. (i) Cytochrome *c* pulse-induced proton pumping. The electrode was calibrated with H_2SO_4 and KOH. COV (0.2 μM externally facing aa_3), 400 nM valinomycin, and 10 nM FCCP were added to 4.4 ml of 64 mM K_2SO_4, pH 7.0, 30°C. Several 30 nmol cytochrome *c* pulses were added as shown. (ii) After the last (fourth) cytochrome *c* pulse 10 mM ascorbate and 150 μM TMPD were added. After anaerobiosis the pH was adjusted to 7.0 and the COV pulsed four times with 50 μl of air-saturated K_2SO_4. 1 μM nigericin was then added and the COV oxidant-pulsed twice more. B: Proton pumping in BSA-treated COV. COV were incubated at 4°C with 150 μM BSA for 16 h. They were then passed down a Sepharose 6B column, equilibrated with 64 mM K_2SO_4, pH 7.0, to remove the BSA. Conditions as in A. (i) Cytochrome *c* pulses. (ii) Oxidant pulses. C: The 'Lazarus' effect: restoration of proton pumping activity. COV were treated with BSA under conditions identical to those in B. They were then incubated for 1 h with 10 mM ascorbate, 10 μM cytochrome *c*, 100 μM C15, 50 μM C17, and 50 μM C13 FFA. COV were then passed down a Sepharose 6B column in 64 mM K_2SO_4, pH 7.0. (i) Cytochrome *c* pulses. (ii) Oxidant pulses.

are clearly biphasic, and the component difference spectra are not identical. This indicates at least two populations of binding sites – a low affinity population with little influence on the binuclear centre and a high affinity population more closely involved with that centre.

A role for carboxylate groups as ligands has been tentatively suggested by Moody et al. [20]; in their model the 'pulsed' (active) form of the enzyme is a form with a red-shifted Soret band. Although BSA treatment also causes a red shift, the resulting enzyme is not a more 'pulsed' form but has a slightly reduced rate of cyanide binding. Nevertheless, conversion of the enzyme from a 'pulsed' to a 'resting'

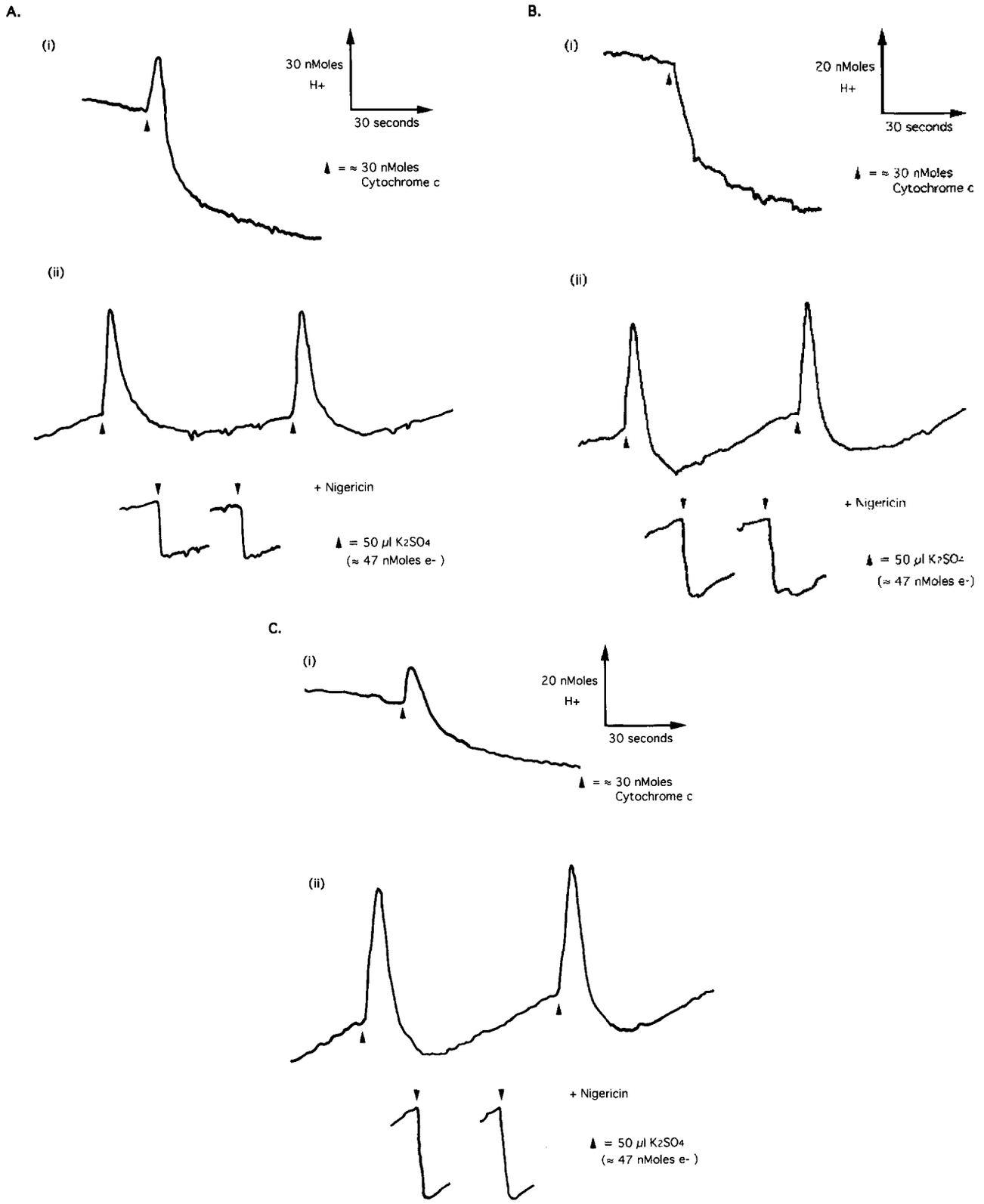


Table 1
Effects of BSA incubation and FFA treatment on the H^+/e^- ratio of cytochrome *c* oxidase-containing proteoliposomes

Pulse type:	H^+/e^- (ferrocytochrome *c*)				H^+/e^- (oxygen)			
Pulse number:	1	2	3	4	1	2	3	4
A. Control COV	1.0±0.1	1.0±0.1	0.7±0.1	0.7±0.1	0.6±0.1	0.7±0.1	0.7±0.1	0.7±0.1
B. BSA-incubated COV	0.2±0.2	0.2±0.2	0.1±0.1	0.1±0.1	0.4±0.1	0.3±0.1	0.3±0.1	0.3±0.1
C. FFA-treated COV from B	0.9±0.1	0.7±0.2	0.5±0.1	0.4±0.1	0.6±0.1	0.5±0.1	0.5±0.1	0.5±0.1

COV were prepared as described in Section 2 and depleted of some internal fatty acids by incubation with BSA, as described in Section 2 and the legend to Fig. 3. Reconstitution with the mixture of C13:0, C15:0 and C17:0 FFA was carried out as described in Section 2. Data were calculated from experiments such as those shown in Fig. 3A–C. Reductant and oxidant pulses were carried out in 64 mM K_2SO_4, at pH 7.0, 30°C. Other conditions are as in the legend to Fig. 3.

form is consistent with the postulated removal of intrinsic fatty acids. If the strongly bound fatty acid(s) were anions, then incubation at acid pH or addition of formate at alkaline pH, both of which cause a pulsed to resting transition [20], would each lead to the loss of intrinsic fatty acids.

The time taken for BSA to inhibit the enzyme was similar to that for it to cause spectral changes. Removal of some intrinsic fatty acids may cause an inhibition as well as the spectral change. BSA-induced inhibition is blocked following palmitate addition; BSA preincubation also increases the steady-state reduction level of cytochrome *a*. Removal of intrinsic fatty acids may thus inhibit electron transfer from cytochrome *a* to a_3.

In the absence of added fatty acids, both uncharged (DOPC/DOPE) and charged (asolectin) COV showed high H^+/e^- translocation ratios. Incubation with BSA abolished this proton pumping. Treatment of depleted COV with ascorbate+cytochrome *c* during fatty acid incubation restored some proton pumping activity. mixtures of fatty acids being the most effective. In our hands the most effective mixture was a combination of C13, C15, and C17 FFA (the 'unphysiological' odd-carbon fatty acids).

A mechanism by which removal of FFA could stop H^+ translocation is lacking. DCCD inhibits H^+ pumping [21] and reacts with carboxylic residues (D90 of bovine subunit III). BSA also abolishes H^+ pumping and causes a small drop in electron transfer rate ([21], this paper). Could abolition of proton pumping by DCCD be due to a reaction with intrinsic FFA? Attempts to restore H^+ pumping in DCCD-treated COV by fatty acid incubation under reducing conditions were unsuccessful, but removal of intrinsic FFA-DCCD adducts may be difficult. We hope to identify the proposed intrinsic fatty acids using chloroform extraction and mass spectrometry. Molecular mass peaks at values between 214 and 360 have been observed, the identity of which will be the subject of a later paper. Other evidence that intrinsic fatty acids modulate respiratory control in the vesicular oxidase is presented elsewhere [12].

Recently, models of proton pumping in cytochrome *c* oxidase have been proposed which rely on proton shuttling along lines of acidic residues gated by H325 of subunit 1 [22,23]. An enzyme acting by such a mechanism ought to be insensitive to BSA action. A model in which the pK_a values of intrinsic FFA are altered by the redox/oxygen intermediate state of the binuclear centre is attractive, but a more likely role for FFA is as an intraenzymic proton carrier, capable of moving protons electroneutrally from the inner aqueous phase to the binuclear centre.

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