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The effect of detergents on bovine cytochrome *c* oxidase: a kinetic approach

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(1) Investigation of the relationship between the detergent concentration and steady-state and pre-steady-state kinetics of cytochrome *c* oxidase proved to be a valid approach in the study of protein-detergent interaction. (2) Laurylmaltoside, sodium cholate and Triton X-100 influenced the kinetics of cytochrome *c* oxidase cooperatively at detergent concentrations near their critical micelle concentration. This mode of interaction reflects disaggregation of the oxidase as a result of cooperative binding of the detergent. (3) Addition of increasing concentrations of Tween-80 to the aggregated enzyme caused a more gradual decrease in aggregation of the oxidase, which did not result in a change in activity of the enzyme. This suggests that aggregation of cytochrome *c* oxidase occurs in a highly regular manner in which no catalytic sites are shielded off. (4) Oxidase aggregates present at detergent concentrations below the critical micelle concentration of laurylmaltoside and Triton X-100 showed considerable activity. Their kinetics were equal to those of the oxidase in Tween-80, suggesting that the protein molecules are aligned in a similar way in all oligomers. Aggregates present in low concentrations of sodium cholate showed turnover rates that were twice as low as those observed with other aggregates. (5) Solubilisation of the oxidase by sodium cholate or Triton X-100 resulted in almost complete inhibition of enzymic activity, whereas the association rate of ferrocytochrome *c* was almost equal to that found for monomeric oxidase in laurylmaltoside. These results are in agreement with a mixed-type inhibition.

Introduction

Cytochrome *c* oxidase (EC 1.9.3.1) is a complex, multisubunit enzyme which catalyses the final step in mitochondrial respiration. Electron-microscopy studies of two-dimensional crystals of bovine heart cytochrome *c* oxidase demonstrated that the enzyme is Y-shaped [1]. Two hydrophobic domains (M_1 and M_2) span the mitochondrial inner membrane and a hydrophilic, so-called C-domain, extends for 5 nm into the inner membrane space [2–5].

Like most amphiphilic proteins, cytochrome *c* oxidase is insoluble in aqueous media. Detergents are used for the extraction of the enzyme from its natural lipid surrounding and for keeping it in solution afterwards (for reviews on detergents, see Refs. 6–8). A variety of detergents has been used in research on cytochrome *c* oxidase. Triton X-100,

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sodium cholate and deoxycholate proved to be suitable for disruption of the mitochondrial membranes and for solubilising the oxidase. The steady-state activity of cytochrome *c* oxidase, however, is strongly inhibited by all three detergents [9,10]. Tween-80 is often used in kinetic studies, since it does not inactivate the oxidase, but it cannot extract the protein from the membrane. In recent years the detergent laurylmaltoside was found to be very suitable for cytochrome *c* oxidase studies [11]. It can be used for purification of the enzyme [11–13] as well as for activity measurements, since a very high steady-state activity was found in its presence [11,14].

Since in all studies of purified cytochrome *c* oxidase the protein is present in complex with detergents and residual phospholipids, conclusions about the enzyme in the native state have to be drawn with the utmost care. Amphiphiles bound to the hydrophobic portion of the enzyme may affect the catalytic activity. Furthermore, many physicochemical techniques, such as for example molecular weight determinations are only valid after careful correction for bound ligand [15]. The lack of consensus on the molecular weight and aggregation state of cytochrome *c* oxidase in the presence of various detergents [16] may partly result from this fact.

The general use of detergents in cytochrome *c* oxidase studies justifies a thorough investigation of detergent effects on the enzyme. In this paper we report on the interaction of cytochrome *c* oxidase with the detergents laurylmaltoside, Tween-80, Triton X-100 and sodium cholate. We chose not to measure detergent binding itself, but the effect of detergent binding on cytochrome *c* oxidase kinetics. In this way we obtained information not only on the factors that determine cytochrome *c* oxidase-detergent interaction, but also on factors that affect the kinetics of cytochrome *c* oxidase.

Materials and Methods

Bovine heart cytochrome *c* oxidase was prepared according to the method of Fowler et al. [17] as modified in our laboratory [18], followed by an additional ammonium-sulphate precipitation step in 50 mM Tris-sulphate (pH 8.0), in order to extract excess cholate.

Cytochrome *c* was prepared from horse heart according to the method of Margoliash and Walasek [19]. Ferrocytochrome *c* was obtained by reduction with ascorbate followed by gel filtration on Sephadex G-50 superfine (Pharmacia) in 100 mM potassium phosphate (pH 7.5)/1 mM EDTA. The absorption coefficients used for cytochrome *c* oxidase and cytochrome *c* were 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm [20] and 21.1 $mM^{-1} \cdot cm^{-1}$ at 550 nm [21], respectively.

Sucrose gradient centrifugation. Sucrose gradients (15–40%, 31 ml total volume) were prepared containing 100 mM potassium phosphate (pH 7.5)/1 mM EDTA and various concentrations of the detergents studied (0.0035–0.2%, w/v). Cytochrome *c* oxidase was incubated for 1 h at 0 °C in 1% (w/v) of the various detergents. On top of the gradients 2 ml samples were applied which contained 5 μM of the oxidase in 100 mM potassium phosphate (pH 7.5)/1 mM EDTA with the same detergent content as the gradients. The gradients were centrifuged for 17 h at $100\,000 \times g$ at 4 °C and were analysed by emptying the tubes from the top using a Densi-flow II-C pump and monitoring the absorbance at 280 nm with a Uvicord S 2138.

Measurements of steady-state reaction rates. The rate of oxidation of ferrocytochrome *c* was measured at 550 nm and at 25 °C with a spectrophotometer built in our laboratory using the optics of a Cary-14 spectrophotometer. Potassium-phosphate buffers were used (100 mM, pH 7.5), containing 1 mM EDTA and various concentrations of detergent (0.0009–0.2%, w/v).

The oxidase was equilibrated with the detergent by incubation for 1 h in the phosphate buffer containing 1% (w/v) detergent, and subsequent dilution to 35 nM protein and the desired detergent concentration. The enzyme was incubated in this medium for 17 h at 4 °C in order to allow comparison with the corresponding experiments with sucrose gradient centrifugation. The final concentration of cytochrome *c* oxidase was 1 nM. In studies on the effect of the detergent concentration on cytochrome *c* oxidase activity the ferrocytochrome *c* concentration was 25 μM. In determinations of K_m and TN_{max} values, a cytochrome *c* concentration range of 8–43 μM was used.

Measurements of pre-steady-state reaction rates. The pre-steady-state reaction of ferrocytochrome *c* with cytochrome *c* oxidase was monitored by following the initial reduction of the oxidase at 444 nm using a Union-Giken RA-401 spectrophotometer. All measurements were performed at 20 °C in 100 mM potassium-phosphate buffers (pH 7.5)/1 mM EDTA, containing various concentrations of detergent. Equilibration with the detergents was performed as described for the steady-state measurements. The final concentration of cytochrome *c* oxidase was 1 μM. Ferrocytochrome *c* concentrations ranged from 4 to 10 μM. Pseudo-first-order reaction-rate constants (k_{obs}) were calculated, after accumulation of 4–6 traces.

Laurylmaltoside was purchased from Boehringer, Tween-80 and Triton X-100 from Sigma, and sodium cholate from Merck. All other chemicals were of analytical grade.

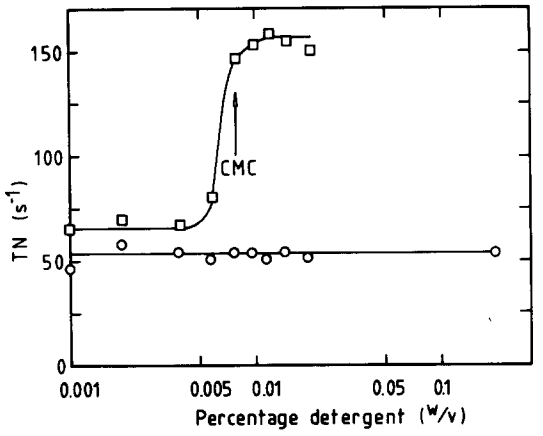


Fig. 1. The relationship between the steady-state activity of cytochrome *c* oxidase and the detergent concentrations. Turnover numbers were determined spectrophotometrically at 25 °C in 100 mM potassium phosphate (pH 7.5)/1 mM EDTA, using 25 μM ferrocytochrome *c* and various concentrations of laurylmaltoside (□———□) or Tween-80 (○———○). Equilibration of the enzyme at the various detergent concentrations was performed as described in Materials and Methods. cmc, critical micelle concentration.

Results

The effect of the non-ionic detergents laurylmaltoside and Tween-80 on cytochrome c oxidase steady-state kinetics

In order to obtain more insight into the interaction of the detergents laurylmaltoside and Tween-80 with cytochrome *c* oxidase, we investigated the relationship between the detergent concentration and the steady-state reaction rate of cytochrome *c* oxidase. The range of detergent concentrations chosen (0.0009–0.2%, w/v) includes the critical micelle concentration of laurylmaltoside (0.008% w/v) and of Tween-80 (0.001–0.006% w/v) [7,11]. After equilibration of the oxidase at the various detergent concentrations (see Materials and Methods) reaction rates were measured spectrophotometrically at 25 μM ferrocytochrome *c* in media of high ionic strength.

At all detergent concentrations studied we found that the oxidation of cytochrome *c* catalysed by cytochrome *c* oxidase showed strictly first-order kinetics [22]. Fig. 1 shows that in the range from 0.0009 to 0.2% (w/v) Tween-80 the rate of ferrocytochrome *c* oxidation by the enzyme was independent of the detergent concentration. For laurylmaltoside, on the other hand, a clear relationship was observed between detergent concentration and steady-state reaction rate. At low laurylmaltoside concentrations, reaction rates were comparable to those in Tween-80. At the critical micelle concentration, however, the rate was found to increase by a factor of 2, reaching the high values generally observed in this detergent [11,14]. These higher values were also found when the activity of samples equilibrated at 0.0009% (w/v) laurylmaltoside or Tween-80 were measured in 0.05% laurylmaltoside. This shows that equilibrium was rapidly attained and that in spite of the 17-h incubation period the process that determines the enzyme activity was fully reversible, thus excluding that the detergent effects originate from alterations in phospholipid content of the oxidase or from denaturation.

The effect of laurylmaltosidase and Tween-80 on the aggregation state of cytochrome c oxidase

The sigmoidal relationship between oxidase activity and laurylmaltoside concentration suggests that the detergent binds to cytochrome *c* oxidase in a strongly cooperative way when the detergent concentration reaches the critical micelle concentration. Consequently, we expected that at a lower detergent concentration the enzyme would

be in an aggregated state.

To test this hypothesis, we performed sucrose gradient centrifugation at various concentrations of either laurylmaltoside or Tween-80. Fig. 2 shows that, after centrifugation in the presence of laurylmaltoside in concentrations above the critical micelle concentration, one sharp band was observed. As shown by Hakvoort et al. [23], our cytochrome *c* oxidase preparation is monomeric under these conditions. We confirmed this observation by partly dimerising our cytochrome *c* oxidase preparation [23]. Sucrose gradient centrifugation of this preparation in the presence of 0.02% laurylmaltoside resulted in two sharp cytochrome *c* oxidase bands (not shown). The position on the sucrose gradient of the lowest molecular-weight form corresponded with the position found for cytochrome *c* oxidase centrifuged at laurylmaltoside concentrations above the critical micelle concentration.

At laurylmaltoside concentrations below the critical micelle concentration, the oxidase precipitated during centrifugation (Fig. 2). This indicated that, as expected from Fig. 1, at low laurylmaltoside concentrations, cytochrome *c* oxidase was present in an aggregated form, a form in which it still had considerable activity.

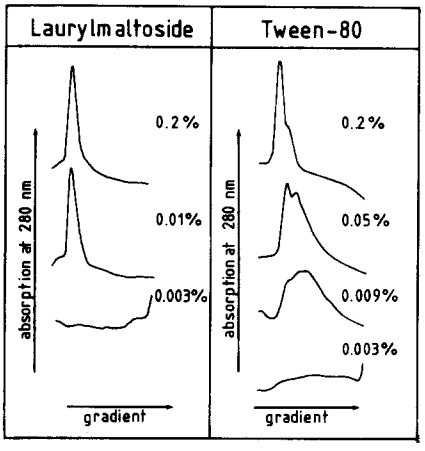


Fig. 2. Sucrose gradient centrifugation of cytochrome *c* oxidase. The enzyme was sedimented through 15–40% sucrose in the presence of 100 mM potassium phosphate (pH 7.5)/1 mM EDTA and the indicated concentrations of laurylmaltoside or Tween-80. Centrifugations were run for 17 h at 4 °C. Prior to the run, the cytochrome *c* oxidase samples were equilibrated with the detergents as described in Materials and Methods. If no A_{280} could be observed, a pellet was formed during centrifugation.

Sucrose gradient centrifugation at various concentrations of Tween-80 (Fig. 2) showed a shift to lower-molecular-weight forms as the Tween-80 concentration increased. In this detergent no sudden disaggregation of the oxidase at the critical micelle concentration was found, but a much more gradual effect which proceeded up to detergent concentrations 40-times the critical micelle concentration.

Unlike the situation in the experiment with laurylmaltoside, we did not know the molecular weight of cytochrome *c* oxidase at any of the Tween-80 concentrations used. Furthermore, direct comparison between gradients run in laurylmaltoside and in Tween-80 could not be made, since the partial specific volume of mixed micelles of cytochrome *c* oxidase and Tween-80 is higher than that of the enzyme and laurylmaltoside [15,16], resulting in slower migration of the enzyme in Tween-80. Thus, it is not known whether the lowest molecular-weight form observed at 0.2% (w/v) Tween-80 is monomeric, dimeric or oligomeric.

The steady-state reaction rate of cytochrome *c* oxidase was found to be independent of the Tween-80 concentration (Fig. 1). This demonstrates that the steady-state activity even of the lowest molecular-weight form (at 0.2% (w/v) Tween-80) corresponded to that of cytochrome *c* oxidase aggregates formed at laurylmaltoside concentrations below the critical micelle concentration. Furthermore, surprisingly, this shows that increased aggregation of the enzyme in Tween-80 did not affect the steady-state activity.

The effect of laurylmaltoside and Tween-80 on various kinetic parameters

To obtain more information about the factors that determine the activity of the enzyme in the monomeric and aggregated states, extended steady-state and pre-steady-state kinetic measurements were performed. Under the applied conditions of high ionic strength the steady-state reaction rate follows simple Michaelis-Menten kinetics [24,25]. The presence of only a high-affinity reaction allows a more straightforward interpretation of the kinetic data. Furthermore, at high ionic

strength, pre-steady-state reaction rates are slowed down to such an extent that the first electron-transfer reaction between ferrocytochrome *c* and the oxidase can be observed [26].

Fig. 3 shows the results of pre-steady-state kinetic measurements. The observed pseudo-first-order rate constants (k_{obs}) are plotted vs. the ferrocytochrome *c* concentration at various detergent conditions. The slopes of the lines represent the second-order rate constants of the association of ferrocytochrome *c* and cytochrome *c* oxidase (k_1). The values of k_1 are listed in Table I. In the presence of 0.05% laurylmaltoside, a detergent concentration at which cytochrome *c* oxidase is monomeric [23], we found a value of $1.2 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ for k_1. A 2-fold lower k_1 value was observed at a laurylmaltoside concentration below the critical micelle concentration (0.005%), where cytochrome *c* oxidase is in an aggregated state. The reversibility of the aggregation of the enzyme was demonstrated by addition of excess (1%) laurylmaltoside to an enzyme preparation that had been incubated for 17 h at 0.0005% laurylmaltoside. The formerly turbid oxidase solution became clear instantaneously and pre-steady-state reaction rates characteristic for the monomeric cytochrome *c* oxidase form were measured (Fig. 3).

Using Tween-80 the association rate constant k_1 appeared to be independent of the detergent concentration. The obtained k_1 values were found to be comparable to the one found for cytochrome *c* oxidase aggregated at laurylmaltoside concentrations below the critical micelle concentration.

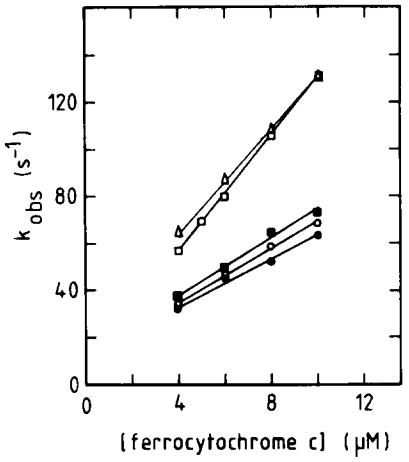


Fig. 3. Rate of cytochrome *c* oxidase reduction as a function of the concentration of ferrocytochrome *c*. Absorption changes at 444 nm were followed at 20 °C in 100 mM potassium phosphate (pH 7.5)/1 mM EDTA. The concentration of cytochrome *c* oxidase was 1 μM, that of ferrocytochrome *c* was 4–10 μM. The buffers contained the same amount of detergent as the corresponding cytochrome *c* oxidase samples had been equilibrated in: □———□, 0.05% (w/v) laurylmaltoside; ■———■, 0.003% (w/v) laurylmaltoside; ○———○, 0.2% Tween-80; ●———●, 0.0005% (w/v) Tween-80; △———△, measurements in a 1% laurylmaltoside buffer with a cytochrome *c* oxidase sample that had been equilibrated at 0.003% (w/v) laurylmaltoside.

The effect of detergent-induced aggregation of the enzyme on the steady-state kinetic parameters K_m and TN_{max} was determined by spectrophotometric measurement of reaction rates, using 8–43 μM ferrocytochrome *c*. Table I shows that the same K_m values were found at laurylmaltoside

TABLE I

THE EFFECT OF THE CONCENTRATION OF VARIOUS DETERGENTS ON STEADY-STATE AND PRE-STEADY-STATE KINETIC PARAMETERS OF BOVINE CYTOCHROME *c* OXIDASE

The values were obtained in 100 mM potassium phosphate (pH 7.5)/1 mM EDTA, as described in Materials and Methods. n.d., not determined; cmc, critical micelle concentration.

Detergent	Percentage detergent (w/v)	$k_1 \cdot 10^{-7}$ ($M^{-1} \cdot s^{-1}$)	$K_m \cdot 10^6$ (M)	TN_{max} (s^{-1})
Laurylmaltoside	0.05 (> cmc)	1.2	30	555
Laurylmaltoside	0.003 (< cmc)	0.7	31	300
Tween-80	0.2 (> cmc)	0.7	38	280
Tween-80	0.0005 (< cmc)	0.6	n.d.	n.d.
Triton X-100	0.2 (> cmc)	0.8	–	–
Triton X-100	0.006 (< cmc)	0.7	–	–
Cholate	1.0 (> cmc)	0.6	–	–
Cholate	0.04 (< cmc)	0.6	–	–

concentrations above (0.05% w/v) and below (0.003% w/v) the critical micelle concentration. At low laurylmaltoside concentrations, however, maximal turnover numbers were found to drop to values comparable to those found in Tween-80.

The inhibitory effect of Triton X-100 and sodium cholate on cytochrome c oxidase kinetics

We also investigated the relationship between detergent concentration and cytochrome *c* oxidase steady-state reaction rate for Triton X-100 and cholate, two detergents that inhibit cytochrome *c* oxidase activity [9,10].

Fig. 4 shows that, again, a change in enzyme activity was observed at the critical micelle concentration of the two detergents (0.6% (w/v) for cholate [8] and 0.015% (w/v) for Triton X-100 [7]). This suggests that, just as for laurylmaltoside, also these detergents bind to the oxidase when the detergent concentration reaches the critical micelle concentration. For Triton X-100 and cholate, however, the formation of protein-detergent complexes resulted in nearly complete inhibition of cytochrome *c* oxidase activity. In parallel with our results in laurylmaltoside, we found cytochrome *c* oxidase to show considerable activity at detergent concentrations below the critical micelle concentration. We did not perform sucrose gradient centrifugation because the enzyme aggregation was obvious as judged from the high turbidity of the solutions.

The reaction rate of the oxidase aggregated in Triton X-100 was comparable to the values found in laurylmaltoside and Tween-80, which are also non-ionic detergents; at low concentrations of the anionic cholate, reaction rates half as low were found. The fact that no denaturation occurred under these circumstances as well as the reversibility of the aggregation was demonstrated by addition of 0.05% laurylmaltoside to samples equilibrated at low cholate and Triton X-100 concentrations: high reaction rates were observed (150 s^{-1}) which are characteristic for the monomeric enzyme in laurylmaltoside.

Finally, we performed pre-steady-state kinetic measurements to determine the association rate constant of ferrocytochrome *c* and cytochrome *c* oxidase (k_1) at Triton X-100 and cholate concentrations above and below the critical micelle concentration. Table I shows that at detergent concentrations above the critical micelle concentration, we found k_1 to be $0.8 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ in Triton X-100 and $0.7 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ in cholate. Although these values are lower than the one found at high laurylmaltoside concentrations ($1.2 \cdot 10^7$ $M^{-1} \cdot s^{-1}$), it can be concluded that the complete lack of steady-state activity under these conditions is not caused by an inhibition of cytochrome *c* binding or electron transfer to the fully oxidised cytochrome *c* oxidase. At low concentrations of cholate and Triton X-100, where we found the protein to self-associate, we measured k_1 values of $0.6 \cdot 10^7$ $M^{-1} \cdot s^{-1}$. This is in the same order as the values found for the oxidase aggregated in Tween-80 and laurylmaltoside.

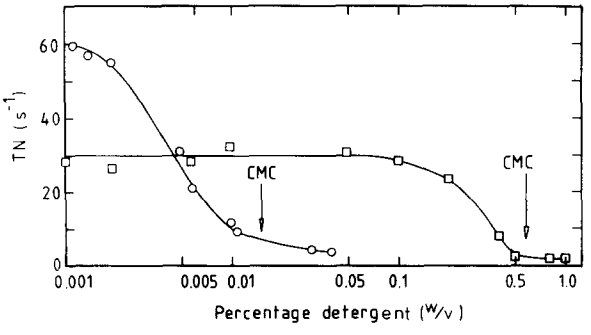


Fig. 4. The relationship between the steady-state activity of cytochrome *c* oxidase and the concentrations of cholate (□———□) and Triton X-100 (○———○). Conditions as in Fig. 1. cmc, critical micelle concentration.

Discussion

Laurylmaltoside, sodium cholate and Triton X-100 show cooperative binding to cytochrome c oxidase

In order to obtain more insight into the interaction of cytochrome *c* oxidase with various detergents, we studied the relationship between the steady-state reaction rate of the enzyme and the detergent concentration. For three of the detergents investigated, namely laurylmaltoside, Triton X-100 and sodium cholate, a sigmoidal relationship was observed between activity and detergent concentration, characterised by a cooperative detergent effect close to the critical micelle concentration of each amphiphile (Figs. 1 and 4). At this detergent concentration laurylmaltoside ex-

hibited a stimulatory effect, whereas Triton X-100 and sodium cholate inhibited the activity of cytochrome *c* oxidase. Sucrose gradient centrifugation and the high turbidity of the enzyme solutions demonstrated that at detergent concentrations below the critical micelle concentration the oxidase self-associated into aggregates. Consequently, we conclude that the change in enzyme activity at the critical micelle concentration directly reflects disaggregation of the oxidase as a result of cooperative detergent binding. These results show that although the three detergents had different effects on the cytochrome *c* oxidase activity, their mode of association to the protein seems to be similar. It should be noted, however, that, although we confirmed that laurylmaltoside disaggregates the oxidase up to a monomeric structure, our results do not demonstrate that this is also true for Triton X-100 and cholate. In fact, several reports have appeared in which oxidase dimers were found at high Triton concentrations [10,27].

A likely mode of interaction of membrane proteins and amphiphylic ligands has been proposed by Tanford [6]. The hydrophobic portion of the protein might act as a nucleus for micelle formation, or bind to an already existing micelle. The criteria for this so-called 'micellar binding' are a highly cooperative binding isotherm and an amount of ligand bound to the protein that is equal to the aggregation number of the detergent.

A perfect example of this type of protein-detergent interaction has been described for the binding of deoxycholate and Triton X-100 to the hydrophobic domain of porcine cytochrome b_5 [28], but deviations have also been reported [29]. In Table II we summarised the literature data on the amount of detergent bound to cytochrome *c* oxidase and on the aggregation number of the detergents studied. It is clear that for all three detergents no relationship exists between detergent binding and micellar aggregation number. Cytochrome *c* oxidase binds more than one micelle of each amphiphile.

Summarising, we conclude that, although the sigmoidal curves (Figs. 1 and 4) suggest that the process of detergent binding to the oxidase is closely related to (but of course slightly favoured over) the process of micelle formation [6], the hydrophobic portion of the enzyme influences the size and shape of the mixed micelles formed.

Highly ordered oxidase aggregates are formed at low detergent concentration

A striking result of our study was the relatively high steady-state activity we found for aggregated forms of cytochrome *c* oxidase. In all experiments the final concentrations were reached by dilution of an oxidase sample containing 1% of the detergent. Our aim in this was to saturate fully the protein with the detergent under study and to dissociate what was left of the cholate used during purification. Since laurylmaltoside, Tween-80 and Triton X-100 were found to affect the kinetics differently from cholate, we conclude that this approach sufficed to overcome possible effects of the residual cholate.

For Tween-80 we found that self-association of cytochrome *c* oxidase did not affect the steady-state activity of the enzyme (Figs. 1 and 2). In our opinion this indicates that upon aggregation of the oxidase no catalytic sites are shielded off. Thus highly ordered protein complexes are likely to be formed.

The ability of cytochrome *c* oxidase to associate into highly regular structures has often been described. Depending on the phospholipid content, detergent and method used, the formation of two-dimensional sheets [1], collapsed vesicles

TABLE II

COMPARISON OF THE NUMBER OF DETERGENT MOLECULES IN MICELLES AND THE MAXIMAL NUMBER BOUND TO ONE MOLECULE OF CYTOCHROME *c* OXIDASE

Detergent	Aggregation number	Refs.	mol detergent per mol aa_3	Refs.
Sodium cholate	3	7	60–100	9
Triton X-100	140	7	150–240	27,29,37
Laurylmaltoside	100–149	11,39	210–362	38,39

[30–33] and 3-dimensional crystals [34,35] have been reported. Our results indicate that also removal of excess Tween-80 by dilution induces a regular association of the protein.

After self association of the oxidase upon dilution from high concentrations of laurylmaltoside and Triton-X-100 to concentrations below the critical micelle concentration, we found kinetic parameters equal to those of the enzyme in Tween-80 ($TN = 50–60\ s^{-1}$, $k_1 = (0.6–0.7) \cdot 10^7\ M^{-1} \cdot s^{-1}$). Therefore, we speculate that all three non-ionic detergents induce the same alignment of the protein in its self-association product.

The only aggregates that showed an activity which was twice as low were those formed at cholate concentrations below the critical micelle concentration. We cannot yet decide whether the lower activities are caused by shielding of catalytic sites or by a true change in catalytic activity.

The interaction of Tween-80 and cytochrome c oxidase

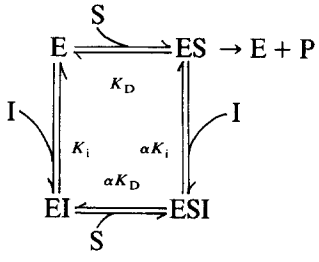
Sucrose gradient centrifugation in Tween-80 revealed a gradual disaggregation of the oxidase with increasing detergent concentration. This effect was found to occur up to concentrations far above the critical micelle concentration. This indicates that the mode of interaction between Tween-80 and the oxidase differs from the cooperative process of mixed-micelle formation observed with the other tested.

Also different is the fact that increased disaggregation of the oxidase in Tween-80 did not result in a concomitant change in activity. Even at 0.2% (w/v) Tween-80, kinetic parameters were measured characteristic for cytochrome *c* oxidase aggregates formed in non-ionic detergents ($TN = 50\ s^{-1}$, $k_1 = 0.7 \cdot 10^7\ M^{-1} \cdot s^{-1}$). Two explanations are possible. The first is that neither Tween-80 binding nor self-association affected the oxidase activity, in contrast to binding of laurylmaltoside, which had a stimulatory effect, and of cholate and Triton, which resulted in inhibition. The alternative is that self-association affected the activity of the oxidase. The low activity at 0.2% (w/v) Tween-80 then indicates that the oxidase was not fully disaggregated. The present data do not allow us to discriminate between the two possibilities.

Triton X-100 and sodium cholate are mixed-type inhibitors

At detergent concentrations above the critical micelle concentration we found that both Triton x-100 and sodium cholate fully inhibited the steady-state activity of cytochrome *c* oxidase. In parallel pre-steady-state kinetic measurements, in which we determined the association rate constant of the ferrocytochrome *c*-cytochrome *c* oxidase complex (k_1), we found a value of $0.8 \cdot 10^7\ M^{-1} \cdot s^{-1}$ for the enzyme solubilised with Triton, and $0.6 \cdot 10^7\ M^{-1} \cdot s^{-1}$ for the oxidase in cholate. Compared to the k_1 value determined for the monomeric enzyme in laurylmaltoside ($1.2 \cdot 10^7\ M^{-1} \cdot s^{-1}$) these values are only slightly lower, indicating that the effects of both detergents on the k_1 are only secondary to their true inhibitory action.

Steady-state kinetic measurements of sodium cholate inhibition of cytochrome *c* oxidase solubilised in Tween-80 performed by Van Buuren et al. [9] revealed that cholate shows a mixed-type inhibition that can be represented as:



in which $1 < \alpha < \infty$ and the presence of inhibitor (I) bound to the enzyme (E) changes the dissociation constant of substrate (S) from K_D to αK_D. Furthermore, the presence of substrate on the enzyme changes the dissociation constant for the inhibitor from K_i to αK_i.

This model for inhibition can explain the results we found for sodium cholate and Triton X-100. At high inhibitor concentrations, the oxidase is completely inactive but each enzyme molecule is still capable of binding and oxidising a cytochrome *c* molecule. The effect of the bound inhibitor of the K_D of this association reaction was seen in the slightly altered association rate constant k_1.

For sodium cholate Van Buuren et al. [9] found

a value of 125 μM for K_i and 190 μM for αK_i, thus $\alpha = 1.5$. Consequently, upon cholate binding to the oxidase the K_D of the ferrocytochrome *c*-cytochrome *c* oxidase complex also changes by a factor of 1.5. The pre-steady-state kinetic measurements we presented in this paper demonstrated that the change in K_D (k_{-1}/k_1) could be completely accounted for by the effect of cholate on the association rate constant k_1.

Finally, we should like to stress that the K_i values determined for cholate inhibition of cytochrome *c* oxidase that is solubilised by Tween-80 [9] are magnitudes lower than the critical micelle concentration of cholate at which we found cholate to bind and to inhibit the oxidase. This indicates that cholate only inhibits when the enzyme preparation has reached a certain level of disaggregation (by the presence of Tween-80 or of cholate itself in a concentration above the critical micelle concentration).

Our results support the notion that the dissociation of ferricytochrome c is rate limiting in steady-state kinetics

Comparison of the kinetic parameters found for the monomeric enzyme in laurylmaltoside and for the aggregates shows that upon self-association the TN_{max} and k_1 values both decreased by a factor of 2, while the K_m value remained unchanged. As discussed above, our results suggest that the decrease in TN_{max} is not caused by a decrease in accessibility of catalytic sites upon self-association of the oxidase. Thus, the effect of enzyme aggregation on the TN_{max} value seems to reflect a change in the rate constant of the rate-limiting step of the steady-state reaction. As proposed before, there is good evidence that the dissociation rate of the ferricytochrome *c*-cytochrome *c* oxidase complex is rate limiting [25,36]. We feel that the concomitant change in TN_{max} and k_1 support this notion. An equal alteration in dissociation (k_{-1}) and association (k_1) rate constants is likely and would require a change in activation energy of enzyme-product complex formation.

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