Protein–Lipid Interactions in Cytochrome Oxidase from *Saccharomyces cerevisiae*

EFFECTS OF DETERGENTS AND RECONSTITUTION OF ENZYME ACTIVITY BY PHOSPHOLIPIDS BY USING CHOLATE-MEDIATED EXCHANGE

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Cytochrome oxidase, purified from the yeast *Saccharomyces cerevisiae*, was shown to have associated phospholipid, cholate or detergent, which could be varied by dialysis or $(\text{NH}_4)_2\text{SO}_4$ precipitation of the protein. Cholate and the detergents Triton X-100 and Tween 80 were shown to differ in their ability to support enzyme activity. Changes in the V_{max} , but not the K_m , for ferrocytochrome *c* as the cholate concentration was varied indicate that cholate increases the number of exposed active sites of the enzyme. Cholate was used to introduce chosen phospholipids into the lipid environment of yeast cytochrome oxidase. Kinetic studies clearly showed that cholate can mediate exchange of exogenous for endogenous phospholipid. All phospholipids screened supported activity up to the basal value for the unsubstituted enzyme, whereas mitochondrial phosphatidylethanolamine and various phosphatidylcholines (except 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) produced enhanced activity. A detailed kinetic examination revealed that the major effect of phosphatidylethanolamine is to increase k_{+1} , whereas the major effect of phosphatidylcholine is to increase k_{+2} in the minimal kinetic scheme

Cardiolipin, although supporting activity, does not give any enhancement of k_{+1} or k_{+2} over the values for the cholate control. The relevance of these observations to protein–lipid interactions in cytochrome oxidase is discussed.

Previous studies have indicated that a single class of phospholipids can reconstitute certain of the functional properties of proteins isolated from biological membranes (Hong & Hubbell, 1973; Warren *et al.*, 1974*a,b,c*; Houslay *et al.*, 1975). There are at least two approaches to the reconstitution of a defined functional structure. The protein may be freed of its lipid during the purification procedure and the pure protein re-activated with exogenous lipid (Hong & Hubbell, 1973). Alternatively, the endogenous lipid can be replaced by a defined lipid with retention of activity throughout the substitution (Warren *et al.*, 1974*a,b,c*). In the latter procedure, replacement of the lipid present with the native enzyme by the defined lipid is mediated by cholate and the reconstituted protein isolated in a form essentially free of cholate by density-gradient centrifugation. A very convenient modification of this procedure, which allows a wide range of lipids to be screened in a short time, is to effect the cholate-

mediated exchange of lipid as described, then to lower the cholate concentration to a very low value by dilution into the assay medium for the enzyme (the 'dilution assay technique'; Warren *et al.*, 1974*a*). In fact the dilution-assay procedure had previously been used to study the role of phospholipids in cytochrome oxidase from ox heart (Tzagoloff & MacLennan, 1965); from these studies, it was concluded that phosphatidylcholine and cardiolipin could reconstitute 60% of the original activity to preparations of the enzyme depleted of phospholipid by repeated $(\text{NH}_4)_2\text{SO}_4$ fractionation in the presence of cholate.

Before using the 'dilution assay technique' to study how various classes of phospholipid are able to support the activity of cytochrome oxidase from *Saccharomyces cerevisiae* (baker's yeast), it is important to establish how cholate itself and other detergents used in the isolation procedure affect the stability and catalytic activity of the enzyme. For the adenosine triphosphatase from sarcoplasmic reticulum (Warren *et al.*, 1974*a,b*), it has been shown that cholate cannot itself support the enzyme activity,

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nor is it an inhibitor; it is, however, an effective reagent for mediating exchange between lipids bound to the enzyme and lipids added externally. Such a simple exchange role for cholate may not hold for other enzymes; indeed it has been reported (Van Buuren & Van Gelder, 1974) that cholate acts as an intrinsic inhibitor of cytochrome oxidase isolated from ox heart, though the yeast enzyme has not similarly been investigated.

The aims of the present investigations are twofold: firstly, to determine the effects of cholate and other detergents used in the isolation procedure on the activity of yeast cytochrome oxidase, and from these results for the effects of cholate, the second aim is to study the effect of various classes of phospholipid on the activity of the enzyme using the dilution assay technique. It is shown below that a detailed kinetic examination of the activation of cytochrome oxidase by phospholipids provides a greater insight into the nature of phospholipid-protein interactions.

Materials and Methods

Materials

Bovine serum albumin (puriss quality) was from Koch-Light Laboratories, Colnbrook, Bucks., U.K.; it was stored over silica gel at 4°C and used as a protein standard without further treatment or drying. Tween 80, cholic acid, Triton X-100 and ferricytochrome *c* (from horse heart, type III) were from Sigma (London) Chemical Co. (Kingston on Thames, Surrey, U.K.); other chemicals (reagent quality) were from BDH Chemicals (Poole, Dorset, U.K.) or from Sigma. The cholic acid was purified by treatment with activated charcoal and subsequent recrystallization (twice) from ethanol; conversion into the sodium salt was effected by adjusting the pH of a 3% (w/v) solution of pure cholic acid in approx. 50% (v/v) aqueous ethanol to 8.3 with NaOH, followed by rotary evaporation and vacuum desiccation to dryness. The uncoupler 1799 [bis(hexafluoroacetyl)acetone] was a gift from Dr. C. I. Ragan, Department of Physiology and Biochemistry, University of Southampton.

Ferrocycytochrome *c* was prepared by anaerobic gel filtration of cytochrome *c* which had been reduced with sodium dithionite by the procedure of Yonetani & Ray (1965). The concentration of cytochrome *c* was determined spectrophotometrically by using the following absorption coefficients: ϵ_{550} (reduced) 27.6 litre $\cdot$ mmol⁻¹ $\cdot$ cm⁻¹ and $\Delta\epsilon_{550}$ (reduced-oxidized) 19.7 litre $\cdot$ mmol⁻¹ $\cdot$ cm⁻¹.

Synthetic phosphatidylcholines were either from Sigma or Fluka (Buchs, Switzerland) and were stored at -25°C. The major phospholipids from the inner membrane of yeast mitochondria (phosphatidylcholine, phosphatidylethanolamine and cardiolipin)

were isolated as follows: electron-transfer particles were prepared from yeast mitochondria by the procedure of Tzagoloff (1969) and extracted by the method of Eichberg *et al.* (1964), to give a crude mixture of phospholipids in chloroform/methanol (2:1, v/v). The mixture was sealed under N₂ in ampoules and sent to Lipid Products, Epsom, Surrey, U.K., where the components phosphatidylcholine, phosphatidylethanolamine and cardiolipin were separated by the procedure of Fleischer *et al.* (1962), and returned again as solutions in chloroform/methanol (2:1, v/v), in ampoules sealed under N₂, which were stored at -25°C before use. Egg phosphatidylcholine was also purchased from Lipid Products and stored at -25°C.

Enzyme isolation

Baker's yeast was from United Yeasts, Morley, Yorkshire, U.K.; mitochondria were prepared from the yeast on the same day by the procedure of Tzagoloff (1969). The mitochondria were stored overnight on ice before preparation of electron-transfer particles by sonication as described by Tzagoloff (1969). The electron-transfer particles were stored in sucrose/Tris buffer (0.25M-sucrose/10mM-Tris/acetic acid buffer, pH7.5 at 20°C) at -25°C before isolation of cytochrome oxidase by the procedure of Eytan & Schatz (1975). This purification uses (NH₄)₂SO₄ fractionation in the presence of sodium cholate; Tween 80 in addition to cholate is used in the later stages of the procedure. The purified enzyme was stored in portions of 1 ml (approx. 3 mg of enzyme) at -70°C in 20mM-Tris/acetic acid buffer, pH7.5 at 20°C, containing 1% Tween 80; the further addition of glycerol (to 30%, v/v) to the storage buffer was made with one batch of the enzyme. Unless otherwise stated, enzyme was used without removal of the Tween 80.

General methods

The purity of all phospholipids was tested by t.l.c. (Hallermeyer & Neupert, 1974). Square silicic acid plates (Merck, Darmstadt, West Germany) were run with chloroform/acetone/methanol/acetic acid/water (28:10:5:5:2, by vol.) in one direction and with chloroform/methanol/25% (v/v) ammonia (13:7:1, by vol.) in the second direction. The plates were first developed with 'Dittmer' reagent (Dittmer & Lester, 1964) to reveal phospholipids and subsequently with iodine vapour to reveal total lipid.

The synthetic phospholipids in chloroform solution (5mg/ml) revealed the presence of a single constituent at a loading of 30 μ l. No impurities were detected in egg yolk phosphatidylcholine at the same loading. The yeast mitochondrial phosphatidylcholine was judged approx. 97% pure, containing

approx. 3% of the lyso degradation product. The yeast phosphatidylethanolamine was approx. 95% pure, the remaining 5% being phosphatidylcholine and the yeast cardiolipin sample contained small percentages of two other unidentified components, which are, however, known not to contain phosphate.

The purity of the isolated cytochrome oxidase was determined from the visible-absorption spectrum before and after reduction with sodium dithionite, by polyacrylamide-gel electrophoresis (using 14% acrylamide gels) in the presence of sodium dodecyl sulphate by the procedure described by Mason *et al.* (1973), and by assay of enzyme activity. According to all these criteria, the enzyme has a purity comparable with that reported by other investigators (Mason *et al.*, 1973; Eytan & Schatz, 1975).

Protein was determined by the method of Lowry *et al.* (1951), as modified by Dulley & Grieve (1975). The phospholipid content of isolated enzyme was measured by phosphate analysis as described by Bartlett (1969). The haem *a* content was measured from the absorption spectrum as described by Yonetani (1961), assuming $\Delta\epsilon_{603-630}$ 16.5 litre·mol⁻¹ cm⁻¹. Cholate was determined by the method of Mosbach *et al.* (1954), as modified by Van Buuren & Van Gelder (1974).

Enzyme-activity measurements

These were done on either a Unicam SP.700 or Unicam SP.1800 recording spectrophotometer fitted with a thermostatic cell housing, the oxidation of ferrocytochrome *c* being followed spectrophotometrically at 550 nm. The standard assay medium (3 ml) contained ferrocytochrome *c* (30 μ M) in 40 mM-sodium phosphate buffer, pH 6.7 at 20°C; reaction was initiated by rapid addition of enzyme. All assays were carried out in unused plastic cuvettes (4 ml capacity; Walter Sarstedt, Leicester, U.K.), which were discarded afterwards to eliminate the possibility of contamination with detergents. The temperature in the spectrophotometer cell was measured to $\pm 0.1^\circ\text{C}$ with an electronic thermometer (Comark model 1604; Comark Electronics, Littlehampton, Sussex, U.K.).

First-order rate constants were determined by the procedure of Smith (1955). Specific activities are given as the first-order rate constant [min⁻¹·(mg of protein)⁻¹]. Initial velocities were obtained by the integral method described by Yonetani & Ray (1965). For the investigations of the intrinsic effector, increasing amounts of the purified enzyme were assayed as described above; the molar ratio of ferrocytochrome *c* to enzyme in these experiments was always greater than 10000:1.

The kinetic data have been fitted to the expression $k = V_{\max}/(K_m + c)$ (Yonetani & Ray,

1965) where k is the apparent first-order rate constant and c is the total concentration of cytochrome *c*; this expression is similar in form to the Michaelis-Menten equation, which has allowed the statistical procedure proposed by Cleland (1967) to be used after appropriate redefinition of terms. The standard errors on K_m and $V_{\max}$ were provided in the computer output.

Removal of cholate from purified enzyme

(a) *By dialysis.* Enzyme (3.5 mg/ml) was dialysed at 0°C for 18 h against a 1500-fold volume excess of 50 mM-Tris/acetic acid buffer, pH 7.5 at 20°C, containing 1% Tween 80.

(b) *By treatment with Amberlite XAD-2 resin.* Enzyme (1.3 mg/ml) was stirred gently with neutral, washed Amberlite XAD-2 resin (0.5 g of resin/mg of protein) at 4°C for either 2 or 18 h. The resin was removed by filtration through glass wool.

Preparation of cytochrome oxidase depleted of phospholipid

Cytochrome oxidase (4.5 mg of protein/ml) was mixed with sodium cholate in 50 mM-Tris/acetic acid buffer, pH 7.5 at 20°C, to a final cholate concentration of 0.5%. Saturated (NH₄)₂SO₄ (pH 7.0 at 4°C) was added dropwise to a final concentration of 35% saturation and the solution stirred at 0°C (on ice) for 15 min, then centrifuged (75000g, 15 min, 4°C). The pellet was resuspended in 50 mM-Tris/acetic acid buffer, pH 7.5 at 20°C, containing sodium cholate (0.5%) and the (NH₄)₂SO₄ precipitation procedure repeated. At each stage, samples were taken for phospholipid, protein and specific-activity assay.

After the final (NH₄)₂SO₄ precipitation, a sample of the enzyme in 50 mM-Tris/acetic acid buffer, pH 7.5 at 20°C, containing sodium cholate (0.5%) was further incubated at 0°C for 16 h before determination of the specific activity.

Removal of cholate from cytochrome oxidase depleted of phospholipid

Enzyme depleted of phospholipid (see the previous section) was subjected to (NH₄)₂SO₄ precipitations as described above except that the pellet was suspended in 50 mM-Tris/acetic acid buffer, pH 7.5 at 20°C, without cholate. The (NH₄)₂SO₄ precipitation was repeated four times, samples being removed for cholate, protein and specific-activity assay at each stage. After the final (NH₄)₂SO₄ precipitation, a sample of the enzyme in 50 mM-Tris/acetic acid buffer, pH 7.5 at 20°C, containing sodium cholate (0.5%) was further incubated at 0°C for 16 h before determination of the specific activity.

Effects of Tween 80, Triton X-100 and cholate on cytochrome oxidase activity: time-dependence

Enzyme was incubated for different times at 0°C in 40 mM-sodium phosphate assay buffer, pH 6.75, containing no detergent, 0.5% Tween 80, 0.5% Triton X-100 or 0.5% sodium cholate. The incubation mixture was warmed to 23°C and the specific activity of the enzyme assayed at this temperature by the addition of ferrocytochrome (30 µM). The amount of Tween 80, added in each case (see under 'Materials'), had a final concentration of 0.0008%.

Effects of Tween 80 on cytochrome oxidase activity: concentration-dependence

(a) Enzyme was incubated for 20 min at 0°C in the presence of various concentrations of Tween 80 and assayed at 25.0°C in the presence of the same concentration of Tween 80.

(b) Enzyme was incubated with various concentrations of Tween 80 at 0°C for 8 h, then assayed in the absence of any detergent at 25°C.

Preparation of samples for reconstitution experiments

Samples of the purified yeast mitochondrial phospholipids and of egg phosphatidylcholine were evaporated to dryness in a stream of high-purity N₂, then vacuum desiccated for 12–18 h. These dried samples were taken up into aqueous solution in one of two ways. For reconstitution experiments in the absence of cholate or in the presence of low concentrations of cholate, sonicated dispersions were prepared by suspension of the dried residues in 50 mM-Tris/acetic acid buffer, pH 7.5 at 20°C, followed by sonication (1–2 min at 4°C using a Dawe Soniprobe fitted with a titanium micro-tip and operating at one-half maximal power) to near clarity and centrifugation (75000g, 15 min, 4°C) to remove undispersed material. For reconstitution experiments in the presence of cholate, the dried residues were suspended in 50 mM-Tris/acetic acid buffer, pH 7.5 at 20°C, containing 0.5% sodium cholate and centrifuged (75000g, 15 min, 4°C) to remove insoluble material. The phospholipid content of the sonicated dispersions and the cholate solutions were determined by phosphate analysis (Bartlett, 1969). Samples of the synthetic phospholipids were similarly converted into sonicated dispersions or cholate solutions.

The phospholipid/cholate ratio was adjusted to the values given in the Figures or Tables (see the Results section) before addition of cytochrome oxidase: the reconstitution mixture was incubated at 0°C for 1 h before assay of the enzyme activity in buffer, 40 mM-sodium phosphate, pH 6.7, containing no cholate or other detergent.

Results

Properties of the purified enzyme

Certain properties of the purified enzyme from two separate preparations (A and B) are summarized in Table 1. Some observations on the stability of the enzyme to storage and to freezing–thawing were made. The specific activities of enzyme (original activity 1100 min⁻¹·mg⁻¹) stored for 2 months at -70°C in 20 mM-Tris/acetic acid buffer, pH 7.5 at 20°C, additionally containing Tween 80 with or without glycerol were as follows: no addition, specific activity 280 min⁻¹·mg⁻¹; addition of 1% (w/v) Tween 80, specific activity 710 min⁻¹·mg⁻¹; addition of 1% (w/v) Tween 80 and 30% (v/v) glycerol, specific activity 920 min⁻¹·mg⁻¹. All these activities refer to enzyme which had been thawed once. These observations, which are similar to those reported by Eytan & Schatz (1975), led to the routine procedure of storing the enzyme in 1 ml portions (protein concn. approx. 3 mg/ml) in 20 mM-Tris/acetic acid buffer, pH 7.5 at 20°C, containing 1% (w/v) Tween 80. The enzyme was never refrozen after thawing.

Investigation of intrinsic effectors in the purified enzyme

The rate of reaction of ferrocytochrome *c* with the purified enzyme was strictly proportional to the enzyme concentration over a 10-fold variation in the latter; this indicates that there is no intrinsic effector present in the purified enzyme.

Effect of cholate removal

Dialysis of the enzyme against buffer containing Tween 80 had little effect on the specific activity

Table 1. *Properties of purified yeast cytochrome oxidase*
For details see the Materials and Methods section.
An average mol.wt. of 775 is used for the phospholipids and a value of 200000 taken for cytochrome oxidase (Caughey *et al.*, 1976).

Property	Preparation A	Preparation B
Specific activity (before freezing) (min ⁻¹ ·mg ⁻¹)	1100	1050
Haem <i>a</i> (nmol/mg of protein)	9.7	8.7
Phospholipid content (mol/mol of protein)	30	25
Cholate content (mol/mol of protein)	500	Not determined
Subunit composition:	42.0	
10 ⁻³ × apparent molecular weight	30.0	
	23.0	
	14.0	
	12.5	
	11.5	
	9.0	

Table 2. *Depletion of phospholipid and cholate from cytochrome oxidase by $(\text{NH}_4)_2\text{SO}_4$ precipitation*
For details see the text.

(a) Phospholipid depletion

No. of precipitations	Phospholipid (mol/mol of protein)	Specific activity at 25°C ($\text{min}^{-1} \cdot \text{mg}^{-1}$)
—	30	545
1	15	367
2	10	330
3	9	303
Enzyme subjected to three $(\text{NH}_4)_2\text{SO}_4$ precipitations, then incubated at 0°C for 16 h in the presence of 0.5% cholate	Not determined	440

(b) Cholate depletion

No. of precipitations	Cholate (mol/mol of protein)	Specific activity at 25°C ($\text{min}^{-1} \cdot \text{mg}^{-1}$)
1	720	440
2	100	307
3	57	217
4	39	104
Enzyme subjected to four $(\text{NH}_4)_2\text{SO}_4$ precipitations, then incubated at 0°C for 16 h in the presence of 0.5% cholate	Not determined	120

or the phospholipid content, which remained at the values shown in Table 1. The cholate content, however, was decreased from 500 to 45 mol/mol of enzyme where the mol.wt. of the enzyme is taken to be 200000 (Caughey *et al.*, 1976).

Removal of cholate by Amberlite XAD-2 treatment allowed only 60% recovery of protein; moreover, the specific activity of the enzyme had decreased to 30% of the original value. No significant increase in the activity of this preparation resulted from inclusion of Tween 80 in the assay medium or preincubation with cholate to a final concentration of 0.5%. Owing to these losses of both protein and enzyme activity, the use of Amberlite XAD-2 to remove cholate was not investigated further.

Effect of phospholipid removal

Repeated $(\text{NH}_4)_2\text{SO}_4$ precipitations in the presence of cholate decreased the bound phospholipid from 30 to 9 mol/mol of enzyme, with a corresponding, though not proportional, decrease in specific activity. The activity of the enzyme subjected to three such $(\text{NH}_4)_2\text{SO}_4$ precipitations could be partially restored by incubation overnight at 0°C in the presence of cholate. The properties of enzyme depleted in phospholipid are shown in Table 2(a).

Effect of cholate removal from enzyme depleted of phospholipid

The properties of enzyme depleted of phospholipid and cholate by $(\text{NH}_4)_2\text{SO}_4$ precipitation in Tris/acetic acid buffer are shown in Table 2(b). The activity of the enzyme after these treatments could not be significantly increased by assay in the presence of Tween 80 or by preincubation overnight at 0°C in the presence of 0.5% cholate.

Effect of detergents on the activity of yeast cytochrome oxidase

(i) *Effect of Tween 80, Triton X-100 and cholate on enzyme activity: time-dependence.* Incubation and assay in the presence of 0.5% Tween 80 had little effect on the enzyme activity, whereas incubation with 0.5% sodium cholate immediately increased the specific activity from 800 to $1200 \text{ min}^{-1} \cdot \text{mg}^{-1}$ at which value it remained constant over a continued incubation period of at least 3 h. Incubation with 0.5% Triton X-100 immediately increased the activity from 800 to $1200 \text{ min}^{-1} \cdot \text{mg}^{-1}$; continued incubation with this detergent, however, resulted in activity decreases to a limiting value of $200 \text{ min}^{-1} \cdot \text{mg}^{-1}$ after 0.5 h.

(ii) *Effect of Tween 80 on enzyme activity: concentration-dependence.* Incubation in the presence and assay in the presence or absence of Tween 80 had little effect on enzyme activities up to detergent concentrations of approx. 1%. However, incubation and assay at very high concentrations of Tween 80 resulted in increased activities; thus 10% Tween 80 caused the activity to increase from 700 to $1200 \text{ min}^{-1} \cdot \text{mg}^{-1}$.

(iii) *Effect of cholate on enzyme activity: concentration-dependence.* Fig. 1 shows the effects of preincubation of the enzyme with different amounts of cholate followed by assay in both the presence and the absence of cholate.

Table 3 shows the results of more detailed kinetic investigations into the effects of cholate in which the concentration of ferrocytochrome *c* was varied; assays were carried out in both the presence and the absence of cholate.

The apparent second-order rate constant for ferrocytochrome *c* binding, k_s , and the apparent first-order catalytic rate constant, k_{cat} , were calculated from K_m and V_{max} by the procedure proposed by Yonetani & Ray (1965).

Effect of cholate concentration on reconstitution of cytochrome oxidase activity by yeast mitochondrial lipids

Fig. 2 shows the effect of cholate concentration on the specific activity of enzyme reconstituted by phospholipids. The specific activity in assay media containing phosphatidylcholine and phosphatidylethanolamine is enhanced by increasing cholate

concentrations, whereas media containing cardiolipin give no such increased activity.

Effects of phospholipids on cytochrome oxidase activity

The results of various enzyme-activity assays carried out at a constant sodium cholate concentration of 0.5% are summarized in Table 4.

To provide further evidence that exchange of phospholipid between the bound state and the added lipid pool is occurring, enzyme was first incubated for 1 h at 0°C in the presence of 0.5% cholate with one class of phospholipid, after which a

second class of phospholipid was added to the cholate medium and the specific activity determined after a further incubation for 1 h at 0°C. The results are shown in Table 5.

Detailed kinetic analysis of cytochrome oxidase reconstituted with different mitochondrial phospholipids in the presence of 0.5% sodium cholate

The apparent second-order rate constant for ferrocytochrome *c* binding, k_s , and the first-order catalytic rate constant, k_{cat} , were calculated by the procedure proposed by Yonetani & Ray (1965) from values of K_m and V_{max} , determined in the presence of the different mitochondrial phospholipids. The results are shown in Table 6.

Fig. 1. Effects of cholate on cytochrome oxidase activity: concentration-dependence

Enzyme was preincubated in the presence of different amounts of cholate and assayed in the presence of the same amount of cholate or in the absence of cholate. ●, Preincubation at 25°C for 60–90s, assay in the presence of cholate; ○, preincubation at 0°C for 16h, assay in the presence of cholate; △, preincubation at 0°C for 16h, assay in the absence of cholate.

Fig. 2. Effect of yeast mitochondrial phospholipids on the activity of yeast cytochrome oxidase

Enzyme was incubated for 1 h at 0°C with a 2500-fold molar excess of the phospholipid in the presence of the concentrations of sodium cholate shown on the abscissa, then assayed in the standard assay medium (3 ml) containing ferrocytochrome *c* (30 μM) and 40 mM-sodium phosphate buffer, pH 6.7, at 24.5°C. The assay buffer contained no additional cholate. △, Control (cholate only); □, phosphatidylcholine; ▽, phosphatidylethanolamine; ○, cardiolipin.

Table 3. Effect of cholate on kinetic parameters for yeast cytochrome oxidase

The kinetic parameters and standard errors were determined by computer assessment of data presented in Fig. 2 and represent the average of two separate experiments which gave closely similar results. The enzyme concentration used was 0.89 nM.

[Cholate] in preincubation (%)	[Cholate] in assay (%)	$10^6 \times K_m$ (M)	$10^8 \times V_{max}$ (M·s ⁻¹)	k_{cat} (s ⁻¹)	$10^{-7} \times k_s$ (M ⁻¹ ·s ⁻¹)
—	—	5.8 ± 0.7	21.4 ± 0.8	242	4.2
0.5	0.5	5.9 ± 0.8	39.7 ± 1.6	449	7.6
1.0	1.0	6.2 ± 0.4	12.3 ± 0.2	140	2.3
0.25	—	6.5 ± 0.7	19.7 ± 0.5	223	3.4
1.0	—	6.6 ± 1.0	28.5 ± 1.8	322	4.9

Table 4. *Effects of various phospholipids on cytochrome oxidase activity*
 The temperature for these assays was 24.5°C. For further details see the Materials and Methods section.

Expt. no.	Phospholipid	Phospholipid/protein (mol/mol)	Specific activity (min ⁻¹ ·mg ⁻¹)	
I	—	—	750	
	Yeast mitochondrial phosphatidylcholine	3000	1685	
	Yeast mitochondrial phosphatidylethanolamine	3000	1370	
	Yeast mitochondrial cardiolipin	3000	780	
II	—	—	No uncoupler 1799 present	40 µM-uncoupler 1799 present
	Phosphatidylcholine	3500	610	650
	Phosphatidylethanolamine	3500	1050	1150
	Phosphatidylethanolamine	3500	860	890
III	Phosphatidylcholine/phosphatidylethanolamine/ cardiolipin (molar proportions)*			
	45:2:1	2000		1650
	4:3:1	2000		1700
	8:3:1	3000		1700
IV	—	—	760	
	Egg-yolk phosphatidylcholine	1500	1500	
	1,2-Dimyristoyl- <i>sn</i> -glycero-3-phosphocholine	1500	1600	
	1,2-Dioleoyl- <i>sn</i> -glycero-3-phosphocholine	1500	1600	
	1,2-Dipalmitoyl- <i>sn</i> -glycero-3-phosphocholine	1500	700	

* The molar proportions of these major phospholipids in yeast inner mitochondrial membrane are 1:2:2 (Fleischer *et al.*, 1961).

Discussion

Properties of the isolated enzyme and the effects of detergents

The phospholipid content of the enzyme as isolated varies from 15 to 35 mol/mol of protein. Similar values have been reported for the yeast enzyme by Tzagoloff and co-workers (Tzagoloff, 1969; Rubin & Tzagoloff, 1973). Successive (NH₄)₂SO₄ precipitations decrease the amount of phospholipid bound to the yeast enzyme to a limiting value of approx. 9 mol/mol of protein. By a similar procedure, Tzagoloff & MacLennan (1965) decreased the phospholipid bound to the ox heart enzyme to 2.5 µg of P/mg of protein, which corresponds to approx. 16 mol of phospholipid/mol of protein, though Yu *et al.* (1975) claim to have obtained a preparation of the ox heart enzyme that contains only 0.01 µmol of P/mg of protein (approx. 2 mol of phospholipid/mol of protein). From spin-labelling studies, Jost *et al.* (1973) have concluded that a complete annulus composed of 50 phospholipid molecules surrounds cytochrome oxidase isolated from ox heart. Since the molecular weights of the yeast and ox enzymes are similar, it appears that our purified yeast enzyme has an incomplete annulus of lipid. However, the purification of yeast enzyme is carried out in the presence of Tween 80 and cholate, so it therefore seems reasonable to suppose that these detergents complete

the hydrophobic envelope needed for enzyme stability. Indeed, the data presented in Table 1 indicate that 500 mol of cholate/mol of protein are associated with the enzyme after purification. Van Buuren & Van Gelder (1974) have reported that this associated cholate acts as an intrinsic inhibitor of ox cytochrome oxidase; for the yeast enzyme, we find no evidence for associated cholate acting as an intrinsic effector. Support for this conclusion comes from two other observations. Firstly, the data in Table 3, lines 1 and 4, show that the activity of enzyme incubated in the presence or absence of cholate is the same; moreover these data establish that the 300-fold dilution of enzyme that has been incubated with 0.05% cholate must remove most of the cholate from association with the enzyme, otherwise enhanced activity would be obtained, as noted when the enzyme was incubated and assayed with cholate (see Fig. 1). Secondly, the results of our experiments to test the effects of cholate removal show that about 90% of the associated cholate can be removed by dialysis against buffer containing Tween 80 with only slight changes in enzyme activity. By contrast, when cholate is removed from yeast cytochrome oxidase depleted of phospholipid, large and irreversible losses of activity result (see Table 2b). Qualitatively, it has been observed that cholate depletion of enzyme already depleted of phospholipid causes aggregation to occur; a plausible explan-

Table 5. *Effects of double exchange of yeast mitochondrial phospholipids on cytochrome oxidase activity*
All incubations were made at 0°C and assays at 24.5°C. For details see the text.

Phospholipid in first substitution	Phospholipid/ protein (mol/mol)	Specific activity after first substitution (min ⁻¹ ·mg ⁻¹)	Phospholipid/protein (mol/mol)			Specific activity after second substitution (min ⁻¹ ·mg ⁻¹)
			Cardio- lipin	Phosphatidyl- choline	Phosphatidyl- ethanolamine	
Cardiolipin	700	940	1480	—	—	900
	700	940	700	1400	—	1480
	700	940	700	—	1400	1200
Phosphatidylcholine	1300	1730	—	2600	—	1800
	1300	1730	700	1300	—	1200
	1300	1730	—	1300	1300	1500

ation for the aggregation and loss of activity is that cytochrome oxidase which is not protected by associated phospholipid or detergent aggregates through protein-protein interactions followed by irreversible protein-conformational changes. The loss of activity observed after treatment with Amberlite XAD-2 is probably also due to such protein-protein interactions, since the resin has an affinity for both cholate and lipid. Therefore we conclude that cholate can provide the hydrophobic environment necessary for enzyme stability, but is itself unable to support enzyme activity. A similar conclusion follows from the observation by Yu *et al.* (1975) that a preparation of the ox heart enzyme depleted of phospholipid yet having residual cholate is essentially devoid of activity.

There are interesting differences between the effects of Triton X-100, cholate and Tween 80 on cytochrome oxidase activity. Triton X-100 initially stimulates enzyme activity, though a longer time of incubation with this detergent results in large decreases in enzyme activity; this effect has not been studied in any detail, though one can speculate that the loss in activity is due to replacement of phospholipid bound to the enzyme by detergent molecules. There is little effect of Tween 80 on enzyme activity over a wide range of preincubation times and Tween 80 concentrations. Very high Tween 80 concentrations stimulate enzyme activity; this effect is also observed with cholate, though at much lower detergent concentrations.

The effect of cholate in stimulating activity can be interpreted in terms of an increase in effective enzyme concentration. In the absence of detergents from the assay medium, dilution of enzyme after preincubation results in aggregates in which some of the sites for interaction with ferrocytochrome *c* are blocked. This aggregation is prevented by the presence of cholate in the assay medium and is even becoming apparent when a 1% cholate concentration is used in the preincubation mixture, but with no cholate in the assay medium. When the assay medium contains cholate at concentrations greater than 0.3%, decreases in enzyme activity result (Fig. 1). These effects of low and high cholate concentration on enzyme activity can be more clearly understood through a comprehensive kinetic examination. The kinetic parameters calculated from these data (Table 3) have been analysed by the model described by Yonetani & Ray (1965), where binding of ferrocytochrome *c* to ox heart cytochrome oxidase and its conversion into ferricytochrome *c* can be considered in terms of a simple Michaelis-Menten equation:

The Yonetani & Ray (1965) analysis requires the

Table 6. Kinetic parameters for ferrocytochrome c reaction with yeast cytochrome oxidase in the presence of different yeast mitochondrial phospholipids

The kinetic parameters and standard errors were determined by computer assessment of the data and represent the average of three separate experiments which gave closely similar results. The molar ratio of phospholipid to enzyme was 2000:1. Further details are given in the text. It should be noted that the analysis of Yonetani & Ray (1965) justifies k_s being assumed equal to k_{+1} and $k_{cat.}$ being assumed equal to k_{+2} for the simple kinetic scheme

Phospholipid	$10^6 \times K_m$ (M)	$10^8 \times V_{max.}$ (M · s ⁻¹)	$k_{cat.}$ (s ⁻¹)	$10^{-7} \times k_s$ (M ⁻¹ · s ⁻¹)	Fractional increase over control value	
					$k_{cat.}$	k_s
—	5.8 ± 0.7	21.4 ± 0.8	242	4.2	1	1
Cardiolipin	5.1 ± 0.6	18.6 ± 0.6	210	4.1	0.87	0.98
Phosphatidylcholine	11.4 ± 1.7	41.8 ± 2.7	472	4.1	1.96	0.98
Phosphatidylethanolamine	2.3 ± 0.5	24.7 ± 0.8	279	12.1	1.15	2.88

assumption that $k_{+2} \gg k_{-1}$; this assumption was justified by the similar values for k_{+1} determined from their analysis and directly from stopped-flow studies (Gibson *et al.*, 1965; Wilson *et al.*, 1975). By using the value reported by Vanderkooi *et al.* (1973) for the binding constant of ferrocytochrome c to the enzyme ($0.5 \times 10^6 M^{-1}$) and the value of k_{+1} of approx. $5 \times 10^6 M^{-1} \cdot s^{-1}$ measured by Wilson *et al.* (1975), k_{-1} can be calculated to be approx. $10 s^{-1}$, which Table 3 shows to be much less than k_{+2} ; this provides further justification for the assumption made by Yonetani & Ray, (1965). It should be noted that the Yonetani & Ray (1965) analysis further justifies k_s being assumed equal to k_{+1} and $k_{cat.}$ being assumed equal to k_{+2} .

Thus from the Yonetani & Ray (1965) model and assuming therefore that $k_{+2} \gg k_{-1}$, it follows that $K_m \approx k_{+2}/k_{+1}$. Since $V_{max.}$ is given by $k_{+2} [E_{total}]$, then an observed increase in $V_{max.}$ without any change in K_m suggests an increase in effective enzyme concentration. Table 3 shows that this indeed occurs up to a cholate concentration of 0.3%. Above 0.3% cholate, a decrease in $V_{max.}$ is observed, whereas K_m remains approximately constant, suggesting that cholate is acting as a non-competitive inhibitor. If cholate is inhibiting, then the results of other experiments shown in Table 3 in which enzyme was incubated with high concentrations of cholate, but assayed in the absence of cholate, clearly indicate that the inhibition is reversible.

Reconstitution of enzyme activity by phospholipids by using cholate-mediated exchange

To summarize the conclusions from the previous section, the evidence indicates that cholate stimulates cytochrome oxidase activity by a disaggregation process yet without itself being able to support activity. An additional role for cholate, namely that

of mediating exchange of exogenous for endogenous phospholipids, has been found with other enzymes (Warren *et al.*, 1974a,b,c; Houslay *et al.*, 1975). The data in Fig. 2 and Table 4 show that phosphatidylcholine and phosphatidylethanolamine in the presence of 0.5% cholate enhance the activity of yeast cytochrome oxidase above that of the enzyme in the presence of cholate alone; cardiolipin, however, does not enhance the activity above that of the cholate control. This is evidence to support the contention that cholate is mediating exchange of exogenous for endogenous phospholipids, which then exert their effect on enzyme activity.

The results of double-substitution experiments presented in Table 5 provide strong evidence that cholate-mediated exchange of phosphatidylcholine, phosphatidylethanolamine and cardiolipin between the enzyme-bound condition and the lipid pool occurs. Thus, for example, the specific activity of cytochrome oxidase that has been incubated with a large excess of phosphatidylcholine in the presence of cholate is virtually unchanged (1800 from 1730 min⁻¹ · mg⁻¹) when further phosphatidylcholine is added, but decreases to 1200 min⁻¹ · mg⁻¹ when cardiolipin is added; it will be remembered that the data in Fig. 2 and Table 4 showed that cardiolipin is unable to enhance cytochrome oxidase activity.

Taken with the observations discussed above that dilution of cholate incubation mixtures decreases the cholate associated with the enzyme to a low concentration, the results in Tables 4 and 5 indicate that the enhancement of activity with phosphatidylcholine and phosphatidylethanolamine is due to the phospholipid rather than the cholate. We cannot rule out the possibility that the enhancement is due to some combined effect of cholate and phospholipid; isolation of enzyme from the dilution assay medium would not permit a reliable estimate of the concentration of cholate associated with the enzyme.

However, our conclusion that the enhancement effects observed are due to the phospholipids is supported by studies made in other laboratories. Thus Racker *et al.* (1975) have found that the dilution procedure is as effective as dialysis in lowering the cholate concentration local to the protein and have used both methods to study the reconstitution of Ca^{2+} -translocation and oxidative-phosphorylation activities. Again, Warren *et al.* (1974*a,b,c*) have provided convincing evidence that similar results are obtained for the reconstitution of activity to the adenosine triphosphatase from sarcoplasmic reticulum when either the dilution assay or direct studies on pure complexes of phospholipids with the enzyme are used.

Considering now in more detail the results summarized in Table 4, all the synthetic and natural phosphatidylcholines tested enhance cytochrome oxidase activity, except 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine. The simplest explanation for this latter result is that 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, whose phase transition is 42°C, would be in the gel phase at the temperature of assay (24.5°C). Table 4 also shows that the uncoupler 1799 does not affect the enzyme activity reconstituted with phosphatidylcholine, which indicates that, under the conditions of the dilution assay, respiratory control is not affecting enzyme activities: this reagent has been shown to be an effective uncoupler of phosphorylation linked to oxidation of succinate under similar dilution assay conditions (Racker *et al.*, 1975).

Further insight into the mode of action of yeast mitochondrial phosphatidylcholine and phosphatidylethanolamine in the reconstitution of cytochrome oxidase activity is obtained by a more detailed kinetic investigation, the results of which are given in Table 6. These phospholipids affect both $V_{\max}$ and K_m relative to a control containing only cholate.

By using the Yonetani & Ray (1965) analysis discussed above, values for k_{+1} and k_{+2} can be calculated from the kinetic parameters. Table 6 shows that the major effect of phosphatidylethanolamine is to increase k_{+1} , whereas the major effect of phosphatidylcholine is to increase k_{+2} . The simplest picture consistent with these results is that phosphatidylethanolamine enhances binding of ferrocycytochrome *c* to the enzyme, whereas phosphatidylcholine exerts an effect on later catalytic steps.

Attempts can be made to correlate our results on the effects of the different classes of phospholipids with those from other investigations. There have been conflicting reports on the ability of cardiolipin to support cytochrome oxidase activity. With ox heart cytochrome oxidase depleted of phospholipids, Tzagoloff & MacLennan (1965) and Yu *et al.* (1975) have reported that cardiolipin has an activity-enhancing effect, whereas Wharton &

Griffiths (1962) found no activity enhancement with cardiolipin under conditions where other phospholipids did enhance activity. A specific role for the negatively charged head group of cardiolipin in binding ferrocycytochrome *c* has been proposed from the results of monolayer (Quinn & Dawson, 1969) and spin-labelling studies (Birrell & Griffith, 1976). Our results suggest that cardiolipin can support the activity of the enzyme, but that, unlike the behaviour with phosphatidylcholine and phosphatidylethanolamine, the activity-enhancing effect is not exhibited. Further, the results in Table 2(*a*) indicate that 9 mol of phospholipid/mol of enzyme remain bound to the enzyme after repeated $(\text{NH}_4)_2\text{SO}_4$ precipitations in the presence of cholate; Robinson & Capaldi (1977) have shown that approx. 10 mol of cardiolipin/mol of ox heart cytochrome oxidase is resistant to detergent exchange.

Thus a plausible explanation for our observations and those reported by other workers is that basal activity of yeast cytochrome oxidase depends on the presence of associated cardiolipin; the basal activity can be selectively enhanced by both phosphatidylcholine and phosphatidylethanolamine. This hypothesis could be tested by detailed studies on isolated complexes of yeast cytochrome oxidase having a known phospholipid composition.

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