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ENZYME BEHAVIOUR AND MOLECULAR ENVIRONMENT

THE EFFECTS OF IONIC STRENGTH, DETERGENTS, LINEAR POLYANIONS AND PHOSPHOLIPIDS ON THE pH PROFILE OF SOLUBLE CYTOCHROME OXIDASE

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Summary

The activity vs. pH profile for the oxidation of ferrocytochrome *c* by purified cytochrome oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) was investigated as a function of ionic strength (from 10 to 200 mM) in the absence and in the presence of various perturbants: Tween 20, linear polyanions (RNA, heparin, polyglutamic acid) and phospholipids (asolectin, phosphatidylcholine, phosphatidic acid and cardiolipin).

The activation induced by Tween 20 and "zero net charge" phospholipid liposomes was not pH dependent. On the other hand, linear polyanions and polyanionic liposomes strongly perturbed the pH profile, mostly at low ionic strength, by shifting the pH optimum about 1.7 pH units towards alkaline pH values. This effect was reversed by increasing ionic strength. These observations are interpreted in the light of polyelectrolyte theory.

Since these results show striking similarities with those obtained under the same conditions of ionic strength with membrane-bound enzyme, it is concluded that in vivo cytochrome oxidase is located within polyanionic sites of the mitochondrial membrane.

The activation brought about by phospholipids may result from two possible processes: creation of a hydrophobic environment by the non-polar tails, preventing autoaggregation; and creation of a suitable polyelectrolytic environment by the polar heads (of non zero net charge), increasing the intrinsic reaction rate.

Introduction

We recently reported [1] that, at low ionic strengths, soluble and membrane-bound cytochrome oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) had different pH optima. These observations were interpreted according to polyelectrolyte theory [2—6], by assuming that the membrane-bound enzyme was located within polyanionic sites of the mitochondrial membrane. Although in agreement with some other recent reports [7,8], this interpretation remained speculative in the absence of a more thorough investigation.

We undertook the present work to clarify this point. Our purpose was 2-fold: firstly, to investigate the perturbations (if any), brought about in the behaviour of soluble cytochrome oxidase as a function of pH and ionic strength, by various reagents providing the enzyme with selected environments (hydrophobic and polyelectrolytic); secondly, to compare these perturbations (if any) with those observed using the mitochondrial membrane-bound form of the enzyme, in order to gather information about its natural environment.

Materials and Methods

Chemicals. Buffers, acetic acid · HCl, cacodylic acid · NaOH, Tris base · HCl, NaCl, $MgCl_2$, Na_2SO_4 were from Merck (Darmstadt, G.F.R.). Tween 20, polyglutamic acid sodium salt (mol. wt. 98 000), heparin sodium salt, asolectin, phosphatidylcholine (in hexane) and cardiolipin sodium salt (in ethanol) were from Sigma (St. Louis, U.S.A.). RNA (from Yeast, highly polymerized) was from Calbiochem (Lucerne, Switzerland) and phosphatidic acid sodium salt (lyophilized) from Koch Light Labs Ltd. (Colnbrook, U.K.). All the other chemicals were of highest quality. Water was deionized and twice distilled.

Submitochondrial particles. The preparation procedure was that described by Crane et al. [9], (method II). Protein concentration was measured by the biuret procedure of Jacobs et al. [10]. Cytochrome oxidase (aa_3) concentration was determined spectrophotometrically using $\Delta\epsilon_{605-630}$ (red-ox) = 24 $mM^{-1} \cdot cm^{-1}$ [11]; a typical value was 0.5 nmol aa_3/mg protein.

Cytochrome oxidase. A purified preparation of cytochrome oxidase (15% (w/w) phospholipid) was made from beef heart according to the method of Yonetani [12,13]. This preparation was dissolved in 10 mM potassium phosphate, 1% Tween 20 and stored at −20°C.

Cytochrome c. Lyophilized cytochrome *c* (type VI, from horse heart) from Sigma (St. Louis, U.S.A.) was used without further purification. Ferrocytochrome *c* was prepared according to Yonetani and Ray [14]. The concentration was determined spectrophotometrically using $\Delta\epsilon_{550}$ (red-ox) = 19.7 $mM^{-1} \cdot cm^{-1}$ [15].

Liposomes. Liposomes were prepared as described by Eytan et al. [16]. In all experiments their concentration is expressed in μatoms phosphorus, per l determined according to the method of Ames and Dubin [17].

Binding studies. Cytochrome *c* (30 μM) and liposomes of phosphatidic acid (10 mg/ml) were incubated in 20 mM sodium cacodylate buffer (pH 6.5) for 1 h at 4°C under various conditions of ionic strength (10, 110 and 210 mM NaCl), and in the absence and in the presence of 2% Tween 20. The suspension

was then centrifuged at 30 000 × *g* for 1 h. The concentration of free and bound cytochrome *c* was determined as recommended by Kimelberg et al. [18].

Kinetic analysis. The initial rate of reaction (over a period from 0 to 30—60 s) was measured spectrophotometrically at 20°C, according to the method of Smith and Conrad [19]. The assay medium (total volume 2 ml) was prepared in a 1 cm path length quartz cell. To 1 ml 20 mM buffer were added appropriate volumes of ferrocytochrome *c* and perturbant solution (containing detergent, polyanions or liposomes) as required. Ionic strength was adjusted with 1 M NaCl. The reaction was initiated by the addition of 2—15 μl cytochrome oxidase. First-order kinetics were initially obtained under all experimental conditions; however, below pH 5.5, the lower the pH the shorter the interval of time within which the kinetics were first order. Kinetic parameters, V and K_m (app), relative to cytochrome *c*, were determined from Lineweaver-Burk plots [20], the concentration of cytochrome *c* varying from 3 to 50 μM. The absolute uncertainty was estimated as ±5% on V and ±10% on K_m (app).

Apparatus. All spectroscopic determinations were carried out with either a Beckman Acta III or an Aminco DW 2 spectrophotometer equipped with a thermostatted cell compartment [21].

Centrifugation was done with a Beckman L3 50 ultracentrifuge (Rotor 50 TI).

pH was determined directly in the assay medium at 20°C with a thin electrode (Radiometer GK 2321) and a Radiometer pH meter 26, previously calibrated with three standard buffer solutions.

Results

Cytochrome oxidase in the presence of a non-ionic detergent and of zero net charge phospholipids

Activity vs. pH profiles at various ionic strengths of purified cytochrome oxidase, preincubated in 1% Tween 20 are reported in Fig. 1, and K_m (app) values for cytochrome *c* at selected pH and ionic strength values, are quoted in Table I. At low ionic strength (10 mM NaCl) the pH optimum seems to lie at 4.8 (no reliable measurements could be performed at pH values lower than 4.5 because of rapid pH-induced enzyme denaturation). As ionic strength increases it is slightly displaced towards higher pH. Between 60 and 210 mM in NaCl, the pH optimum does not change significantly from 5.5, in reasonable agreement with earlier reports [22,23]. We can see (Fig. 1 inset) that, as ionic strength increases at constant pH, the reaction is first stimulated and then inhibited after passing through a maximum. These results, in agreement with others [8,24], show that no ion specificity is involved in this process. As already observed [8,24], K_m (app) was found to increase markedly with ionic strength (a 20-fold increase between 10 and 200 mM).

No modification of this behaviour was observed when the enzyme was assayed in the presence of low net charge liposomes prepared from phosphatidylcholine or asolectin (0—20 μM). Table I shows that the presence of these liposomes does not change K_m (app) from its control value.

When cytochrome oxidase is incubated and assayed in the absence of either

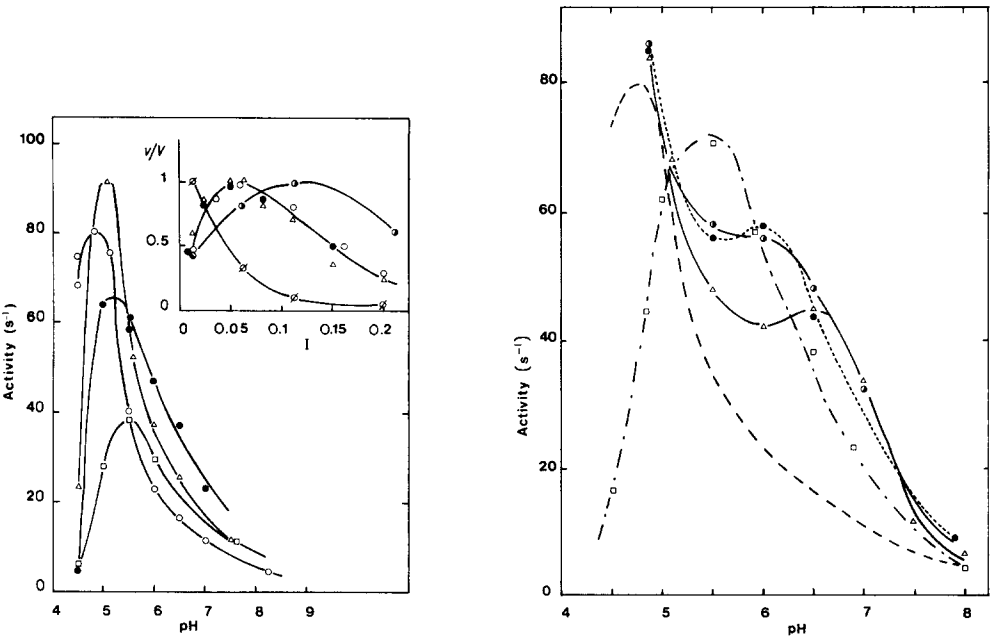

Fig. 1. Activity (V/[E]) of purified cytochrome oxidase (aa_3) as a function of pH at various ionic strengths in NaCl: —○—, 10 mM; —△—, 60 mM; —●—, 110 mM; —□—, 210 mM. The enzyme was diluted to 3.95 μM in 100 mM phosphate buffer, (pH 7.0) and 1% Tween 20. After 1 h incubation at 4°C, 2-μl aliquots were added to the assay medium (10 mM buffer: acetate pH 4.5—5.5, cacodylate pH 5.5—7.0, Tris pH 7.5—9.0; ferrocytochrome c: 3—50 μM) to start the reaction. Insert: Normalized curves of activity as a function of ionic strength at various pH. Ionic strength was adjusted with: —○—, NaCl; —▽—, KCl; —●—, Na_2SO_4; —△—, $MgCl_2$ at pH 5.2. —ϕ—, NaCl at pH 4.5 and —◑—, NaCl at pH 6.0.

Fig. 2. Activity (V/[E]) of cytochrome oxidase (aa_3) as a function of pH and ionic strength in NaCl, in presence of linear polyanions. —△—, polyglutamic acid (10 μg/ml); —◑—, heparin (10 μg/ml); —●—, RNA (10 μg/ml), ionic strength 10 mM. pH profile of the soluble enzyme for comparison (- - - - - -) at 10 mM NaCl, and (—·—·—·—·—) at 110 mM NaCl (from Fig. 1). —□—, RNA ionic strength 110 mM NaCl. The enzyme was diluted to 4 μM in 100 mM phosphate buffer (pH 7.0), 1% Tween 20. Linear polyanions were added to the assay medium (10 mM buffer: acetate pH 4.5—5.5, cacodylate pH 5.5—7.0, Tris pH 7.5—9.0; ferrocytochrome c: 3—50 μM); then the reaction was started by addition of 2-μl aliquots of the enzyme solution.

Tween 20 or phospholipids the results are qualitatively similar to those Fig. 1, but the activity of the enzyme is extremely low (less than one-hundredth of the activity shown in Fig. 1) and K_m (app) values are high (Table I).

TABLE I

K_m (app) RELATIVE TO CYTOCHROME c, FOR SOLUBLE CYTOCHROME OXIDASE ASSAYED AT pH 6.5, IONIC STRENGTH 10 mM NaCl, UNDER VARIOUS EXPERIMENTAL CONDITIONS

TW 20, experimental conditions as in legend Fig. 1. PC, phosphatidylcholine liposomes (15 μM). pGlu, polyglutamic acid: PA (1), experimental conditions as in legend Fig. 4; PA (2), experimental conditions as in legend Fig. 4 with a high concentration of phosphatidic acid: 86 μM. CL, cardiolipin: experimental conditions as in legend Fig. 4.

Perturbants	None	TW 20	PC	pGlu	PA (1)	PA (2)	CL
K_m (app) at pH 6.5, I = 10 mM	20.5	5.2	4.1	6.1	5.2	20.0	5.05
		6.5		4.3	4.3	12.6	5.95
		4.7		4.5	4.85		4.2

Cytochrome oxidase in the presence of linear polyanions

pH profiles obtained at different ionic strengths in the presence of RNA, polyglutamic acid and heparin are reported in Fig. 2. By comparison with Fig. 1, it is clear that all the polyanions tested modify the pH profile of the enzyme at low ionic strength. Whereas the pH optimum remains at 4.8, a second optimum or shoulder appears at 6.0—6.5. As ionic strength increases the perturbation disappears and the "normal" pH profile is restored. At low ionic strength and at pH values lower than 5.5, the effect of the polyanions no longer occurs.

The activation by these polymers has been studied under various conditions of enzyme, cytochrome *c* and detergent concentration. The results obtained with polyglutamic acid are presented in Fig. 3 (identical observations were made with the other polyanions). The activation first increases linearly and then reaches a plateau, so that it is possible to define a polyglutamic acid concentration giving 50% maximal activation (50% PG). When cytochrome *c* concentration is kept constant, 50% PG decreases with enzyme concentration; on the other hand when enzyme concentration is kept constant, 50% PG decreases with cytochrome *c* concentration (see Table II). These results indicate that both enzyme and cytochrome *c* interact with polyglutamic acid. In view of their relative concentrations in the assay medium, cytochrome oxidase is probably saturated with polyglutamic acid and cytochrome *c* can act as a competitor towards this process. Table II also shows that 2% Tween 20 has no effect on 50% PG.

The "activation ratio" (rate at maximal activation divided by rate in the absence of polyglutamic acid) is not dependent on the cytochrome *c* concentration and is slightly increased when the enzyme concentration decreases (Table III). K_m (app) is thus not affected by the linear polyanions as was confirmed by direct Lineweaver-Burk plots (Table I). Table III shows that as ionic strength increases the "activation ratio" decreases strongly. Tween 20 has no effect on this ratio.

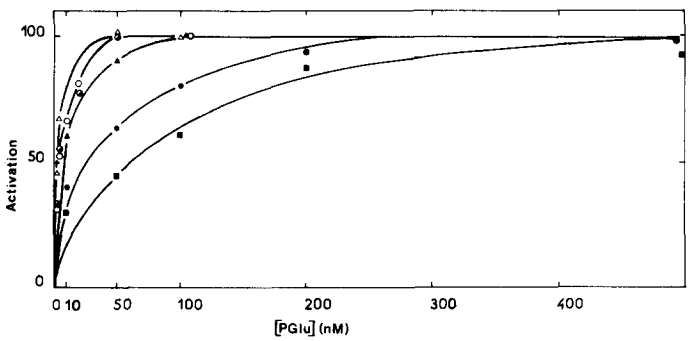

Fig. 3. Polyglutamic acid-induced activation at pH 6.5 and 10 mM NaCl as a function of polyglutamic acid concentration in the assay medium (experimental procedure described in legend Fig. 2). Enzyme concentration in the assay medium 0.4 nM: —△—, 5 μM cytochrome *c*; —○—, 25 μM cytochrome *c*; —◑—, 25 μM cytochrome *c*, 2% Tween 20. Enzyme concentration in the assay medium 4 nM: —▲—, 5 μM cytochrome *c*; —●—, 25 μM cytochrome *c*; —■—, 50 μM cytochrome *c*.

TABLE II

ACTIVATION OF PURIFIED CYTOCHROME OXIDASE BROUGHT ABOUT AT pH 6.5 AND 10 mM NaCl BY POLYGLUTAMIC ACID AND ANIONIC LIPOSOMES

Polyglutamic and phosphatic acids and cardiolipin (perturbant concentrations inducing 50% of maximal activation are taken from Figs. 3 and 5, under various conditions of concentration of enzyme, cytochrome *c* and detergent.

Cytochrome *c*	Polyglutamic acid		Phosphatidic acid		Cardiolipin	
	0.4 nM	4 nM	0.4 nM	4 nM	0.4 nM	4 nM
5 μM	2.5 nM	9 nM	3.0 μM	2.5 μM	0.5 μM	
25 μM	4.5 nM	25 nM	6.0 μM	6 μM	1.9 μM	1.2 μM
	4.5 nM		5.5 μM			
50 μM		60 nM		8.3 μM		
25 μM Tween 20 (2%)	4.5 nM		42.5 μM			

Cytochrome oxidase in the presence of anionic phospholipids

Fig. 4 presents the pH profiles for the enzyme assayed at high and low ionic strength in the presence of liposomes composed of cardiolipin and phosphatidylcholine in variable proportions. As the proportion of cardiolipin is increased the pH profile at low ionic strength is progressively more perturbed; with pure cardiolipin or pure phosphatidic acid liposomes the pH optimum of the reaction is 6.5 instead of 4.8. As ionic strength is increased to 210 mM NaCl, the "normal" pH profile is restored (pH optimum 5.5). When the final amounts of phospholipid in the assay medium do not exceed the equivalent of 10—20 μM, the K_m (app) for cytochrome *c* is the same as with soluble enzyme. However as the amount of phospholipid concentration is increased beyond this range, there is an increase in K_m (app) (Table I).

The effects of anionic phosphatidic acid liposomes under various conditions

TABLE III

ACTIVATION OF PURIFIED CYTOCHROME OXIDASE BROUGHT ABOUT AT pH 6.5 BY POLYGLUTAMIC ACID AND ANIONIC LIPOSOMES

Values of the activation ratio *r* defined as: rate at maximal activation/rate in the absence of activator, under various conditions of concentration of enzyme, cytochrome *c* detergent and NaCl. (1) 10 mM NaCl; (2) 10 mM NaCl + 2% Tween 20; (3) 110 mM NaCl; (4) 210 mM NaCl.

Cytochrome *c*	Polyglutamic acid		Phosphatidic acid		Cardiolipin	
	0.4 nM	4 nM	0.4 nM	4 nM	0.4 nM	4 nM
5 μM (1)		2.4	3.22	2.2	3.4	2.1
			3.0	2.1		
25 μM (1)	2.8	2.5	2.8	2.2	3.05	2.2
			3.25			
50 μM (1)		2.2		2.1		
25 μM (2)	2.9	2.3	—	—	—	—
25 μM (3)	1.0		1.4			
25 μM (4)	1.0		1.0		1.0	

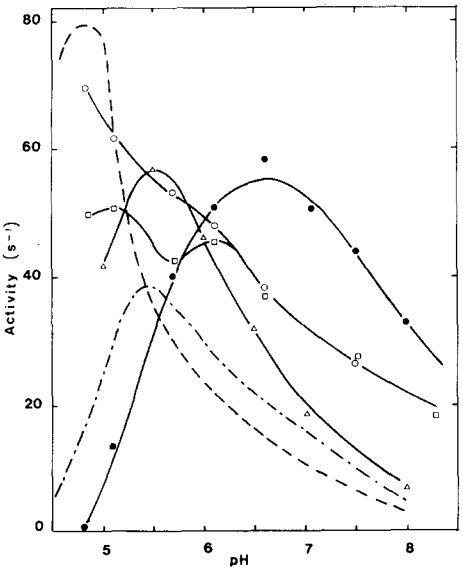

Fig. 4. Activity ($V/[E]$) of cytochrome oxidase (aa_3) in the presence of polyanionic liposomes as a function of pH and ionic strength in NaCl. Liposomes of phosphatidylcholine/cardiolipin: 82.4: 17.6 (—○—); 65: 35 (—□—); 0: 100 (—●—), ionic strength 10 mM. —△—, liposomes of 100% cardiolipin, ionic strength 210 mM. pH profile of soluble enzyme for comparison (· · · · · ·) at 10 mM NaCl and (—·—·—) at 210 mM NaCl. Enzyme and liposomes were, respectively, diluted to 4 μM and 10 mg/ml in 20 mM sodium cacodylate (pH 7.0), 50 mM NaCl or 10 mM $MgCl_2$. After incubation, 2-μl aliquots were added to the assay medium (10 mM buffer: acetate pH 4.5—5.5, cacodylate pH 5.5—7.0, Tris pH 7.5—9.0; ferrocytochrome *c*: 3—50 μM) to start the reaction.

are reported in Fig. 5. Liposomes composed of cardiolipin behaved in the same way.

The amount of phosphatidic acid producing 50% maximal activation (50% PA) increases with increasing cytochrome *c* concentration (Table II). The cardiolipin level giving 50% maximal activation (50% CL) is lower than the 50%

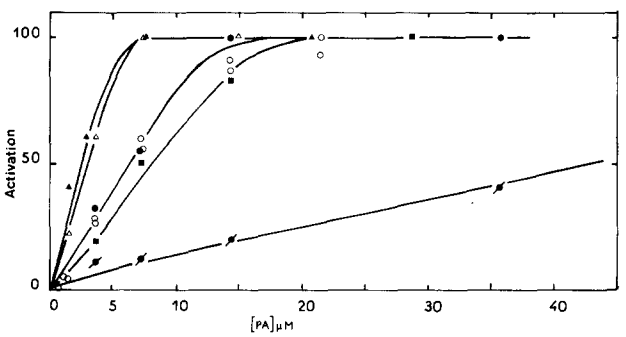

Fig. 5. Phosphatidic acid induced activation at pH 6.5 and 10 mM NaCl as a function of phosphatidic acid concentration in the assay medium. The enzyme was diluted to 0.4 μM in 100 mM phosphate buffer (pH 7.0), 1% Tween 20. Liposomes in 20 mM cacodylate buffer (pH 7.0) were added to the assay medium in the presence of cytochrome *c* prior to the enzyme; then the reaction was started by addition of 2-μl aliquots of the enzyme solution. Enzyme concentration in the assay medium 0.4 nM: —△—, 5 μM cytochrome *c*; —○—, 25 μM cytochrome *c*. Enzyme concentration in the assay medium 4 nM: —▲—, 5 μM cytochrome *c*; —●—, 25 μM cytochrome *c*; —✗—, 25 μM cytochrome *c*, 2% Tween 20; —■—, 50 μM cytochrome *c*.

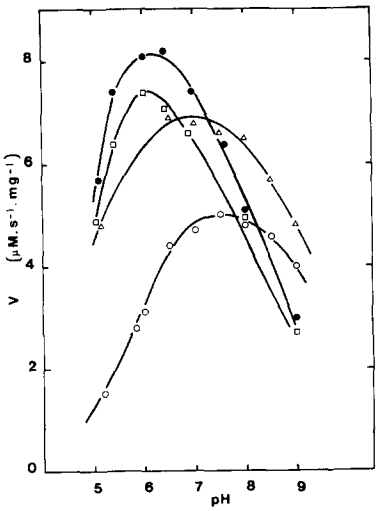

Fig. 6. V (in μM of cytochrome c oxidized/s per mg protein) vs. pH profile of submitochondrial particle at various ionic strength in NaCl: —○—, 10 mM; —△—, 60 mM; —●—, 110 mM; —□—, 210 mM. 5 μl of submitochondrial preparation in 8.5% saccharose were added to the assay medium (10 mM buffer: acetate pH 4.5—5.5, cacodylate pH 5.5—7.0, Tris pH 7.5—9.0; ferrocytochrome c: 3—50 μM) to start the reaction.

PA. The "activation ratio" is very similar to that found with polyglutamic acid (Table III). It decreases slightly as enzyme concentration is reduced and it is not dependent on cytochrome c concentration; i.e. K_m (app) is not affected by liposomes in the concentration range explored (Table I). Table II suggests that the activation ratio decreases to 1.0 as the ionic strength increases to 210 mM NaCl. On the other hand, Fig. 5 shows that, in contrast to the observations made with the linear polyanions, 2% Tween 20 strongly inhibits the effect of liposomes on the reaction. These observations were supported by binding experiments which showed that, whereas at low ionic strength (10 mM) 60% of the cytochrome c in the incubation medium was tightly bound to phosphatidic acid liposomes (see Materials and Methods), less than 10% was bound at high ionic strength (200 mM) or at low ionic strength (10 mM) in the presence of 2% Tween 20.

Submitochondrial preparations

Activity vs. pH profiles of a submitochondrial preparation (from beef heart) at various ionic strengths are reported in Fig. 6. As already observed by two of us [1], as well as by others [25,26] with rat and human liver preparations and with beef heart preparations, at low ionic strength, the pH optimum lies in the range 7.5—8.0. At higher ionic strength it is shifted towards 5.5—6.0.

Discussion

In contrast to the effects produced by Tween 20 (a non-ionic laurate ester) and by low net charge liposomes of phosphatidylcholine or asolectin (containing less than 10% charged phospholipids [27]), the activation of purified cyto-

chrome oxidase at low ionic strength by anionic phospholipid liposomes (cardiolipin and phosphatidic acid) is accompanied by a modification of the V vs. pH profile similar to that produced by non-specific linear polyanions such as RNA or polyglutamic acid.

The following characteristics appear to be common to the perturbations brought about by these reagents: (a) The perturbations are related to their polyanionic rather than their chemical nature: Fig. 5 shows that, when the liposomes are dispersed by Tween 20, the enzyme behaves just as if phospholipids were absent, whereas the same level of Tween 20 does not modify the effect of polyglutamic acid (Fig. 3). On the other hand Fig. 4 demonstrates that the higher the charge density on the liposomes surface (i.e. the larger the content of doubly charged cardiolipin molecules) the greater the effect. Table II shows that the cardiolipin concentration inducing 50% maximal activation is smaller than the corresponding amount of phosphatidic acid, which only bears one negative charge. (b) The perturbation disappears as ionic strength reaches 100—200 mM.

These observations can be satisfactorily interpreted in terms of polyelectrolyte theory [2—6]. Linear polyanions and polyanionic liposomes interact either with enzyme or with cytochrome *c* or with both, thereby providing a microenvironment within which the reaction proceeds under the influence of a strong negative electrostatic potential. This potential decreases the local pH and therefore induced a shift of the observed V vs. pH profile towards more alkaline pH values (a change of the pH optimum from 4.8 to 6.5). The activation observed at pH 6.5 and at low ionic strength in the presence of either linear polyanions or anionic liposomes (Figs. 3 and 5) results from this pH profile shift. As the ionic strength is increased the electrostatic potential vanishes (the screening effect) so that the "normal" pH profile, i.e. that of soluble enzyme, is restored (with a pH optimum 5.5), and the "activation ratio" at pH 6.5 decreases to 1 (Table III). The disappearance of the linear polyanion effect as the pH is decreased below 5.0 (Fig. 2) could reflect weakening of interactions with enzyme and cytochrome *c*.

Since, as shown in Fig. 6 and elsewhere [1,25,26], the apparent optimum in the V vs. pH profile of a mitochondrial preparation is displaced from pH 7.5 to pH 6.0 as ionic strength is increased from 10 to 210 mM, it can be concluded that membrane-bound cytochrome oxidase is located within a polyanionic microenvironment of the mitochondrial membrane.

It is apparent from Table I that activation of purified cytochrome oxidase, either by Tween 20 or by zero net charge and anionic phospholipids, is accompanied by a decrease in K_m (app). This could reflect the ability of the hydrophobic environment provided by these additives to prevent enzyme autoassociation [12,28—31]. Data given in Table I also show that K_m (app) for cytochrome *c* increases with the amount of anionic phospholipid present in the assay medium. Polyanionic liposomes may "concentrate" the positively charged molecules of cytochrome *c* in their neighborhood, through electrostatic interactions [18,32]. At least two different populations of bound cytochrome *c* molecules may then exist: population I tightly bound at the surface of the liposomes and population II less firmly bound but remaining in the vicinity of the liposomal surface. Because of its higher mobility, population II

will be more reactive than population I, so that, as I increases with respect to II, K_m (app) will increase and vice versa.

If the total concentration of cytochrome *c* is kept constant, any increase in liposome concentration will involve an increase of population I with respect to II and therefore produce an increase in K_m (app). This is indeed what we observed, and this interpretation is supported by a recent report [26]. On the other hand the value of K_m (app) is not modified with respect to the control value by polyanionic liposomes as their concentration remains low.

Finally it may be suggested that phospholipid molecules are able to activate cytochrome oxidase and presumably other enzyme systems by two distinct processes. The first, common to all phospholipids, is brought about by the non-polar part of their molecule. Phospholipid tails (as well as non-ionic detergents) provide the enzyme with a hydrophobic environment which prevents the formation of inactive aggregates and eventually induced a conformational change or rearrangement of the enzyme [28—31]. The second, more specifically studied in the present paper, is only brought about by those phospholipids whose polar head bears a non-zero net charge. Such phospholipid head groups are able to generate and maintain an environment which can significantly affect the activity of the enzyme at low ionic strength in the neutral pH range.

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