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Inhibition of the Phosphate-Stimulated Cytochrome *c* Oxidase Activity by Thiophosphate[1]

Stephen Manon,[2] Nadine Camougrand,[2] and Martine Guerin[2,3]

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Abstract

Yeast and mammalian cytochrome *c* oxidase activity is inhibited by thiophosphate. This inhibition was observed when using either whole mitochondria or the isolated or reconstituted enzyme. The kinetics of the reduction reaction enabled us to demonstrate that thiophosphate acted on the electron transfer between hemes *a* and a_3. With whole mitochondria, phosphate alone stimulated respiration. The inhibition induced by thiophosphate was suppressed by phosphate only in mitochondria, but not when the isolated enzyme was used. The possibility of a kinetic regulation is discussed.

Key Words: Cytochrome *c* oxidase; thiophosphate; phosphate; kinetic regulation.

Introduction

Cytochrome *c* oxidase, the terminal and universal member of the mitochondrial electron transport chain, is a multipeptide complex containing four electron acceptors, which include two heme groups denoted *a* and a_3, at least two copper atoms (Azzi, 1980; Denis, 1986) and some other metals (Yewey and Caughey, 1987). Whether the total number of subunits (varying from 9 to 13) depends on the cellular origin of the enzyme (Kadenbach *et al.*, 1985; Power *et al.*, 1984), the three largest subunits that support the catalytic activity are always encoded by the mitochondrial genome, whereas the others (possibly the regulatory subunits (Kadenbach, 1986)) are encoded by the nucleus. Among the prosthetic groups, heme *a* and Cu_A are low-potential

[1] Abbreviations: CCCP, *p*-carbonylcyanide *m*-chlorophenylhydrazone; TMPD, *N,N,N′,N′*-tetramethyl *p*-phenylenediamine; SPi, thiophosphate.

[2] Institut de Biochimie Cellulaire et de Neurochimie du Centre National de la Recherche Scientifique, 1 rue Camille Saint-Saens, F-33077 Bordeaux Cedex, France, or Université de Bordeaux II, 146 rue Leo Saignat, Bordeaux, France.

[3] To whom correspondence should be addressed.

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centers and the acceptors of the first pair of electrons to enter the complex. Heme a_3 and Cu_B are high-potential centers, probably forming the oxygen-binding site (Babcock *et al.*, 1981). Hence, cytochrome *c* oxidase catalyzes the transfer of four electrons from the reduced cytochrome *c* to oxygen according to the following sequence of reactions (Hill *et al.*, 1986):

$$4 \text{ cyt.}c \rightarrow \text{cyt.}a \rightarrow Cu_A \rightarrow Cu_B\text{-cyt.}a3 \rightarrow O_2$$

The coupling mechanism between the electron and proton flows is not yet fully understood. Although the existence of a protein proton pump is generally accepted (Sigel and Carafoli, 1979; Krab *et al.*, 1984; Krab and Wikström, 1987; Williams, 1987), new models involving a transfer of hydroxyl ions have been proposed recently (Mitchell, 1987).

Cytochrome *c* oxidase is a key enzyme in the control of the ATP synthesis flow (Groen *et al.*, 1982; Mazat *et al.*, 1986). Recent results have led to the hypothesis of an allosteric regulation of this enzyme (Kadenbach, 1986). Indeed, using the isolated enzyme incorporated into liposomes, it was shown that phosphate, ADP, and ATP modulated the activity (Ferguson-Miller *et al.*, 1976; Smith *et al.*, 1980; Kadenbach *et al.*, 1985; Kadenbach, 1986; Malatesta *et al.*, 1987). It was also shown that some subunits contain adenine nucleotide-binding sites (Montecucco *et al.*, 1986). Binding of ATP to these sites promotes conformational changes of the complex (Bisson *et al.*, 1987). Recently, working on isolated yeast mitochondria, Rigoulet *et al.* (1987) proposed that the ATP-induced changes of the oxidoreduction level of cytochrome *c* oxidase could explain the different relationships observed between the oxygen consumption and the electrochemical proton gradient when variations of the latter are induced by the addition of CCCP or ADP and Pi.

Previous studies of isolated mitochondria showed that thiophosphate, a phosphate analog, promotes inhibition of respiration by acting on complex IV, and that this inhibition is removed by the addition of phosphate (Manon and Guérin, 1988).

This report deals with measurements of the phosphate and thiophosphate effects in experiments performed using isolated mitochondria and purified cytochrome *c* oxidase, incorporated or not into phospholipid vesicles. It is proposed that thiophosphate acts at a level situated between cytochrome *a* and cytochrome a_3. The comparison of the effects of both anions has led us to propose the existence of phosphate-binding sites that induce an activation of the cytochrome *c* oxidase.

Materials and Methods

Origin of Mitochondria. Mitochondria were isolated by classic methods (Guérin *et al.*, 1979; Klingenberg and Slenczka, 1963) either from *Saccharomyces cerevisiae* (strain Yeast Foam) or from rat liver mitochondria.

Mitochondrial Respiration. This was monitored at 28°C by using a Clarke electrode connected to a computer giving an on-line display of rate values. Yeast mitochondria were suspended in a 0.65 M mannitol, 0.36 mM EGTA, and 10 mM Tris–Maleate buffer, pH 6.7, and rat liver mitochondria were suspended in a 0.2 M sucrose, 10 mM KCl, 1 mM EDTA, 40 mM Tris–maleate buffer, pH 6.7.

Cytochrome aa_3 Difference Spectra and Kinetics of Cytochrome aa_3 Reduction. These were recorded on a double-beam/double-wavelength spectrophotometer Aminco DW2, at 4°C.

Preparation of Cytochrome c Oxidase. Cytochrome *c* oxidase was purified according to the method described by Camougrand *et al.* (1987) for the yeast enzyme and according to the method described by Kadenbach *et al.* (1985) for the mammalian enzyme. In both cases, the purification procedure involved the following steps: extraction of matrix proteins and most other membrane proteins with nonionic detergents, chromatography on DEAE–cellulose in the presence of Triton X-100, and ammonium sulfate fractionation in the presence of sodium cholate. For reconstitution experiments, the DEAE–cellulose chromatography step was omitted. The cytochrome *c* oxidase pellet was suspended in a small volume of a 0.25 M sucrose, 2 mM EDTA, 10 mM Tris–HCl buffer, pH 6.7.

Cytochrome c Oxidase Reconstitution in Liposomes. This was done by the cholate dialysis method (Buge and Kadenbach, 1986): 27.5 mg of purified asolectin in 700 μl of a 1.5% sodium cholate, 50 mM KCl, 100 mM HEPES buffer, pH 7.2, was sonicated to clarity in an ultrasonic bath (Branson 1200, Bioblock). After addition of 0.3 mg of cytochrome *c* oxidase [corresponding to a lipid/protein ratio of 95 (wt/wt)], the solution was dialyzed for 48 h against a 50 mM KCl, 100 mM HEPES buffer, pH 7.2, at 4°C. The orientation of the cytochrome *c* oxidase within the membrane was determined by the method of Casey *et al.* (1982).

Activity of Isolated Cytochrome c Oxidase. This was measured polarographically at 25°C in a buffer solution composed of 50 mM KCl, 1 mM laurylmaltoside, 7 mM Tris–ascorbate, 0.014 mM EDTA, 0.7 mM TMPD, 40 μM cytochrome *c*, 100 mM HEPES, pH 7.2 (Buge and Kadenbach, 1986). The cytochrome *c* oxidase activity of proteoliposomes was measured under the same conditions except that laurylmaltoside was omitted from the buffer.

Reagents. Sodium thiophosphate (Ventron, GmbH) was washed twice with absolute ethanol. After centrifugation, the pellet was dissolved in a 0.6 M mannitol, 2 mM EGTA, 10 mM Tris–maleate buffer, pH 6.7, to prevent spontaneous hydrolysis, which produces hydrogen sulfide, a potent inhibitor of cytochrome *c* oxidase (Nicholls and Kum, 1982). *N,N,N′,N′*-tetramethyl *p*-phenylenediamine (TMPD) and ascorbic acid were from

Merck. DEAE-cellulose was from Fluka. Laurylmaltoside was from Calbiochem. Yeast cytochrome *c* and CCCP were from Sigma.

Results

Effect of Thiophosphate on Oxygen Consumption by Isolated Mitochondria from Different Origins

Thiophosphate, a phosphate analog, has already been shown to inhibit the transition from state 4 to state 3 in both rat liver mitochondria (Tavares De Sousa *et al.*, 1972) and yeast mitochondria (Manon *et al.*, 1988). Although no effect was observed on the state 4 of respiration in mammals (Tavares De Sousa *et al.*, 1972), thiophosphate slightly diminished the level of this steady state in yeast mitochondria (Manon *et al.*, 1988), suggesting that thiophosphate inhibits the electron transfer between the respiratory substrates and oxygen.

The effect of thiophosphate was assayed on uncoupled respiration monitored with different electron donors, in the presence of CCCP, or KCl + valinomycin (Table I). Whatever the substrate used (NADH, ethanol, ascorbate + TMPD, lactate + antimycin A), thiophosphate inhibited respiration to the same extent, showing that this product acts on the electron transfer between cytochrome *c* and oxygen.

Furthermore, it appeared that the effect of thiophosphate was pH dependent (Fig. 1), the maximal effect occurring at pH 6.0. Above pH 7.0, the effect was weak, which explains the fact that thiophosphate did not act on mammalian mitochondria (Tavares De Sousa *et al.*, 1972; Manon and

Table I. Effect of Thiophosphate on Yeast Mitochondria Respiration with Various Electron Donors[a]

Substrate	Respiration rates (nat · 0 · min^{-1} · mg^{-1})		% Inhibition
	−SPi	+SPi	
Ethanol	282	105	63
NADH	432	141	68
Lactate + antimycin A	28	14	50
Ascorbate + TMPD	1120	520	54

[a] Yeast mitochondria were suspended in a 0.65 M mannitol, 0.36 mM EGTA, 10 mM Tris-maleate buffer, pH 6.7, added to 4 μM CCCP, in the presence or in the absence of 0.3 mM thiophosphate. Concentrations of substrates were: ethanol 4 mM, NADH 2 mM, Tris-ascrobate 15 mM + TMPD 0.7 mM, Tris-lactate 10 mM + antimycin A 0.1 μg/mg. It was verified that, in the absence of mitochondria, addition of thiophosphate ± substrate did not induce any oxygen consumption.

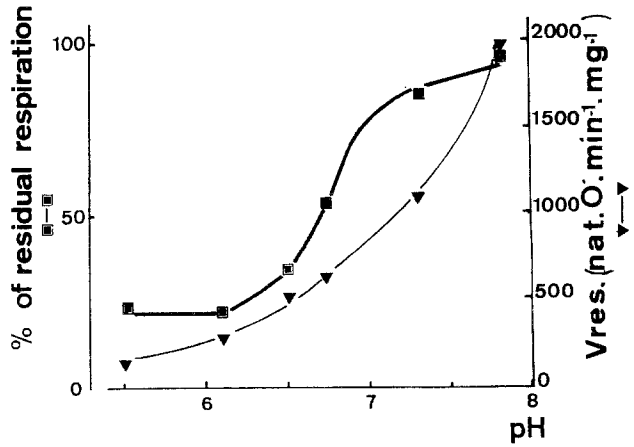

Fig. 1. Effect of pH on thiophosphate inhibition. Yeast mitochondria (0.5 mg/ml) were suspended in a 0.65 M mannitol, 0.36 mM EGTA, 10 mM Tris–maleate buffer adjusted to different pH values with Tris and HCl; 0.25 mM thiophosphate was added 1 minute before the substrate (Tris–ascrobate 15 mM, TMPD 0.7 mM, in the presence of 4 μM CCCP): (▼—▼) actual respiratory rate and (■—■) normalization to 100 (control without thiophosphate) of the residual respiratory rate.

Guérin, 1988), whose respiration was monitored at pH 7.4. However, when experiments were performed on rat liver mitochondria at pH 6.7, although respiration was lowered at this pH, thiophosphate induced an inhibition of respiration in the same range of concentrations as for yeast mitochondria (Fig. 2).

Consequently, all of the following experiments were performed at pH 6.7 using ascorbate–TMPD as the electron donor. Under these conditions, respiration of yeast mitochondria was maximally (75%) inhibited with a $K_i = 0.26 \pm 0.04$ mM (Fig. 2a). It should be noted that the inhibition was removed, or prevented, by the addition of phosphate in both yeast (Fig. 2a) and rat liver mitochondria (not shown).

Effect of Thiophosphate on Oxygen Consumption by the Isolated and Reconstituted Cytochrome c Oxidase

Cytochrome *c* oxidase was isolated from either yeast or mammalian mitochondria as indicated in *Materials and Methods*. The enzyme was either dissolved in laurylmaltoside or reconstituted in asolectin vesicles. In the latter case, it was verified that 80% of the enzyme was right-side-out and that the respiration was activated by CCCP.

The effect of thiophosphate on cytochrome *c* oxidase activity was monitored polarographically for both the isolated enzyme and the

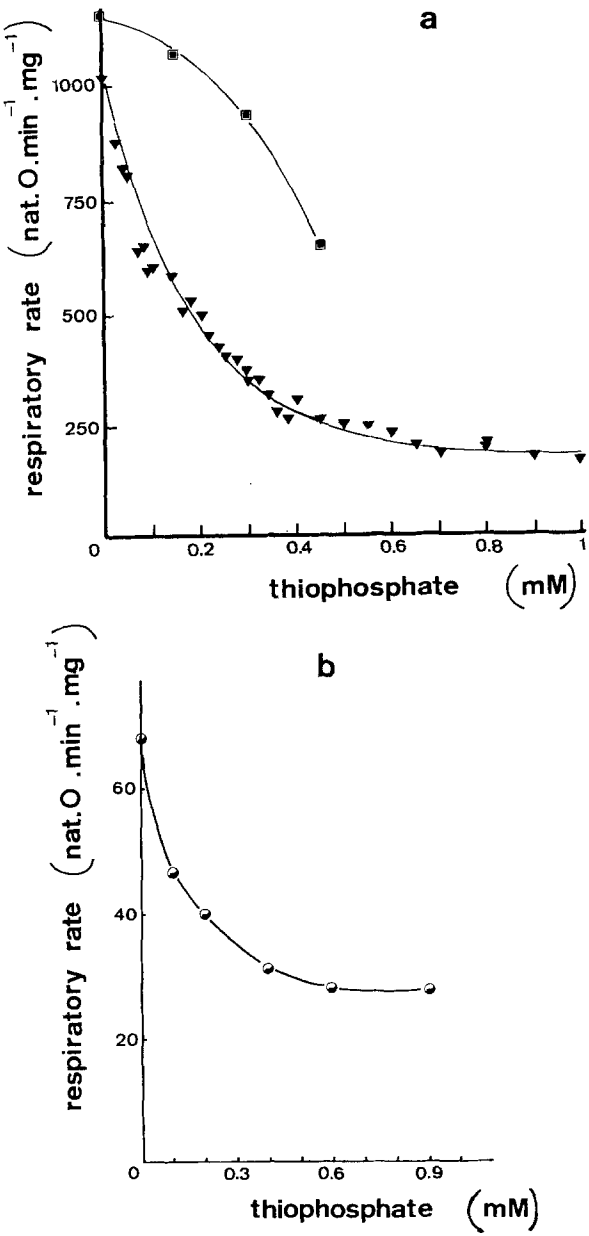

Fig. 2. Inhibition of (a) yeast and (b) mammalian mitochondria respiration by thiophosphate. (a) Yeast mitochondria (0.5 mg/ml) were suspended in a 0.65 M mannitol, 0.36 mM EGTA, 10 mM Tris–maleate buffer, pH 6.7, to which was added 4 μM CCCP and the indicated concentrations of thiophosphate in the absence (▼—▼), or in the presence (□—□), of Tris-phosphate 5 mM. (b) Mammalian mitochondria (0.5 mg/ml) were suspended in a 0.25 M sucrose, 10 mM KCl, 1 mM EGTA, 40 mM Tris–maleate buffer, pH 6.7, to which were added 4 μM CCCP and the indicated concentrations of thiophosphate. In both cases, the substrate (added 1 min later) was Tris–ascorbate 15 mM + TMPD 0.7 mM.

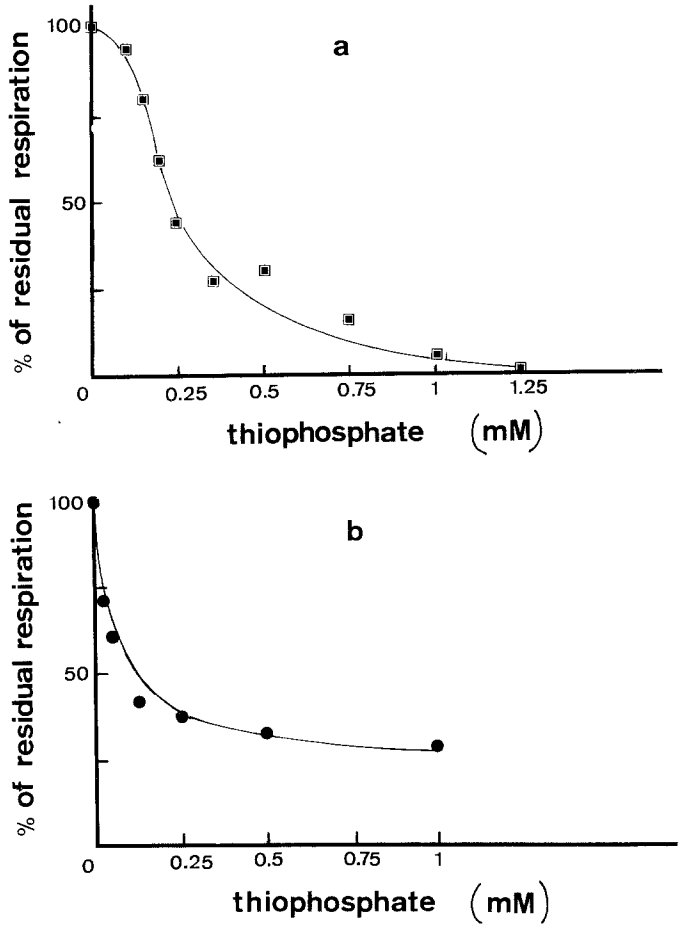

Fig. 3. Effect of thiophosphate on (a) isolated and (b) reconstituted yeast cytochrome *c* oxidase. (a) Cytochrome *c* oxidase was isolated according to the method described by Camougrand *et al.* (1987) and suspended (at a concentration of 20 μg/ml) in a 50 mM KCl, 100 mM HEPES buffer, pH 6.7, at 25°C, containing 1 mM laurylmaltoside, 7 mM Tris-ascrobate, 0.014 mM EDTA, 0.7 mM TMPD, and 40 μM cytochrome *c* and the indicated concentrations of thiophosphate. (b) Cytochrome *c* oxidase was purified and reconstituted as described by Buge and Kadenbach (1986) and suspended (at a concentration of 5–10 μg/ml) in the same buffer as above, without laurylmaltoside.

enzyme-containing vesicles. Figure 3 shows that thiophosphate acts in the same concentration range as that observed for mitochondria.

However, in contrast to that observed for the mitochondria, the inhibition was not prevented by the addition of phosphate. The isolated enzyme was completely inhibited by thiophosphate, while the reconstituted enzyme was not. This could be related to the monomeric structure of the isolated

enzyme (Rosevaer *et al.*, 1980), the reconstituted one being a variable mixture of more than one distinct molecular form of the enzyme (Naqui *et al.*, 1984), while the native enzyme is probably in a dimeric form (Henderson *et al.*, 1977).

At What Level Does Thiophosphate Act on the Electron Transfer Pathway?

The effect of thiophosphate was assayed on the cytochrome *c*-binding activity of the enzyme solubilized in laurylmaltoside (Camougrand *et al.*, 1987). In the absence of thiophosphate, the Eadie–Hofstee representation of the cytochrome *c* dependence of the turnover of the enzyme can be resolved into two kinetic systems with high (K_m = 0.04 μM) and low (K_m = 0.4 μM) affinities.

From measurements monitored in the presence of thiophosphate, it appeared that thiophosphate did not affect the first step of the electron transfer, since it did not change the affinities of either system for cytochrome *c* (not shown). The V_m of the high-affinity system seemed to be less affected by the inhibitor than that of the low-affinity system.

The effect of thiophosphate on the oxidoreduction spectra of mitochondria was then assayed. In the visible region of the spectrum, thiophosphate only induced a 15% decrease in the absorption level (without a shift of the maximal absorption wavelength), whereas, in the Soret band region (between 444 and 465 nm), where each of heme accounts for ~ 50% of the total absorption (Wikstrom *et al.*, 1976), thiophosphate provoked a 50% decrease in the reduction level induced by the substrate (see below); after 5 min, however, this spectrum tended toward the control spectrum.

Consequently, the reduction kinetics of the cytochrome aa_3 were measured in the Soret band region (Fig. 4). Mitochondria were vigorously stirred at 0°C in order to promote maximal oxidation of the cytochromes (it was calculated that the oxidation level reached in this way represented 85% of that obtained with hydrogen peroxide).

In the absence of thiophosphate, the addition of ascorbate–TMPD did not change the steady-state reduction of the enzyme. The establishment of anaerobiosis that occurred a few minutes latter led to a reduction level that was 85% of that occurring in the presence of dithionite (Fig. 4, curve a). As a control experiment, we compared the effects of cyanide and thiophosphate. The addition of cyanide, in the presence of ascorbate–TMPD, promoted a reduction equivalent to 50% of that due to anaerobiosis. It is known that cyanide binds to the oxidized form of heme a_3, preventing its reduction. The 50% reduction obtained by the addition of cyanide corresponds to the reduction of heme *a*. The addition of thiophosphate before or after the

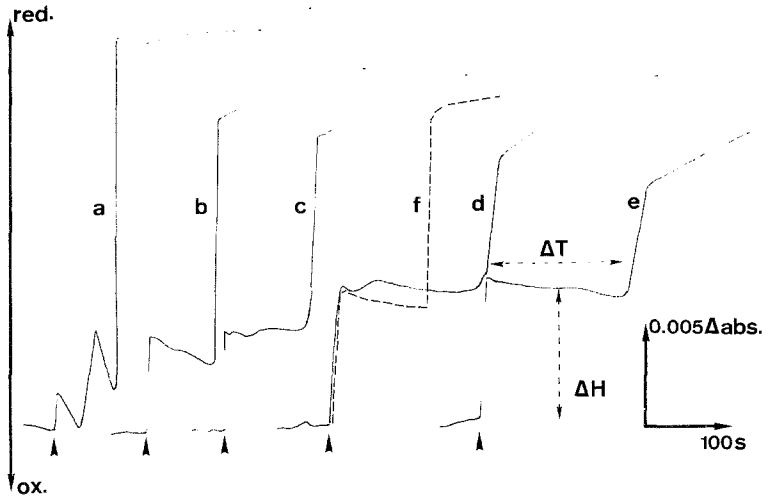

Fig. 4. Effect of thiophosphate on the reduction kinetics of cytochrome aa_3, monitored in the Soret band (444–475 nm). Yeast mitochondria (5 mg/ml) were suspended in the 0.65 M mannitol, 0.36 mM EGTA, 10 mM Tris–maleate buffer, pH 6.7, containing 4 μM CCCP, and vigorously stirred at 0°C. An aliquot of the suspension was placed in the cuvette of a double-wavelength spectrophotometer, in the absence (a), or in the presence (b–f), of thiophosphate; 2 min later, ascrobate–TMPD (2 mM/0.17 mM) was added (▲) to induce the reduction of hemes. Curves b–e correspond to thiophosphate concentrations of 0.15, 0.3, 0.6, and 0.9 mM, respectively; curve f corresponds to 0.6 mM thiophosphate + 5 mM phosphate.

cyanide did not lead to any change in the reduction level obtained in the presence of cyanide alone, indicating that the reduction of heme *a* was not affected (now shown).

In the presence of thiophosphate, the reduction kinetics were greatly modified (Fig. 4, curves b–e):

1. Contrary to control experiments, the addition of ascorbate–TMPD induced a fast, but partial, reduction of oxidase. The reduction level was dependent on the thiophosphate concentration, but the maximal reduction never exceeded 45% of the control (ΔH).
2. Anaerobiosis was established after a lag that was dependent on the thiophosphate concentration (ΔT).
3. The reduction rate during anaerobiosis decreased when thiophosphate concentration increased.

Taking into account all of these data, it can be suggested that the addition of thiophosphate promotes a decrease in the electron transfer rate between heme *a* and heme a_3, inducing a partial reduction of heme *a*.

However, in contrast with cyanide, the effect of thiophosphate is not total, since anaerobiosis can be obtained; the electron transfer to oxygen is diminished, but not fully inhibited. The fact that the reduction level obtained during anaerobiosis is only slightly affected demonstrates that heme a_3 is fully reducible in the presence of thiophosphate. Thiophosphate only affects the rate of the reduction.

Reversibility of Thiophosphate Inhibition

It could be argued that the effects of the thiophosphate observed in the preceding experiments are in fact due to the contamination of the thiophosphate solution by sulfide, a well-known inhibitor of cytochrome *c* oxidase (Nicholls and Kim, 1982). Indeed, within 50 days, thiophosphate is spontaneously hydrolyzed to an extent of $\sim 50\%$, yielding phosphate and hydrogen sulfide (Lamotte *et al.*, 1965). To ensure that the observed inhibition was effectively due to thiophosphate and not to sulfide, the effects of the two inhibitors were compared. Several lines of evidence excluded the possibility that the observed effects were due to sulfide: (a) The inhibition by thiophosphate was slow, while that by sulfide was instantaneous (not shown). (b) Thiophosphate induced no more than 75–80% inhibition, while sulfide, like cyanide, promoted a complete inhibition (Table II). (c) The inhibition by thiophosphate can be removed, or prevented, by the addition of phosphate (Fig. 2a and Table II), while phosphate has no effect on the inhibition by sulfide (Table II).

Contrary to that of numerous other respiratory chain inhibitors, the inhibition induced by thiophosphate is reversible. Indeed, the addition of phosphate before (or after) thiophosphate prevented (or removed) the inhibition of the respiration (Fig. 2a). Other anions were tested for their ability to affect thiophosphate inhibition (Table II). Only arsenate and pyrophosphate presented the same effect as phosphate, while ADP, ATP (+ oligomycin), and acetate were ineffective.

Table II. Effect of Various Anions on the Release of Thiophosphate Inhibition[a]

Addition	None	Phosphate	Pyrophosphate	Arsenate	ATP + oligomycin	ADP	Acetate
None	100	130	100	120	103	ND	ND
SPi	30	90	78	66	30	40	30
Sulfide	7	14	ND	ND	ND	ND	ND

[a]Yeast mitochondria were suspended as in Table I in the presence of 3 mM of the indicated anions and in the presence or the absence of thiophosphate, 0.3 mM, or sodium sulfide, 10 μM. Substrate was ascorbate 15 mM + TMPD 0.7 mM. Results are expressed as % of the control without any addition.

Effect of Phosphate on Mitochondrial Oxygen Consumption

From Fig. 2a, it appears that the addition of phosphate, in the absence of thiophosphate, stimulates the respiration. To determine on which side of the inner membrane phosphate was effective, experiments were performed in the presence of mersalyl so as to prevent phosphate influx into mitochondria. The results reported in Table III show that phosphate always stimulated the oxidation of ascorbate (+TMPD) even with mitochondria preincubated in the presence of mersalyl. This indicates that the stimulatory site of the cytochrome *c* oxidase activity was on the external side of the inner mitochondrial membrane. The stimulatory effect of Pi was measured (in the absence of mersalyl) as a function of the anion concentration: the Lineweaver-Burk representation (Fig. 5) indicated the existence of two systems presenting low and high activation constants, respectively. Unfortunately, the actual values of these kinetic constants cannot be determined under these experimental conditions, since the effective concentration at the binding site is not known (the addition of mersalyl to mitochondria results in a too large scattering of the experimental points); in our conditions, the apparent activation constants can be estimated at Ka1 = 10 ± 7 μM and Ka2 = 0.3 mM, respectively.

Table III. Effect of Mersalyl on the Phosphate Activation of the Oxygen Consumption

Addition	−mersalyl	+mersalyl
None	980	880
Pi 0.5 mM	1040	1080
Pi 5 mM	1260	1190

[a] Aliquots of mitochondria (1 mg protein) were preincubated with 20 nmol mersalyl and then suspended in the respiration medium. Respiration rate was expressed in nat $O \cdot min^{-1} \cdot mg^{-1}$.

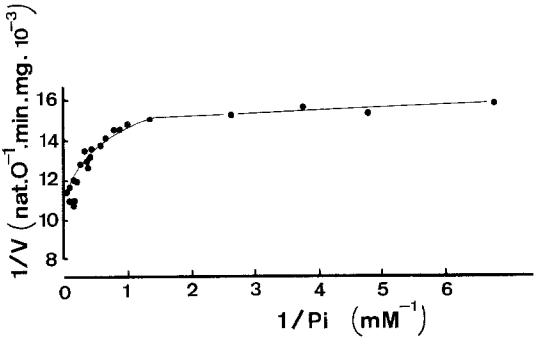

Fig. 5. Lineweaver-Burk representation of the phosphate activation of yeast mitochondrial respiration. Yeast mitochondria were suspended, as in Fig. 2a, in the presence of the indicated concentrations of Tris–phosphate.

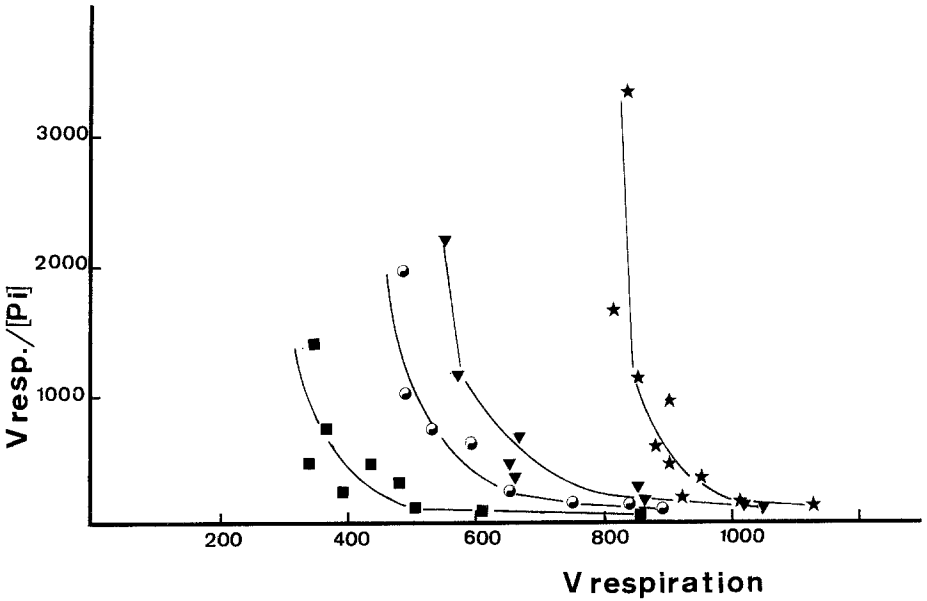

Fig. 6. Effect of phosphate, at different thiophosphate concentrations, on the respiration rate. Yeast mitochondria were suspended, as in Fig. 2a, at different thiophosphate concentrations: (★—★) 0 mM SpI, (▼—▼) 0.15 mM SPi (○—○) 0.3 mM SPi and (■—■) 0.45 mM SPi. Then, the effect of concentration range of phosphate was assayed, and the the ratio respiration rate (nat · 0 · min^{-1} · mg^{-1})/Pi (mM) was plotted against the respiration rate.

In a second set of experiments, the effect of phosphate and thiophosphate, added simultaneously, on the oxygen consumption was assayed. The effect of phosphate at different thiophosphate concentrations was reported following the Eadie–Hofstee representation (Fig. 6). Although it was difficult under these conditions to calculate the actual kinetic constants, it appeared clearly that thiophosphate diminished the V_m corresponding to the high-affinity phosphate-activation site, but did not affect the V_m of the low-affinity system.

The effect of phosphate on the kinetics of reduction of cytochrome aa_3 by ascorbate–TMPD was also assayed in the presence or in the absence of thiophosphate (Fig. 4, f). In both conditions, the addition of phosphate did not change the reduction level by ascorbate–TMPD, but shortened the lag necessary to obtain anaerobiosis (ΔT), in accordance with the phosphate effect on oxygen consumption.

From these results, it appears that the enzyme has a high-affinity site for phosphate; the binding of the anion to this site, which is probably saturated in our experimental conditions, induces the normal actvity of the enzyme, monitored on isolated mitochondria. Thus, it could be proposed that thiophosphate has two effects:

1. It displaces phosphate from the high-affinity binding site, thereby inducing a dramatic reduction of the oxygen consumption rate by the complex.
2. It competes with phosphate at the low-affinity binding-site, but high phosphate concentrations restore an activated oxygen consumption.

Discussion

The data presented in this report have enabled us to demonstrate the existence of a new type of inhibition of cytochrome *c* oxidase. The results support the hypothesis that thiophosphate is not a classic inhibitor acting by a chemical reaction: its effect was small, partial, and reversible.

Experiments performed to determine the localization of the effect showed that thiophosphate acted on the rate of electron transfer between the two hemes without affecting the capacity of these hemes to be reduced or oxidized.

The most interesting aspect of this work is the reversibility of the thiophosphate inhibition by the addition of phosphate to the system. Many groups have described the effects of phosphate on the cytochrome *c* oxidase activity. In all cases, however, high phosphate concentrations were used (Kadenbach, 1986; Malatesta *et al.*, 1987) and the effects on whole mitochondria were never described. Changes in the ionic environment of the enzyme were supposed to be involved in these effects (Kadenbach *et al.*, 1988) as was already described for other ions (Roberts and Hess, 1977; Brooks and Nicholls, 1982; Sinjorgo *et al.*, 1986). Cytochrome *c* binding to the complex was often supposed to be the primary event affected by phosphate (and also ADP and ATP) (Roberts and Hess, 1977; Smith *et al.*, 1980; Huther and Kadenbach, 1986). Our experiments show that thiophosphate does not affect the binding of cytochrome *c*.

From our data, the following hypothesis can be proposed:

1. The cytochrome *c* oxidase had a high-affinity phosphate binding site located on the external side of the inner mitochondrial membrane, and that allows a normal functioning of the enzyme.

The binding of thiophosphate induces a reduction of both the V_m and the affinity for phosphate, which could be interpreted as a displacement of phosphate by the thiophosphate from its high-affinity binding site, thereby lowering the electron transfer rate between heme *a* and heme a_3. Under these latter conditions, the rate of electron transfer appeared to be reduced (but electron transfer was not fully inhibited). Thiophosphate might not be a real inhibitor, but could act by displacing the bound activator, phosphate.

2. The existence of a second system, with a low affinity for phosphate, is also suggested. The binding of phosphate at this site promotes a stimulation of the respiration rate and of the electron transfer to oxygen. Thiophosphate only slightly alters the phosphate effect, explaining the apparent protective effect of phosphate.

According to this hypothesis, the cytochrome *c* oxydase would manifest three functional states:

- In the "normal" conditions, i.e., in the presence of low concentrations of phosphate, the enzyme presents the activity that is normally monitored in whole mitochondria.
- In the presence of thiophosphate (in the absence of phosphate?), the activity is reduced, due to a low electron transfer rate between hemes *a* and a_3; this state cannot be obtained with the isolated enzyme, suggesting that it needs a specific enzyme conformation and environment.
- In the presence of high phosphate concentrations, and independent of the presence or the absence of thiophosphate, the activity is enhanced, but the reduction steady state is not modified; this state cannot be obtained with the isolated enzyme or with the reconstituted one.

In conclusion, we have demonstrated effects of phosphate at physiological conditions on the cytochrome *c* oxidase activity *in situ* in isolated mitochondria. At present, we cannot attribute a physiologicl role to these effects, but they could be related to the recently observed effects of ATP (Rigoulet *et al.*, 1987). Experiments are under way to determine the functional and topological localizations of the thiophosphate and phosphate effects.

Acknowledgments

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