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Reaction of Cytochrome *c* Oxidase with Endogenous and Exogenous Cytochrome *c*†

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ABSTRACT: The reaction of the membrane-bound cytochrome *c* oxidase of beef heart submitochondrial particles with added exogenous cytochrome *c*, which can diffuse off into the assay medium, was compared with the reaction with the endogenous cytochrome *c*, which is restrained on or within membrane vesicles. Our suspensions of particles appeared to contain about equal quantities of vesicles with the oxidase sites exposed (right-side out) and with the oxidase sites within the vesicles (inside out). Both showed similar variations in activity, measured polarographically with *N,N,N′,N′*-tetramethylphenylenediamine (TMPD) plus ascorbate, with an increase in pH of nonbinding buffers between pH 6 and pH 8. At pH 7.8 low concentrations of exogenous cytochrome *c* (0.05–0.1 μM) appeared to form a tight combination with the exposed oxidase sites, similar in reactivity to that with the endogenous cytochrome *c*. The data confirm our previous postulate [Smith, L., Davies, H. C., & Nava, M. E. (1979) *Biochemistry 18*, 3140] of the formation of an especially reactive combination of cytochrome *c* with cytochrome *c* oxidase under these conditions. In phosphate buffers the reaction with exogenous, but not with endogenous, cytochrome *c* was inhibited at all pH values above 6. We saw no evidence for accumulation of Würster's Blue within the vesicles under our experimental conditions when concentrations of TMPD below 0.75 mM were used in the polarographic assays. However, increased rates of O_2 uptake were observed with the reaction with endogenous cytochrome *c* but not that with exogenous cytochrome *c* at higher concentrations of TMPD. Concentrations of exogenous cytochrome *c* above 0.1 μM gave increased rates of O_2 uptake with the exposed vesicular oxidase similar to those seen previously with nonvesicular oxidase preparations, but higher concentrations of cytochrome *c* within the vesicles did not lead to a significant increase in O_2 uptake rates.

We have made detailed studies of the kinetics of reaction of soluble beef cytochrome *c* with several different kinds of preparations of beef cytochrome oxidase, using both spectrophotometric and polarographic methods (Smith et al., 1979a,b). These gave evidence for a combination of cytochrome *c* with cytochrome oxidase with a high turnover rate at low concentrations of cytochrome *c* (0.05–0.2 μM) when the assays were run polarographically with TMPD[1] plus ascorbate in Tris–cacodylate buffer, pH 7.8. Under other experimental conditions the polarographic assays and under all conditions the spectrophotometric assays gave lower turnover rates. In the spectrophotometric measurements of oxidase activity with soluble ferrocytochrome *c*, the ferricytochrome *c* formed must dissociate away into solution in order for another molecule to react.

To obtain more insight into the cytochrome *c*–cytochrome oxidase combination, we have compared the reaction of the oxidase with soluble exogenous cytochrome *c*, which can diffuse off into the suspending medium, with the endogenous cytochrome *c* of mitochondrial membrane vesicles, which cannot. The preparation of submitochondrial particles, made by sonication of heart mitochondria, contains a mixture of vesicles, some with the cytochrome *c* reaction site exposed and others with the sites within the interior.

Polarographic assays described here with 0.75 mM TMPD plus ascorbate of the reactions with endogenous and with added exogenous 0.1 μM cytochrome *c* show similar activities and also similar changes with variation of pH of Tris–cacodylate or Hepes buffers. Both reactions have low activity at pH 6 and maximal activity at pH 7.8. Ions which bind to cytochrome *c*, such as phosphate (Stellwagen & Shulman, 1973), inhibit the reaction with exogenous but not with endogenous

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[1] Abbreviations used: SMP, submitochondrial particles; TMPD, *N,N,N′,N′*-tetramethylphenylenediamine; Tris, tris(hydroxymethyl)-aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid.

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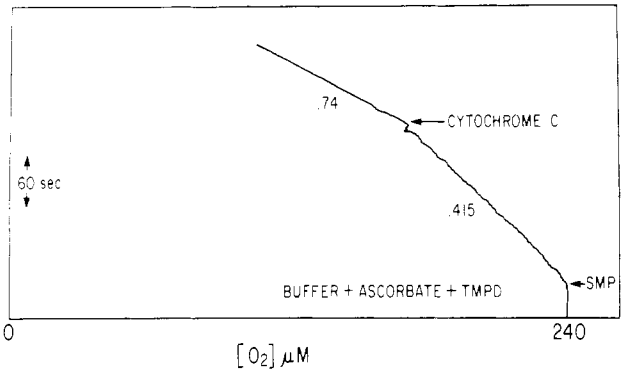

FIGURE 1: Polarographic measurement of oxidase activity with endogenous and 0.1 μM exogenous cytochrome *c*. Assays were run in 25 mM Tris–cacodylate buffer, pH 7.8, with 0.75 mM TMPD plus 10 mM ascorbate, with SMP containing 0.048 μM cytochrome aa_3 and 0.03 μM cytochrome *c*.

cytochrome *c* at pH values above 6. The observations agree with the postulate (Smith et al., 1979a,b) that cytochrome *c* and cytochrome oxidase can form different kinds of combinations and that one with an especially high turnover rate is formed under some conditions.

Experimental Section

Preparations. Submitochondrial particles (SMP) were derived from beef heart mitochondria by the method of Lee & Ernster (1967). Other preparations were made by sonicating the mitochondria in the presence of added cytochrome *c*, following the method of Lee (1971). The content of cytochrome aa_3 in the SMP preparations was calculated from the reduced plus NADH minus oxidized difference spectrum following the procedure of Vanneste (1966) and that of cytochrome *c* by the method of Williams (1968).

Cytochrome *c* was isolated from beef heart by the method of Margoliash & Walasek (1967) and further purified by isoelectric focusing (Smith, 1978). The concentration of cytochrome *c* in solution was determined from the absorbance of the reduced compound at 550 nm, with 27.6 as the millimolar extinction coefficient (Margoliash & Frohwirt, 1959).

Assays. Cytochrome oxidase activity was assayed polarographically with a Clark-type O_2 electrode in a 2.6-mL chamber at 26 °C in the presence of TMPD plus ascorbate (rate with endogenous cytochrome *c*) and then after addition of cytochrome *c* (additional rate with exogenous cytochrome *c*). The rates are expressed as μM O_2 uptake s^{-1}. Figure 1 is a typical recording with SMP before and after addition of 0.1 μM cytochrome *c*. The assay conditions are given in the legends to the figures and tables.

The oxidation–reduction state of cytochrome *c* in the aerobic state in the presence of TMPD plus ascorbate was measured in the Aminco Dual-Wavelength DW2a spectrophotometer as described in a previous publication (Smith et al., 1979b).

Chemicals. TMPD was a Sigma Chemical Co. product; it was neutralized to pH 6 with NaOH. Sodium ascorbate was recrystallized from hot water.

Results

The cytochrome content of three separate SMP preparations is listed in Table I. The data are not very precise, since they are derived from difference spectra, where the absorption peaks of cytochromes *b*, c_1, and *c* overlap. In each, the content of cytochrome *c* was about the same or slightly less than that of cytochrome aa_3; thus, some cytochrome *c* was apparently lost, presumably from vesicles oriented right-side out. As seen in Table I, the O_2 uptake rates with endogenous cytochrome *c*

Table 1: Comparison of Oxidase Activity of Several SMP Preparations with Endogenous and Exogenous Cytochrome *c*[a]

prepn	cyt aa_3 in assay (μM)	endogenous cyt *c* in assay (μM)	O_2 uptake (μM s^{-1}) with endogenous cyt *c*	O_2 uptake (μM s^{-1}) with exogenous 0.1 μM cyt *c*
1	0.058	0.043	0.35	0.32
2	0.020	0.024	0.16	0.15
3	0.051	0.033	0.24	0.18

[a] Assays were run in 25 mM Tris-cacodylate buffer, pH 7.8, with 0.5 mM TMPD plus 10 mM ascorbate. The SMP preparations were prepared by sonication in the absence of added cytochrome *c*. cyt, cytochrome.

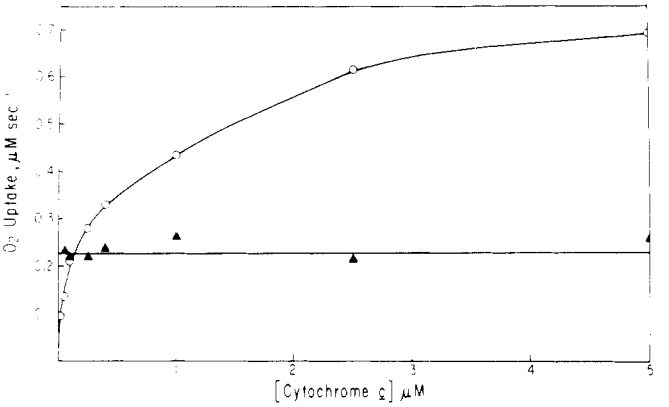

FIGURE 2: Comparison of oxidase activity with endogenous and exogenous cytochrome *c*. Assays were run in 25 mM Tris–cacodylate buffer, pH 7.8, with 0.5 mM TMPD plus 10 mM ascorbate and SMP containing 0.04 μM cytochrome aa_3 and 0.04 μM endogenous cytochrome *c*. (▲) Endogenous cytochrome *c*; (○) exogenous cytochrome *c*.

were roughly proportional to the content of endogenous cytochrome *c*.

Figure 2 plots rates of O_2 uptake of one preparation with the endogenous cytochrome *c* and the increased rates observed after addition of increasing concentrations of added exogenous cytochrome *c*. The rate with 0.1 μM exogenous cytochrome *c* was about equal to the rate with endogenous cytochrome *c*. This was true with all of the preparations described in Table I, where the level of endogenous cytochrome *c* varied between 0.024 and 0.043 μM in the assay mixture. A further 50-fold increase in the concentration of exogenous cytochrome *c* only increased the exogenous rate 2.5-fold. These assays were run in 25 mM Tris–cacodylate buffer, pH 7.8, where the O_2 uptake rates were maximal (see Figure 4).

The data of Figure 3 show that the O_2 uptake rates with endogenous cytochrome *c* increased linearly with an increase in the content of SMP (expressed as the cytochrome aa_3 content). However, the increased O_2 uptake with low concentrations of exogenous cytochrome *c* leveled off with increasing content of SMP, approaching saturation at a content of cytochrome aa_3 around that of the added cytochrome *c*.

When the pH of the Tris–cacodylate buffers was varied, the rates with endogenous cytochrome *c* and with 0.1 μM exogenous cytochrome *c* showed parallel changes with increasing pH between 6.0 and 7.8, and similar changes were found with the rates with endogenous cytochrome *c* in phosphate buffers (Figure 4). However, the effect of changing pH with phosphate buffers on the reaction with 0.1 μM exogenous cytochrome *c* was quite different.

The effect of variation of the concentration of TMPD in the assays in the presence of 10 mM ascorbate is shown in Figure 5. The rates with endogenous and with 0.1 μM ex-

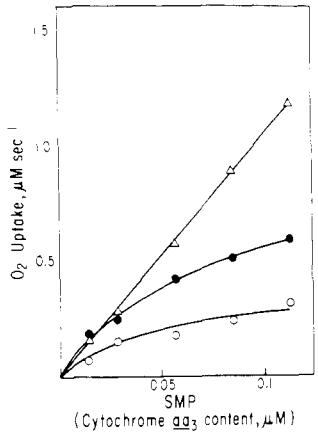

FIGURE 3: Oxidase activity with endogenous and exogenous cytochrome *c* (0.05 and 0.2 μM) with increasing quantities of SMP. Assays were run in 25 mM Tris–cacodylate buffer, pH 7.8, with 0.75 mM TMPD plus 10 mM ascorbate. (△) Endogenous cytochrome *c*; (●) exogenous 0.2 μM cytochrome *c*; (○) exogenous 0.05 μM cytochrome *c*.

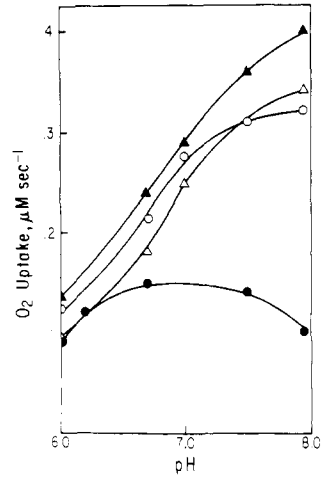

FIGURE 4: Effect of pH on oxidase activity with endogenous and exogenous cytochrome *c*. Assays were run in 25 mM Tris–cacodylate or phosphate buffers with 0.77 mM TMPD plus 10 mM ascorbate with and without 0.1 μM cytochrome *c* and with SMP containing 0.11 mg of protein/mL. (△) Endogenous cytochrome *c*, Tris–cacodylate buffer; (○) exogenous cytochrome *c*, Tris–cacodylate buffer; (▲) endogenous cytochrome *c*, phosphate buffer; (●) exogenous cytochrome *c*, phosphate buffer.

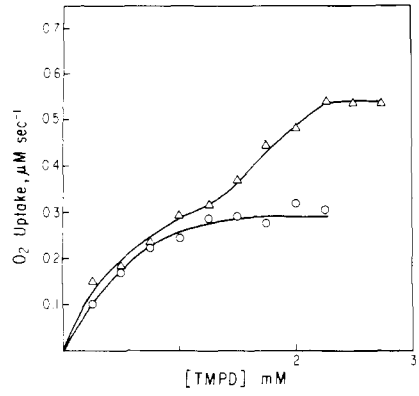

FIGURE 5: Effect of concentration of TMPD on oxidase activity with endogenous and exogenous cytochrome *c*. Assays were run in 25 mM Tris–cacodylate buffer, pH 7.8, with and without 0.1 μM cytochrome *c*, 10 mM ascorbate, and SMP containing 0.028 μM cytochrome aa_3. (△) Endogenous; (○) exogenous.

ogenous cytochrome *c* were similar with the preparation tested at concentrations of TMPD up to ~0.75 mM. Then the rates

Table II: Effect of Poly-L-lysine on Oxidase Activity with Exogenous and Endogenous Cytochrome *c*[a]

	% inhibn with	
	0.039 μM PL	0.077 μM PL
with endogenous cyt *c*	0	0
with 0.1 μM exogenous cyt *c*		100
with 0.25 μM exogenous cyt *c*	81	100

[a] Assays were run in 25 mM Tris–cacodylate buffer, pH 7.8, with 0.75 mM TMPD plus 10 mM ascorbate and SMP containing 0.035 μM cytochrome aa_3 and 0.024 μM endogenous cytochrome *c*. cyt, cytochrome.

Table III: Oxidase Activity of SMP Preparations Loaded with Cytochrome *c*[a]

cyt *c* during sonication (μM)	endogenous cyt *c* in assays (μM)	cyt aa_3 in assays (μM)	O_2 uptake (μM s^{-1})
0	0.018	0.020	0.22
13	0.032	0.016	0.25
52	0.080	0.016	0.34

[a] O_2 uptake was measured in 25 mM Tris–cacodylate buffer, pH 7.8, with 0.75 mM TMPD plus 10 mM ascorbate. No cytochrome *c* was added to the assays. The endogenous cytochrome *c* is that which was not removed by washing. cyt, cytochrome.

Table IV: Steady State of Cytochrome *c* and O_2 Uptake Rates of Preparations with Different Content of Endogenous Cytochrome *c*[a]

concn of added cyt *c* during sonication (μM)	cyt *c* content in assays (μM)	O_2 uptake (μM s^{-1})/ cyt aa_3	oxidized cyt *c* in aerobic state (μM)/cyt aa_3
0	0.037	8.7	0.25
18	0.065	12.3	0.80
52	0.16	18.3	1.15

[a] Assays were run in 25 mM Tris–cacodylate buffer, pH 7.8, with 0.75 mM TMPD plus 10 mM ascorbate. O_2 uptake rates and steady-state levels of cytochrome *c* are expressed in terms of the content of cytochrome aa_3 in the assays. cyt, cytochrome.

with exogenous cytochrome *c* leveled off with increasing concentrations of TMPD, while those with the endogenous cytochrome *c* showed a second increase until a concentration of 2.25 mM TMPD was reached before leveling off.

Poly-L-lysine has been shown previously to inhibit the reaction of cytochrome oxidase with exogenous cytochrome *c* (Davies et al., 1964). Table II shows that poly-L-lysine had no inhibitory effect on the reaction with endogenous cytochrome *c* in concentrations which completely inhibit the reaction with exogenous 0.1 μM cytochrome *c*.

Several SMP preparations made by sonication in the presence of added cytochrome *c* contained increased levels of "endogenous" cytochrome *c*, i.e., cytochrome *c* that was not removed by washing (Table III). In spite of the 4.5-fold increase in the levels of this endogenous cytochrome *c* in the assays with similar content of cytochrome aa_3, the rate of O_2 uptake with 0.75 mM TMPD plus 10 mM ascorbate increased only slightly (1.5-fold) (Table III). Thus, these observations differ from those made with the SMP preparations sonicated in the absence of cytochrome *c*, where the O_2 uptake rates correlated with the content of endogenous cytochrome *c* (Table I). Figure 6 shows the reduction state of this endogenous cytochrome *c* in the aerobic state in the presence of TMPD plus ascorbate and then after anaerobiosis with SMP preparations sonicated in the presence of 0, 13, and 52 μM cytochrome *c*. The concentrations of oxidized cytochrome *c* in the

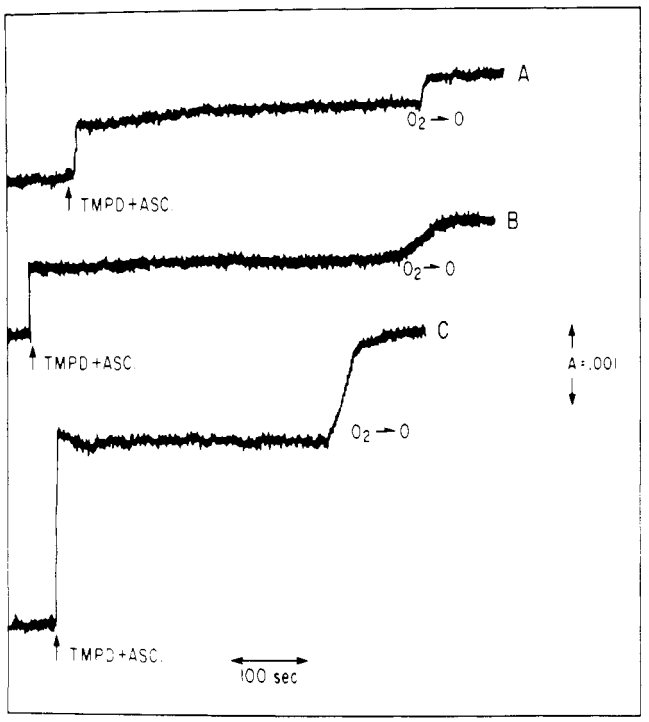

FIGURE 6: Oxidation–reduction state of endogenous cytochrome *c* after addition of 0.75 mM TMPD plus 10 mM ascorbate and then after exhaustion of O_2. SMP were suspended in 25 mM Hepes buffer, pH 7.8, and contained 0.059, 0.033, and 0.031 μM cytochrome aa_3 in A, B, and C, respectively. Preparations in A, B, and C contained 0.055, 0.065, and 0.16 μM endogenous cytochrome *c*.

aerobic state are listed in Table IV, along with the simultaneously measured rates of O_2 uptake under the same experimental conditions. The data are expressed in terms of the content of cytochrome aa_3 in the assays. Both increase with increasing content of endogenous cytochrome *c*, but the rates of O_2 uptake increase to a smaller extent than the increase in oxidized cytochrome *c* in the aerobic state. The measurements of the concentration of oxidized cytochrome *c* in the aerobic state with the preparation sonicated in the absence of added cytochrome *c* are not very accurate, since the cytochrome *c* content is so low. The concentration of oxidized cytochrome *c* in the aerobic state was greater in Tris–cacodylate buffer, pH 7.8, than at pH 7.0 and was hardly measurable at pH 6.1 except in the preparation with the highest level of cytochrome *c* (data not shown). The presence of varying levels of endogenous cytochrome *c* in the preparations had little or no effect on the reaction of the different preparations with exogenous cytochrome *c*, particularly at low concentrations of cytochrome *c* (data not shown).

Discussion

SMP preparations such as those we worked with are composed of vesicles, some of which are oriented "right-side out" and others which are oriented "inside out" with respect to the sidedness of the membrane of intact mitochondria. We found that several preparations contained about equal amounts of cytochrome aa_3 and cytochrome *c*, which means that some cytochrome *c* was lost, since heart mitochondria contain about twice as much cytochrome *c* as cytochrome aa_3. The cytochrome *c* would have dissociated off from the right-side-out vesicles into the assay buffer. Our data suggest that rather similar amounts of the two kinds of vesicles were present in the preparations. In Tris–cacodylate buffer, pH 7.8, there seems to be a tight combination of exogenous cytochrome *c* with the exposed reaction sites, and low concentrations of cytochrome *c* appear to be mostly bound (Figure 3). The tight combination can be repeatedly reduced by TMPD plus ascorbate, as previously demonstrated (Ferguson-Miller et al., 1978; Smith et al., 1979b). The reactivity of the bound exogenous cytochrome *c* is similar to that of the endogenous cytochrome *c*, which cannot diffuse away into the reaction medium, and the content of the two is similar at low concentrations of cytochrome *c* (Figure 3 and Table I). The O_2 uptake rates with endogenous cytochrome *c* of the different SMP preparations were roughly proportional to the content of endogenous cytochrome *c* (Table I).

The observation that an appropriate concentration of poly-L-lysine can completely inhibit the reaction with exogenous cytochrome *c* without affecting that with endogenous cytochrome *c* (Table II) agrees with the supposition that these reactions are independent and of similar magnitude.

TMPD in the reduced form can penetrate membranes, but the oxidized form (Würster's Blue) cannot, and Würster's Blue has been observed to accumulate within closed SMP vesicles under some conditions (Sagi-Eisenberg & Gutman, 1979). Most of our previous experiments were made with cytochrome *c* deficient SMP treated with detergent or with purified cytochrome oxidase, where closed vesicles were not involved. In the present work we found no evidence for the accumulation of Würster's Blue in the vesicles in Tris–cacodylate buffer, pH 7.8. Anaerobiosis always resulted in a further increase in the absorbance at 550–535 nm rather than the decrease observed by Sagi-Eisenberg & Gutman (1979) (Figure 6). Also, we observed no steady increase in the absorbance at 550–535 nm during the aerobic state, as they did, and the total change in absorbance (anaerobically reduced minus oxidized) was the same as that found in the anaerobic plus NADH minus oxidized difference spectrum. Most important, the data of Figure 5 show that the response of the O_2 uptake rate to variation of added TMPD concentration was identical with that of endogenous cytochrome *c* and with that of exogenous 0.1 μM cytochrome *c* up to 0.75 mM. All of our experiments comparing endogenous and exogenous reactions were made with 0.75 mM TMPD or less; thus, the comparisons were made under comparable conditions.

The O_2 uptake rates with endogenous cytochrome *c* showed additional increases at concentrations of TMPD above $\sim$1 mM, but those with exogenous cytochrome *c* did not. An additional reaction with the endogenous system apparently takes place at the higher concentrations. The identity of this reaction is not known.

The lack of knowledge of the rate of penetration of reduced TMPD across membranes and the observation of the accumulation of Würster's Blue under some conditions suggest uncertainties in the use of the polarographic method with TMPD as reductant with closed vesicles not deficient in cytochrome *c*. The experiments of Miller et al. (1979) with ferricyanide-containing liposomes lead to the suggestion of a further oxidation or dismutation of TMPD past the Würster's Blue stage. We have pointed out previously other experimental difficulties involved in this assay method (Smith et al., 1979a,b).

The significant observation here is the similarity of the properties of the reaction of the membrane-bound oxidase with the endogenous cytochrome *c* of inside-out vesicles and with low concentrations of added exogenous cytochrome *c* with the right-side-out vesicles. The reactions showed similar variations with changes of pH in Tris–cacodylate buffers (which are nonbinding ions to cytochrome *c*); both were low at pH 6 and highest at pH 7.8 (Figure 4). This would disagree with the

conclusion that binding constants are being measured (Ferguson-Miller et al., 1976, 1978) and supports our postulate (Smith et al., 1979a,b) that at pH 7.8 a combination of cytochrome *c* and cytochrome oxidase is formed with a high rate of electron transfer or a high rate of reaction with oxidant and reductant. The complex with the high turnover rate is also formed at pH 7.8 but not at pH 6 in the reaction with detergent-treated SMP or with purified oxidase (Smith et al., 1979a,b).

Similar reactivity of the oxidase with endogenous cytochrome *c* was seen in phosphate buffers as in Tris–cacodylate buffers of the same pH, but in phosphate the reaction with the exogenous cytochrome *c* was less at all pH values above 6 (Figure 4). The binding of phosphate to exogenous cytochrome *c* (Stellwagen & Shulman, 1973) must prevent its reaction with the exposed oxidase sites. We have previously shown that lower concentrations of ATP, ADP, or pyrophosphate will similarly inhibit the reaction with exogenous, but not with endogenous cytochrome *c* of SMP (Smith et al., 1980). ATP and ADP also bind to cytochrome *c* (Stellwagen & Shulman, 1973). Apparently the combination of the endogenous cytochrome *c* with the oxidase cannot be dissociated by phosphate ions in the assay medium.

The reaction of the oxidase of SMP with increasing concentrations of exogenous cytochrome *c* resembled that seen previously with cytochrome *c* deficient SMP, with or without detergent treatment (Smith et al., 1979b); increasing concentrations above $\sim$0.1–0.2 μM gave relatively small increases in the O_2 uptake rate in assays with TMPD plus ascorbate. Higher concentrations of "endogenous" cytochrome *c* added by sonication in the presence of cytochrome *c* stimulate the O_2 uptake rates to only a minor extent (Table III). Thus, unlike the observations made with preparations sonicated in the absence of cytochrome *c* (Table I), the O_2 uptake rates were not proportional to the content of cytochrome *c*. Also, the concentration of cytochrome *c* which remained oxidized in the aerobic state in the presence of TMPD plus ascorbate was not proportional to the O_2 uptake rates, as was found previously with the reaction of exogenous cytochrome *c* with detergent-treated membrane preparations or with purified cytochrome oxidase (Smith et al., 1979a,b). The lack of reactivity of the higher concentrations of added cytochrome *c* within the vesicles with the oxidase points to a lack of availability of internal reaction sites in the inside-out vesicles or to some differences in the internal environment.

In summary, our data indicate that cytochrome *c* and cytochrome oxidase form a tight and highly reactive complex in the presence of nonbinding ions at pH 7.8, whether the oxidase site for reaction with cytochrome *c* is exposed to the suspending medium or is restrained on or within membrane vesicles. This reactive form has a high turnover rate and remains largely oxidized during the aerobic state in the presence of TMPD plus ascorbate. At lower pH values or in the presence of ions that bind to exogenous cytochrome *c*, the formation of the highly reactive complex is prevented and some other kind of combination is formed which lacks the ability to turn over at such a high rate.

Acknowledgments

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