Evidence for Binding Sites on Cytochrome *c* for Oxidases and Reductases from Studies of Different Cytochromes *c* of Known Structure†

Lucile Smith,* Helen C. Davies, and María Elena Nava

ABSTRACT: Assays of cytochrome *c* oxidase and succinate- and NADH-cytochrome *c* reductase activities indicate that cytochromes *c* from beef, *Paracoccus denitrificans,* and *Rhodospirillum rubrum* all possess effective binding sites for reaction with eucaryotic and *P. denitrificans* reductase systems. *P. denitrificans* and beef cytochromes *c* have binding sites for the oxidase of both species, but *R. rubrum* cytochrome c_2 does not. Since the tertiary structures of the three cytochromes are known, the data give evidence for localization of the oxidase and reductase binding sites on the surface of the molecule. These appear to be separate sites, both of which are in the area around the heme crevice. The cytochrome *c* from *P. denitrificans* can bind poly(L-lysine), presumably because of a localized area of negative charge on the surface of the molecule. This binding can stimulate the oxidation of the *P. denitrificans* cytochrome *c* by the oxidase of either beef or *P. denitrificans,* giving additional evidence for the involvement of charged groups in the reaction of cytochrome *c* with the oxidase.

Cytochrome *c* functions as an intermediary electron transporter in the membrane-bound respiratory chain system of the mitochondrial inner membrane or the cytoplasmic membrane of bacteria, receiving electrons from the reductase segment and passing them on to the oxidase segment. The mechanism of electron transport is not known. X-ray crystallographic studies have shown that the cytochromes *c* of eucaryotes and of the bacteria *Rhodospirillum rubrum* and *Paracoccus* (formerly *Micrococcus*) *denitrificans* have nearly identical internal structures with the heme in a crevice surrounded by hydrophobic side chains, and with only one edge of the heme exposed (Dickerson et al., 1971; Swanson et al., 1976; Salemme et al., 1973; Timkovich and Dickerson, 1976). However, the number of side chains on the surface of the two bacterial cytochromes differs markedly from that of the eucaryotic type, accounting for the large difference in isoelectric point.

Earlier studies suggested that different areas of the surface of the eucaryotic cytochromes *c* were involved in binding to the reductase and oxidase segments of the electron transport chain (Smith et al., 1973, 1974; Margoliash et al., 1973). Since the binding sites must be on the surface of the molecule, we have compared the reactivity of eucaryotic cytochrome *c* and the two bacterial cytochromes *c* of known structure with the oxidase and reductases of eucaryotic mitochondria and of the *P. denitrificans* cytoplasmic membrane. The data indicate that separate binding sites for the oxidase and reductase systems are both in the area around the heme crevice.

Poly(L-lysine) competitively inhibits the oxidation of eucaryotic cytochrome *c* by the eucaryotic oxidase (Davies et al., 1964); we find that it also inhibits the oxidation of eucaryotic cytochrome *c* by the *P. denitrificans* oxidase. However, *P. denitrificans* cytochrome *c*, because of the distribution of charged groups on the surface, can also bind poly(L-lysine), to compete with its binding to the oxidase. We have found that addition of different amounts of poly(L-lysine) to the *P. denitrificans* cytochrome *c* can result in either stimulation or inhibition of its oxidation by the *P. denitrificans* oxidase. The observations confirm the involvement of charged groups in the reaction of cytochrome *c* with the oxidase (Davies et al., 1964; Smith and Minnaert, 1965).

Experimental Section

Methods. Cytochromes *c* were prepared from beef heart by the method of Margoliash and Walasek (1967) and from *P. denitrificans* by the method of Scholes et al. (1971). Both cytochromes were further purified by isoelectric focusing in Ampholine 8100 on an LKB column.[1] We are indebted to Drs. R. Bartsch and M. Cusanovich for gifts of ferricytochrome c_2 from *R. rubrum.* The concentrations of the cytochrome *c* solutions were calculated from the absorbance at 550 nm of the pigments reduced with $Na_2S_2O_4$, applying extinction coefficients of 27.6 cm^{-1} mM^{-1} for beef and *P. denitrificans* cytochromes (Margoliash, 1954; Scholes et al., 1971) and 28.1 cm^{-1} mM^{-1} for the *R. rubrum* pigment (Horio and Kamen, 1961). The cytochromes *c* were reduced with sodium borohydride (Smith et al., 1973) for use in the oxidase assays.

The source of the mammalian oxidase and reductases was the usual submitochondrial particle preparation from beef heart mitochondria (SMP[2]) (Lee and Ernster, 1967), treated where indicated with sodium deoxycholate (1 mg per mg of protein) to remove the endogenous cytochrome *c* and to "expose" maximally the oxidase and reductase sites (Smith and Camerino, 1963b). Fragments of cytoplasmic membrane of *P. denitrificans* were isolated by osmotic shock of spheroplasts as described by Scholes and Smith (1968a). These fragments were treated with deoxycholate in the same manner as were the SMP from beef heart. The protein content of membrane

† From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755, and the Department of Microbiology, University of Pennsylvania Medical School, Philadelphia, Pennsylvania 19119. *Received July 28, 1976.* This research was supported by Grant GM-06270 from the National Institutes of Health and by General Research Support Grant RR-05392 to L.S., and by Grant CC-00310 from the Center for Disease Control, Atlanta, Georgia, to H.C.D.

[1] G. McLain and L. Smith, manuscript in preparation.

[2] Abbreviations used: SMP, submitochondrial particle; Tris, tris(hydroxymethyl)aminomethane.

TABLE I: Oxidation and Reduction of Cytochrome *c* from Beef, *P. denitrificans* and *R. rubrum* by Beef, and *P. denitrificans* Oxidases and Reductases.[a]

Membrane Fragments	Cytochrome *c*	% of Reaction with Beef Cytochrome *c*				
		Cytochrome *c* Oxidase	Succinate-Cytochrome *c* Reductase		NADH-Cytochrome *c* Reductase	
			Zero Order	1st Order[c]	Zero Order	1st Order[c]
Particles treated with deoxycholate						
Beef	Beef	100	100	100	100	100
	P. denitrificans	<1	75	15	*b*	14
	R. rubrum	0	100	20	*b*	14
P. denitrificans	Beef	100	100	100	100	100
	P. denitrificans	40	95	20	*b*	25
	R. rubrum	<1	*b*	12	*b*	10
Particles not treated with deoxycholate						
Beef	Beef	100	100	100	100	100
	P. denitrificans	<1	*b*	17	*b*	21
	R. rubrum	0	35	19	*b*	21
P. denitrificans	Beef	100	100	100	100	100
	P. denitrificans	45	106	38	75	50
	R. rubrum	1	79	28	*b*	38

[a] Oxidase and reductase activity was measured in 0.05 M Tris-maleate buffer, pH 6.0 for oxidases and pH 7.6 for reductases, using beef SMP containing 0.005 mg of protein or *P. denitrificans* membrane fragments containing 0.008 mg of protein in 2 ml. All cytochromes *c* were 4.3 μM. [b] There was no measurable zero order portion of the curve. [c] First order rate constants ÷ mg of protein were 24.7, 7.9, and 10.7 for oxidase and succinate and NADH reductases of beef SMP + deoxycholate with beef cytochrome *c* and 10.0, 4.7, and 24 for *P. denitrificans* particles + deoxycholate with beef cytochrome *c*.

fragments was measured by the biuret method (Gornall et al., 1949) in the presence of 0.2% sodium deoxycholate.

Poly(L-lysine) hydrobromide (mol wt 16 500) was obtained from Miles-Yeda, Ltd. This polypeptide was converted to the free base and the Br^- ions were removed by chromatography on Sephadex G-75 at pH 11.5 followed by dialysis against weak NaOH (pH 9.0). The final concentration of the solution was 4 mg/ml as determined by either dry weight measurements or the biuret reaction. At pH 9.0 this amount of poly(L-lysine) is soluble.

Assay Methods. Cytochrome *c* oxidase was assayed spectrophotometrically at 25 °C as described previously (Smith, 1954; Smith and Conrad, 1956); the activities are expressed as the first-order-rate constants. The oxidase was also assayed polarographically with a Clark O_2 electrode after addition of 30 mM sodium ascorbate (Smith and Camerino, 1963a), where the activities are expressed as μM O_2 uptake per second.

The reduction of ferricytochrome *c* by the membrane-bound succinate- and NADH-cytochrome *c* reductases was assayed by following the increase in absorbance at 550 nm with a Cary spectrophotometer (Smith et al., 1974). Some reductase reactions were first-order with respect to ferricytochrome *c* for the entire sequence; others showed both zero-order and first-order parts. Both the initial zero-order velocities (μM cytochrome *c* reduced per second) and the rate constants for the first-order portions were calculated as described previously (Smith et al., 1974).

The buffers and the concentrations of cytochromes and enzymes used in the different assays are documented in the tables and figures.

Results

Table I compares the activities of cytochrome *c* oxidase and NADH- and succinate-cytochromes *c* reductases of membrane fragments from beef mitochondria and *P. denitrificans* with the cytochromes *c* from beef, *P. denitrificans*, and *R. rubrum;* the activities are related to those with beef cytochrome *c* as 100%. These activities vary somewhat with different membrane preparations. The assays were run with membrane fragments both before and after treatment with deoxycholate, as described in Methods. The relative reactivities of the different cytochromes *c* are similar with treated and untreated membranes, although any activities present were increased by detergent treatment (Smith et al., 1974). The data show that all three cytochromes *c* are reducible at reasonable rates by the reductases of either beef or *P. denitrificans*. With some combinations the reductase reactions were entirely first-order in ferricytochrome *c;* in these the reaction with the soluble cytochrome *c* was always rate limiting (Smith et al., 1974). As expected, when zero-order parts were apparent [i.e., when some other reaction in the chain was rate limiting (Smith et al., 1974)], they were similar in magnitude with the different cytochromes *c* in most instances. The first-order-rate constants for the reaction of the two bacterial cytochromes *c* with either beef or *P. denitrificans* reductases were always lower than those found with beef cytochrome *c*. At any rate, the cytochromes *c* from all three species apparently possess sites for binding to the reductase systems as well as proper structures for reduction of the cytochromes.

In contrast, cytochrome c_2 from *R. rubrum* is not oxidized by the beef oxidase and the *P. denitrificans* cytochrome *c* is oxidized at a very low rate. The *P. denitrificans* oxidase oxidizes beef cytochrome *c* more rapidly than its own cytochrome, but does not oxidize *R. rubrum* cytochrome c_2 at a significant rate. The two bacterial cytochromes *c* either cannot bind to or cannot react rapidly with the beef oxidase.

To test for the possible presence of oxidase binding sites on the two bacterial cytochromes *c*, evidence was sought for competition of either cytochrome with beef cytochrome *c* for

TABLE II: Effect of Poly(L-lysine) on the Oxidation of Beef Cytochrome *c* by *P. denitrificans* Oxidase.[a]

	Oxidase Rate, k (s^{-1})	
Poly(L-lysine) (μM)	0.0025 mg of Protein	0.005 mg of Protein
0	0.040	0.072
0.025	0.025	0.052
0.050	0	0.0027
0.075		0

[a] The assays were run in 0.05 M Tris-maleate buffer, pH 6.0, with 4.3 μM beef cytochrome *c* and *P. denitrificans* membranes containing 0.0025 or 0.005 mg of protein in 2 ml.

reaction with the beef oxidase. Such measurements are difficult to interpret using the spectrophotometric method since the cytochromes have similar absorption spectra and they interact with varying rates in solution. However, in the polarographic method for oxidase assay the cytochromes *c* are maintained in the reduced state by 30 mM ascorbate (Smith and Camerino, 1963a), thus minimizing interaction in solution. Oxidase assays by both methods indicate that *R. rubrum* cytochrome c_2 does not compete measurably with beef cytochrome *c* for the binding site on the oxidase. *R. rubrum* cytochrome c_2 was not oxidized at a measurable rate by beef oxidase, while beef cytochrome *c* was rapidly oxidized, the rate increasing with increasing concentration of cytochrome *c* in a nonlinear fashion, as previously shown (Smith and Conrad, 1956). Addition of *R. rubrum* cytochrome c_2 did not affect the rate of oxidation of the beef cytochrome *c*, as would be expected if there were competitive binding. Evidence will be presented later for the ability of the cytochrome *c* from *P. denitrificans* to interact with the beef oxidase.

The *P. denitrificans* cytochrome *c* is characterized by an asymmetric charge distribution on the surface of the molecule. All of the lysines are on one side of the molecule, 15 of the 17 being grouped in a collar around the exposed heme edge. The acidic groups show a uniform distribution on the other hemisphere of the molecule (Timkovich, 1975). The cytochrome is a "real dipole" (Timkovich, 1975). Because of this we tested the effect of poly(L-lysine) on the oxidases and reductases.

The data of Table II show that the oxidation of *beef* cytochrome *c* by the oxidase of *P. denitrificans* shows the same kind of inhibition by poly(L-lysine) that we found with the beef oxidase (Davies et al., 1964). The extent of inhibition with a fixed concentration of cytochrome *c* depends upon the concentration of oxidase protein present. The poly(L-lysine) apparently binds tightly to the oxidase since when poly(L-lysine) is added to give 100% inhibition, further addition of a similar quantity of oxidase yields the initial activity. In contrast to these observations with beef cytochrome *c*, the oxidation of *P. denitrificans* cytochrome *c* by the *P. denitrificans* oxidase was not inhibited by the same concentrations of poly(L-lysine), as shown in Figure 1. Addition of still higher concentrations of poly(L-lysine) stimulated the activity. We then found that, when an appropriate concentration of poly(L-lysine) is added to *P. denitrificans* cytochrome *c* before addition of the *beef* oxidase, it can be oxidized by the beef enzyme (Figure 2). The final rate achieved, however, is not as great as that seen with beef cytochrome *c*. Finally, addition of even higher concentrations of poly(L-lysine) gives inhibition of the reaction with either oxidase; Figure 3 shows these effects with the *P. denitrificans* oxidase.

As with the oxidase, the reduction of *beef* cytochrome *c* by

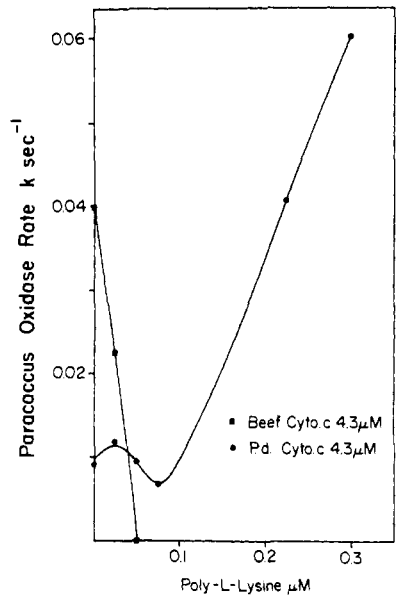

FIGURE 1: Effect of poly(L-lysine) on the oxidation of beef and *P. denitrificans* cytochromes *c* by *P. denitrificans* oxidase. The assays were run in 0.05 M tris-maleate, pH 6.0, with either 4.3 μM beef or *P. denitrificans* cytochrome *c* and *P. denitrificans* membranes containing 0.0025 mg of protein. Poly(L-lysine) was added to the buffered cytochrome *c* and the reactions were initiated by addition of the membranes containing the oxidase.

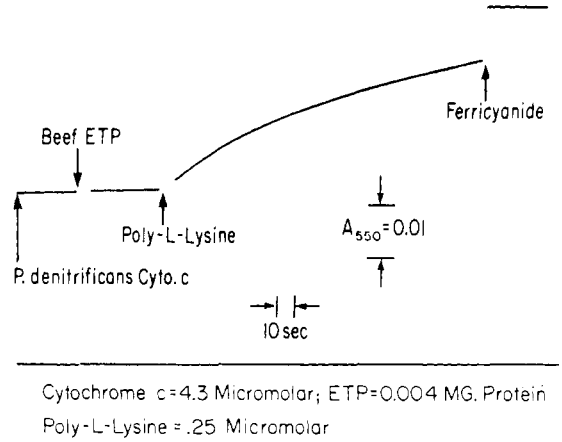

FIGURE 2: Effect of poly(L-lysine) on the oxidation of *P. denitrificans* cytochrome *c* by beef oxidase. Tracing from a Cary spectrophotometer showing the oxidation of 4.3 μM *P. denitrificans* cytochrome *c* (decrease in absorbance at 550 nm) by beef oxidase (membranes containing 0.004 mg of protein) after the addition of poly(L-lysine) to a concentration of 0.25 μM.

the reductases of *P. denitrificans* membranes was completely inhibited by 0.1 μM poly(L-lysine) under the usual conditions of assay. However, the effect of poly(L-lysine) on the succinate- and NADH-cytochrome *c* reductases of *P. denitrificans* membranes with the *P. denitrificans* cytochrome *c* was different from that described above with the oxidase reaction. The differences were apparent both when the oxidase and reductase reactions were run at their pH optima and when all assays were run at pH 7.0. Poly(L-lysine) had no stimulatory effect on the first-order reaction of either NADH- or succinate-cytochrome *c* reductase. Some, usually small, effects were apparent on the zero-order portions, where some other step than the reaction with the cytochrome *c* is rate limiting. These effects will be described in a subsequent publication.

In the experiments of Figures 1 and 3, the poly(L-lysine) was

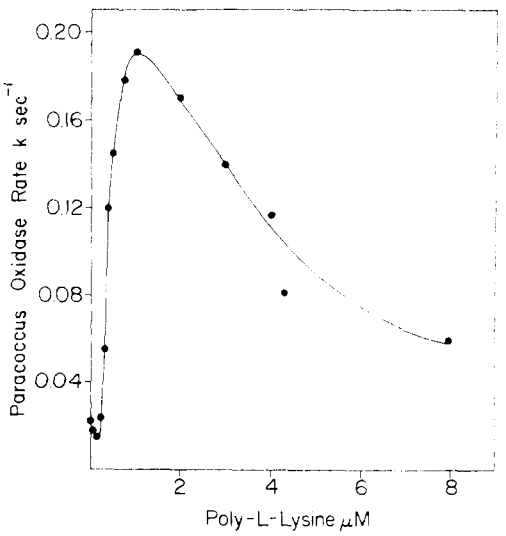

FIGURE 3: Effect of poly(L-lysine) on the oxidation of *P. denitrificans* cytochrome *c* by *P. denitrificans* oxidase. The oxidase assays were run spectrophotometrically in Tris-maleate buffer, 0.05 M, pH 6.0, with 4.3 μM ferrocytochrome *c* and *P. denitrificans* membranes containing 0.006 mg of protein. Poly(L-lysine) was added to the cytochrome *c* and the reaction initiated by addition of the membranes.

added to buffered *P. denitrificans* cytochrome *c* and then the reaction initiated by addition of the *P. denitrificans* membrane fragments. If the order of addition was changed, different effects were seen. In contrast to the lack of effect seen when 0.05 μM poly(L-lysine) was added first to the cytochrome *c*, 90% inhibition was seen when the poly(L-lysine) was added to the oxidase and then the reaction initiated by the addition of cytochrome *c*. The observations suggest that the *P. denitrificans* cytochrome *c* can bind the poly(L-lysine) to prevent its binding to the membrane-bound oxidase. Further evidence for this is seen in the protection by the *P. denitrificans* cytochrome *c* against poly(L-lysine) inhibition of the oxidation of beef cytochrome *c* by the beef oxidase (Table III). Also, a precipitate is formed with higher concentrations of *P. denitrificans* cytochrome *c* and poly(L-lysine) than those used in the experiments reported here.

Poly(L-lysine) did not stimulate the reaction of *R. rubrum* cytochrome *c* with either beef or *P. denitrificans* systems at any of the concentrations which were effective with the *P. denitrificans* cytochrome *c*.

Discussion

The succinate- and NADH-cytochrome *c* reductase systems of both beef and *P. denitrificans* can reduce the *c* cytochromes from beef, *P. denitrificans*, and *R. rubrum* at appreciable rates. The relevant reactions here are the first-order sequences, where the reaction with the cytochrome *c* is the rate-limiting step (Smith et al., 1974). During the zero-order parts, some other step is rate limiting, and these usually proceed at similar rates with all of the cytochromes *c*. The data show that in spite of the large differences in surface structure, all three cytochromes *c* possess sites for binding to and reacting with the two reductase systems of beef and *P. denitrificans*. Similar conclusions were reached by Davis et al. (1972) concerning the reaction of *R. rubrum* cytochrome c_2 with a purified beef NADH-cytochrome *c* reductase. However, they report what appear to be initial rates and do not specify whether they observed zero- or first-order kinetics.

Some hypotheses on the mechanism of reduction of cytochrome *c* by the reductases postulate electron transport into

TABLE III: Relief by *P. denitrificans* Cytochrome *c* of Poly(L-lysine) Inhibition of Oxidation of Beef Cytochrome *c* by Beef Oxidase.[a]

Reaction Components	Initial Rate of Cytochrome *c* Oxidation (μM s^{-1})
Oxidase + beef cyto. *c*	0.172
Oxidase + *P. d.* cyto. *c*	0
Oxidase + beef cyto. *c* + poly(L-lysine)	0.011
Oxidase + *P. d.* cyto. *c* + poly(L-lysine)	0.0025
Oxidase + beef cyto. *c* + *P. d.* cyto. *c* + poly(L-lysine)	0.108

[a] Assays were run in 0.05 M Tris-maleate buffer, pH 6.0, with beef membranes containing 0.0032 mg of protein. Both beef and *P. denitrificans* cytochromes *c* were 4.3 μM, and poly(L-lysine) was 0.075 μM. The reactions were initiated in each case by the addition of the oxidase. Initial rates were calculated, rather than first-order rate constants, because with the combination of both cytochromes *c* the reaction was not first-order throughout because of the interaction of the cytochromes in solution.

the protein via several aromatic amino acids (Dickerson et al., 1972), including tyrosines 67 and 74 (eucaryotic numbering). However, the amino acid which is equivalent to tyrosine 74 in *P. denitrificans* cytochrome *c* is leucine (Timkovich and Dickerson, 1976). Thus mechanisms involving an aromatic amino acid in this position are questionable since *P. denitrificans* cytochrome *c* is reducible by both bacterial and eucaryotic systems.

In contrast to the observations with the reductase systems, the two bacterial cytochromes *c* were not rapidly oxidized by the beef oxidase, and the *R. rubrum* cytochrome c_2 was not oxidized by the oxidase of *P. denitrificans*. The *R. rubrum* cytochrome c_2 does not possess an effective binding site for the beef oxidase, as shown by the lack of competition with beef cytochrome *c*. The *P. denitrificans* cytochrome does have an oxidase binding site, which is only revealed in the presence of poly(L-lysine).

Previous evidence documented the competition of poly(L-lysine) with eucaryotic cytochrome *c* for binding to the eucaryotic oxidase (Davies et al., 1964; Smith and Minnaert, 1965). The oxidase of *P. denitrificans* is similarly inhibited by poly(L-lysine) in its reaction with eucaryotic cytochrome *c*, which is additional evidence for the resemblance of the respiratory chain system of these bacteria with that of eucaryotic cells (Scholes and Smith, 1968b; John and Whatley, 1975). Several lines of evidence show that the *P. denitrificans* cytochrome *c* can also bind the poly(L-lysine) less strongly, presumably to the negatively charged surface area. This can prevent the inhibitory binding of poly(L-lysine) to both beef and *P. denitrificans* oxidases when the poly(L-lysine) is added to a great excess of the cytochrome *c*. The concentration of cytochrome *c* in the assays was as much as 1000-fold greater than that of the oxidases. Appropriate concentrations of poly(L-lysine) added to the *P. denitrificans* cytochrome *c* can stimulate its oxidation by both beef and *P. denitrificans* oxidases. The stimulatory effect could result from masking of negative charges on the *P. denitrificans* cytochrome *c*, thus promoting binding to the negatively charged group on the oxidase. We have previously reported evidence for the importance of charged groups in the interaction of cytochrome *c* with cytochrome *c* oxidase (Davies et al., 1964; Smith and Minnaert, 1965). For the present purpose, the significant observation is that a binding site on the *P. denitrificans* cyto-

chrome *c* for the beef oxidase is revealed in the presence of appropriate concentrations of poly(L-lysine).

In contrast to its stimulatory effect on the reaction of *P. denitrificans* cytochrome *c* with either beef or *P. denitrificans* oxidase, similar concentrations of poly(L-lysine) do not stimulate the first-order reductase reactions. This is good evidence that the binding of cytochrome *c* to the reductases is different from its binding to the oxidase, as suggested from other kinds of observations (Smith et al., 1973, 1974; Margoliash et al., 1973) and also from the presence of a binding site for the reductases, but not the oxidase, on *R. rubrum* cytochrome c_2.

The data presented on the reactivity of the different cytochromes *c* give some evidence about the localization of the binding sites on cytochrome *c* for reaction with the eucaryotic and *P. denitrificans* oxidase and reductase systems. *P. denitrificans* cytochrome *c*, which can react with all of the oxidases and reductases, has several extra loops of amino acids and a 15-residue tail which are not present in the eucaryotic cytochrome *c*. These loops and tail would constitute large perturbations on the surface of the pigment which would make these regions unlikely binding sites for the oxidases and reductases. Looking into the heme crevice as the front of the molecule, they are on the right side (residues 20–40, *P. denitrificans* numbering), on the bottom (residues 55–70 and 85–98) and on the back (tail at C terminus of residues 120–135) (Timkovich and Dickerson, 1976). The sequence from 88 to 98 corresponds to an insertion into eucaryotic cytochrome *c* between positions 75 and 76 and is in intimate proximity to the exposed heme edge (Timkovich and Dickerson, 1976). In addition the acidic side chains form a slight grouping on the back surface of the molecule (Timkovich, 1975), and it is presumably here where poly(L-lysine) binds. Since the *P. denitrificans* cytochrome *c* with poly(L-lysine) bound can be both oxidized and reduced by the eucaryotic systems, this is additional evidence against binding sites on the surface of the molecule opposite to the exposed heme edge. With these various surface areas eliminated, what remain as possibilities are the top of the molecule and the heme crevice. Since there are now several different kinds of evidence that separate binding sites are involved with the oxidase and reductase systems, an obvious possibility is that they are on either side of the heme crevice. Timkovich and Dickerson (1976) have pointed out that the heme crevice is the most structurally invariant surface feature among the different cytochromes *c*.

Our conclusion that both oxidase and reductase binding sites are most likely in the area around the heme crevice is in disagreement with the conclusions of Margoliash et al. (1973), based on observations with chemical derivatives of cytochromes *c*. They found that formation of derivatives of horse cytochrome *c* on tyrosine 74, tryptophan 59, tyrosine 67, or methionine 80 resulted in the loss of nearly all of the ability to react with succinate–cytochrome *c* reductase. However, the derivatives of tryptophan 59, tyrosine 67, and methionine 80 were autooxidizable, were not reducible by ascorbate, and had altered absorption spectra. More recently it has been found that the lack of activity of the monoiodotyrosine derivative resulted from polymerization upon lyophilization, and that the unpolymerized derivative reacts like native cytochrome *c* (Feinberg and Brautigan, 1975).

Attempts have been made to relate reactivities of cytochromes *c* from different species to evolutionary sequences (Yamanaka, 1972). The present observation that *P. denitrificans* cytochrome *c* reacts rapidly with the eucaryotic oxidase only in the presence of poly(L-lysine) introduces a note of caution into this kind of speculation.

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