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EFFECTS OF MONOCLONAL ANTIBODIES TO BOVINE AND *PARACOCCUS DENITRIFICANS* CYTOCHROMES *c* ON REACTIONS WITH OXIDASE, REDUCTASE AND PEROXIDASE

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The effects of monoclonal antibodies to bovine and *Paracoccus denitrificans* cytochromes *c* (Kuo, L.M. and Davies, H.C. (1983) Mol. Immunol. 20, 827–838) in the reactions of the cytochromes *c* with cytochrome *c* oxidase, reductase and peroxidase were studied. Spectrophotometric assays were employed, under conditions where binding of cytochrome *c* to the enzymes appears to be rate-limiting. Less than stoichiometric amounts of antibodies to *P. denitrificans* cytochrome *c* added to the cytochrome rendered some of it nonoxidizable or nonreducible by the *P. denitrificans* membrane-bound electron transport system and decreased the rate constant with the remaining cytochrome *c*. The antibodies appear to affect both electron transport reactions (blocking effects) with the oxidase and reductase and binding effects (effects on rate constants) and to distinguish between the two. Different ratios of antibody site to cytochrome *c* gave different extents of blocking of the reductase as compared with the oxidase reaction. Differences were also apparent in the effect of these antibodies on the reaction of yeast peroxidase and the oxidase with the *P. denitrificans* cytochrome *c*. Antibodies to bovine and *P. denitrificans* cytochromes *c* had considerably less effect on the reactions of the bovine cytochrome with bovine oxidase and reductase. One antibody was inhibitory to the oxidase reaction with bovine cytochrome *c*, but not to that with the reductase. Also, an antibody which inhibited the oxidase reaction had no effect on the reaction with yeast peroxidase. The data give evidence that the interaction areas on cytochrome *c* for oxidase and reductase and peroxidase are not identical, although they may be nearby.

Introduction

We have prepared a number of monoclonal antibodies to cytochromes *c* from bovine heart and from *P. denitrificans* [1,2]. Some could bind to bovine cytochrome *c*, some to *P. denitrificans* cytochrome *c* and some to both species of cytochrome, although widely different binding constants were observed for those tested [1]. We have now studied the effects of these antibodies on the cytochrome oxidase and reductases of bovine heart mitochondria and *P. denitrificans* cytoplasmic membrane and on yeast cytochrome *c* peroxidase. The effects were tested under assay conditions where the binding of cytochrome *c* to the electron transport systems is rate-limiting [3]. The advantage of using monoclonal antibodies is that they are chemically homogeneous and react with constant avidity to single antigenic determinants.

Previous work on the interaction of cytochrome *c* with the electron transport chain has led to disagreements. Some experimenters using a variety

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

of experimental approaches have concluded that binding and/or reaction sites on cytochrome *c* for the oxidase, reductases and peroxidase are the same [4–7]; while others have indicated that the sites are not identical [8–14]. A resolution of this question would lead to a better understanding of the mechanism of interaction of cytochrome *c* with the adjoining members of the electron-transport chain and in fact the interaction of all such membrane-bound electron-transport pigments.

The two antibodies specific for the *P. denitrificans* cytochrome *c* strongly affected the reactions with the *P. denitrificans* oxidase and reductases. The antibody can bind to the cytochrome *c* so that it can distinguish electron-transport reactions with the oxidase or reductase from binding effects, as shown by complete blockage of some cytochrome in the reactions and a decreased rate of reaction of the remainder. Different ratios of antibody site to cytochrome *c* were required for blocking of the oxidase as compared to the reductase, pointing to differences in the reaction sites for the two enzyme systems.

Only one of the antibodies had much effect on the reaction of bovine cytochrome *c* with the bovine oxidase, and that was only a decrease in rate without complete blocking. This antibody had little or no effect on the bovine reductase reaction. Apparently, none of the antibodies binds strongly to the bovine cytochrome *c* in the area of the reaction sites.

Some of the antibodies showed quite different effects on the rate constants of the peroxidation of cytochrome *c* by cytochrome *c* peroxidase as compared with the oxidation of cytochrome *c* by its oxidase.

The observations reported here, together with previous work seem to show that the sites on cytochrome *c* for reaction with the oxidase, reductases and peroxidase are not the same, although they may be close or even overlapping.

Methods

Preparations

Cytochromes c. The cytochromes of bovine and *P. denitrificans* were isolated and purified by the methods of Margoliash and Walasek [15] and of Scholes et al. [16], respectively; they were then further purified by isoelectric focusing [17]. Cytochrome concentrations were assayed by measuring the A_{550} of the reduced form, using 27.6 mM^{-1} and 26.8 mM^{-1} as extinction coefficients for the bovine and *P. denitrificans* pigments [16,18]. The $\Delta\varepsilon_{550\,nm}$ (reduced minus oxidized) for the bovine cytochrome was assumed to be 19.1 mM^{-1}. The cytochromes were reduced with $NaBH_4$ for use in the oxidase assays [9] and oxidized for the reductase assays by adjusting the pH briefly to 2.5 with HCl, then bringing it back to pH 7.0 with NaOH, or by treatment with $K_3Fe(CN)_6$ followed by removal of the latter by passage of the solution through a column of Sephadex G-25 (Pharmacia).

Yeast cytochrome *c*, prepared by the method of Yonetani [19], was generously supplied by him. The concentration of the reduced form was calculated assuming that the $\varepsilon_{550\,nm}$ is 29.0 mM^{-1} [20]. The $\Delta\varepsilon_{550\,nm}$ (reduced minus oxidized) was assumed to be 21.2 mM^{-1}.

Cytochromes used in the peroxidase assay were reduced with $NaBH_4$, then chromatographed on Sephadex G-25 equilibrated with water, to ensure that H_2O_2 or free radicals were completely absent from these solutions.

Monoclonal antibodies. In a previous communication [1] we have described the production, isolation and characterization of the hybridoma monoclonal antibodies against cytochromes *c* of bovine and *P. denitrificans*; these were used in this study. Briefly, BALB/c mice were immunized either with native cytochrome *c*, succinylated hemocyanin-conjugated cytochrome *c* or bovine-heart cytochrome *c* polymerized with glutaraldehyde. The spleen cells were fused with the SP2/0-Ag 14 myeloma cell line by centrifugation in the presence of 30% poly(ethylene glycol) 1000, as detailed in our prior publication [1]. Table I lists those antibodies utilized in tests of their effects on enzymatic activities. Of the ten antibodies studied, F3-10.2 and F3-29.4, which bound only to *P. denitrificans* cytochrome *c*, were proteins purified with a *P. denitrificans* cytochrome *c* Affi-Gel 10 affinity column and F12-29.23 and F12-266.20, which bound only bovine-heart cytochrome *c*, were purified with a bovine cytochrome *c*-BSA-Affi-Gel 10 affinity column [1]. As seen in Table I, not all of the antibodies specific for cytochrome *c* were able to bind that cytochrome on the Affi-Gel

column. The sites on cytochrome *c* necessary for binding to some of the specific antibodies may be coupled to the Affi-Gel 10 [1]. The monoclonal antibodies were freshly prepared and used within a few days. The other six antibodies bound both types of cytochrome *c* and were IgG or IgM pools from a Sephadex column. For tests with kinetic assays, all antibodies were dialyzed against 0.05 M Tris-maleate (pH 7.0), and concentrated by a vacuum system with dialysis tubing with a molecular weight cutoff of 12000.

The Fab′ fragments were prepared by our modification of the methods of Nisonoff et al. [21], Noble et al. [22] and Warr et al. [23]. The IgG pool from a Sephadex G-200 column was dialyzed against 0.05 M Tris-buffered saline (pH 8.0), and concentrated to about 5 mg/ml, then 1.7 M sodium acetate buffer (pH 4.0) was added to a final concentration of 0.2 M. The pepsin was added in the amount of 2% of the total protein weight for 18–24 h at 37°C. The digestion reaction was stopped by dialysis against 0.05 M Tris-buffered saline (pH 8.0). $(Fab')_2$ fragments, obtained by passage of this solution through a Sephadex G-200 column, were reduced under nitrogen with dithiothreitol (Sigma; final concentration, 20 mM) at 22°C for 1 h and alkylated with a 2.5-fold molar excess of iodoacetamide (Sigma) at 22°C for another h. The resulting Fab′ fragments were separated from excess reagents by gel filtration on Sephadex G-150.

A nonspecific plasmacytoma protein, prepared from ascites fluid and treated similarly to the specific protein was used as a control protein.

The association constants for binding of cytochrome *c* to several antibodies, measured by the fluorescence-quenching method, were $1.10 \cdot 10^6$ and $1.27 \cdot 10^6$ for F12-29.23 and F12-266.20 to bovine cytochrome *c*, and $1.57 \cdot 10^7$ and $1.16 \cdot 10^8$ for F3-10.2 and F3-29.4 to *P. denitrificans* cytochrome *c* [1]. All of these antibodies were IgG isotypes.

Radioimmunoassay and analytical isoelectric focusing were performed as described previously [1].

Oxidase and reductase preparations. P. denitrificans: As a source of cytochrome *c* oxidase and NADH : cytochrome *c* reductase, fragments of cytoplasmic membranes from aerobically grown bacteria were prepared by osmotic shock of spheroplasts [24]. These contain the electron-transport chains of these cells [24] and are stable on storage at −20°C for several months. The protein content was measured with the Biuret method [25]

TABLE I

ANTI-CYTOCHROME *c* MONOCLONAL ANTIBODIES USED FOR KINETIC ASSAYS

Monoclonal antibody cell line	Antigen for immunization	Antibody class	Binding activity to cytochrome *c*	Purified by affinity column
F5-162.38	HC-bovine [a]	IgM	*P.d.* and bovine	non-binding [b]
F9-499.24	*P.d.*	IgM	*Pd.* and bovine	non-binding
F10-253.10	HC-bovine	IgG	*P.d.* and bovine	non-binding
F12-443.4	HC-bovine	IgG_1	*P.d.* and bovine	non-binding
F12-505.6	HC-bovine	IgG_1	*P.d.* and bovine	bovine-BSA-Affi-Gel [c]
F12-505.15	HC-bovine	IgG_1	*P.d.* and bovine	bovine-BSA-Affi-Gel [c]
F12-29.23	HC-bovine	IgG_1	bovine	bovine-BSA-Affi-Gel [d]
F12-266.20	HC-bovine	IgG_1	bovine	bovine-BSA-Affi-Gel [d]
F3-10.2	*P.d.* [e]	IgG_{2a}	*P.d.*	*P.d.*-Affi-Gel [f]
F3-29.4	*P.d.*	IgG_1	*P.d.*	*P.d.*-Affi-Gel [f]

[a] Succinylated hemocyanin-conjugated bovine-heart cytochrome *c*.

[b] The antibodies could not be purified by either of the affinity columns (*P.d.* Affi-Gel [f] and bovine-BSA-Affi-Gel [c,d]).

[c,d] The antibodies were adsorbed on bovine-heart cytochrome *c* BSA-Affi-Gel 10 affinity column and eluted by 6 M guanidine HCl [c] or by 3 M KSCN [d].

[e] *P. denitrificans* cytochrome *c*.

[f] *P. denitrificans* cytochrome *c* Affi-Gel 10 affinity column.

P.d., *Paracoccus denitrificans*; HC, hemocyanin; BSA, bovine serum albumin.

in the presence of 0.1% sodium deoxycholate [24]. Immediately prior to use, concentrated suspensions of the membrane vesicles were treated with deoxycholate, 1 mg/mg protein, then diluted with cold water [26]. 10 μl of the diluted membrane fragments containing 0.0345 mg protein were used in each of the oxidase and reductase assays with either bovine or *P. denitrificans* cytochrome *c*. The reductase activity was 98% inhibited by 2.32 μg antimycin A/mg protein.

Bovine: some oxidase assays were made with submitochondrial particles prepared from bovine-heart mitochondria which had been made deficient in cytochrome *c* [3]. The protein content was measured with the Biuret method [25] in the presence of 0.2% deoxycholate and the cytochrome aa_3 content following the method of Vanneste [27]. These particles were also treated with deoxycholate immediately before use as described above for the *P. denitrificans* membranes, and 0.019 or 0.037 mg protein were used in the assays.

Oxidase assays were also run with purified cytochrome aa_3 prepared by the method of Yonetani [28], a generous gift from him, using 10 μl of a 6 μM suspension in the assays.

Succinate cytochrome *c* reductase was kindly supplied by Trumpower and Edwards [29]. The activity decreased upon freezing but could be restored as described by them [29]. Aliquots containing 0.008 or 0.015 mg protein were used in the assay mixtures.

Cytochrome *c* peroxidase prepared from yeast by the method of Yonetani and Ray [30] was a gift of Dr. T. Yonetani. Its concentration was estimated spectrophotometrically assuming that $\varepsilon_{408\,nm}$ was 93 mM^{-1}. For the assays, 5, 25 or 50 μl of a 0.1 μM solution was employed.

Assay methods

Cytochrome *c* oxidase was assayed spectrophotometrically by the method of Smith and Conrad [31], using a Dual Wavelength Filterphotometer (Technical Consulting Service, South Hampton, PA) with a water-jacketed cuvette containing a sample of 2.8 ml at 26°C. Assays were run in 0.05 M Tris-maleate (pH 7.0) and the first-order rate constants calculated [31]. When the effects of antibodies were tested, they were added to cytochrome *c* in buffer before the addition of the enzyme. Since the rate constants decrease with increasing total cytochrome *c* in the oxidase assays [31], those in the presence of antibody were compared with assays in its absence at the same concentration of cytochrome *c*.

NADH: cytochrome c reductase. NADH: cytochrome *c* reductase of *P. denitrificans* and succinate cytochrome *c* reductase of bovine heart were assayed by recording the increase in absorbance at 550 nm as described previously [10]. After the reaction stopped, sodium dithionite was added and the absorbance recorded. The reactions were initiated by the addition of KCN (final concentration 0.4 mM) and substrate to the mixture of cytochrome *c*, enzyme, and buffer as described in the figure legends. As with the oxidase, activities in the presence of antibody were compared with those in its absence with the same concentration of cytochrome *c*. First-order rate constants were calculated for the first-order part of the time course [10], where the reaction with cytochrome *c* is rate-limiting.

The oxidase and reductases were all assayed in the same buffer so that the comparative effects of antibodies were independent of pH or ionic strength variations.

Cytochrome c peroxidase. This peroxidase was assayed spectrophotometrically with cytochromes *c* from yeast, bovine or *P. denitrificans* in the same buffer used for the oxidase and reductase assays with 35.7 μM H_2O_2, using 1.8 nM peroxidase with *P. denitrificans* cytochrome *c* and 0.89 nM peroxidase with bovine cytochrome *c*.

Results

Oxidase

P. denitrificans. Increasing amounts of purified antibody to *P. denitrificans* cytochrome *c* (F3-29.4) were added to cytochrome *c* in buffer before addition of the membrane preparation (Fig. 1). The rate of absorbance change is decreased in the presence of the antibody, and the reaction stops before complete oxidation. When the reaction stops, the addition of more oxidase has no effect, but added cytochrome *c* can be oxidized, indicating that the oxidase is uninhibited. The amount of non-oxidizable cytochrome *c* as a function of the ratio of antibody to cytochrome *c* in the assays is

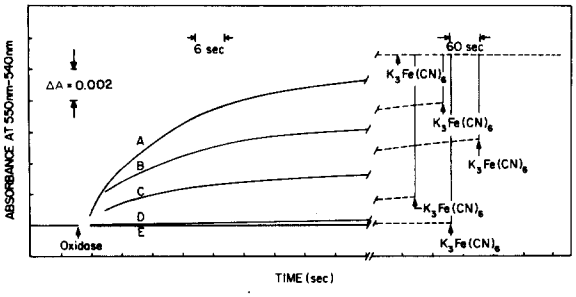

Fig. 1. Recording of the change in absorbance at 550–540 nm as 0.466 μM *P. denitrificans* cytochrome *c* is oxidized by *P. denitrificans* cytochrome *c* oxidase in the presence of F3-29.4 antibody (IgG). The different experiments were run with the same concentration of cytochrome *c* (0.466 μM) in 0.05 M Tris-maleate buffer (pH 7.0), and with the same concentration of oxidase containing 0.0345 mg of protein in 2.8 ml, but with varying concentrations of antibody. At the arrow marked oxidase, 10 μl diluted *P. denitrificans* membrane fragments were added. At arrow marked $K_3Fe(CN)_6$, 2 μl of a 10-fold dilution of saturated solution were added. (———) Running time at 6 s/unit; (- - - - -) running time at 60 s/unit. Curve A, no antibody added; curve B, 0.130 mol antibody/mol cytochrome *c*; curve C, 0.260 mol antibody/mol cytochrome *c*; curve D, 0.519 mol antibody/mol cytochrome *c*; curve E, 1.040 mol antibody/mol cytochrome *c*.

given in Table II, which shows that 0.5 mol of IgG renders 1 mol of cytochrome *c* non-oxidizable by the oxidase. That is, one antibody binding site is sufficient to block the oxidation of one molecule of cytochrome *c*. Table II also shows that the effects of antibody on blocking the reaction and on decreasing the rate constant of the reaction were correlated with the ratio of antibody site to cytochrome *c* rather than to the total cytochrome *c* concentration. Similar results to these obtained with IgG (F3-29.4) were also seen with Fab′ derived from this antibody (data not shown).

Similar observations were obtained with a second monoclonal antibody to *P. denitrificans* cytochrome *c*, which could be adsorbed and purified on *P. denitrificans* cytochrome *c* Affi-Gel 10 affinity columns (F3-10.2 of Table I). This is a different protein from F3-29.4 as shown by different K_a values for cytochrome *c* and differences in radioimmunoassay and isoelectric focusing data, but it was not examined in kinetic studies in as great detail as was F3-29.4. Other antibodies which bound *P. denitrificans* cytochrome *c* (generated by either bovine or *P. denitrificans* cytochrome *c*) either had no effect with equivalent ratios of antibody site to cytochrome *c* or produced decreases in the first-order rate constant (Table III), but did not result in non-oxidizable cytochrome *c*.

Bovine. The effect of the antibodies tested on the oxidation of bovine cytochrome *c* by the bovine oxidase was negligible or small (Table IV). No

TABLE II

RATIO OF F3-29.4 ANTIBODY ADDED TO NONOXIDIZABLE *P. denitrificans* CYTOCHROME *c* FORMED

Antibody added/mol cytochrome *c* [a] (mol)	Nonoxidizable cytochrome *c* (%)	Antibody added/mol non-oxidizable cytochrome *c* (mol)	Percent of initial rate constant remaining (%)
0.104	19.6	0.530	83.8
0.130	30.0	0.433	80.3
0.208	43.1	0.483	67.5
0.260	51.0	0.509	42.1
0.416	81.5	0.511	13.3
0.519 [b]	93.4	0.556	6.8
0.520 [c]	90.2	0.577	9.3
1.038 [d]	100.0	↑	0
1.040 [e]	100.0	Complete | inhibition	0
2.081	100.0		0
4.151	100.0	↓	0

[a] Concentrations of cytochrome *c* varied between 0.058 and 2.32 μM.
[b] 0.466 μM cytochrome *c*.
[c] 0.116 μM cytochrome *c*.
[d] 0.058 μM cytochrome *c*.
[e] 0.466 μM cytochrome *c*.

TABLE III

EFFECTS OF ANTIBODIES ON THE OXIDATION OF *P. DENITRIFICANS* CYTOCHROME *c* WITH *P. DENITRIFICANS* CYTOCHROME *c* OXIDASE

Antibody cell line	Antibody added/ mole cytochrome *c* (mol)	Percent of initial rate constant remaining(%)
F5-162.38	5	100
	10	100
F9-499.24	3	25.8
	4.5	17.7
	5	15.6
F10-253.10	10	69.0
	10	57.6
F12-443.4	5	30.7
F12-505.6	10	23.1
	20	18.2
F12-505.15	5	41.1
	10	33.7
F12-29.23, F12-266.20	Did not bind to *P. denitrificans* cytochrome *c*	
F3-10.2, F3-29.4	(i) inhibited oxidation; (ii) formed nonoxidizable cytochrome *c*	

TABLE IV

EFFECTS OF ANTIBODIES ON THE OXIDATION OF BOVINE-HEART CYTOCHROME *c* WITH BOVINE-HEART OXIDASE

Antibody cell line	Antibody added/ mol cytochrome *c* (mol)	Percent of initial rate constant remaining (%)
F5-162.38	5	87.8
F9-499.24	4.5 [a]	32.3
	5	34.8
F10-253.10	5	100
	10 [a]	100
F12-443.4	5	81.8
	10 [b]	78.3
	10 [a]	76.7
F12-505.6	5 [a]	82.2
	10	84.8
F12-505.15	5	100
	10	100
F12-29.23	1	100
F12-266.20	2	100
F3-10.2, F3-29.4	Did not bind to bovine-heart cytochrome *c*	

[a,b] 10 μl [a] or 5 μl [b] of the diluted submitochondrial particle (0.0185 or 0.037 mg) were the source of bovine oxidase. For the other experiments, cytochrome aa_3 was used.

non-oxidizable cytochrome *c* was seen, and only one (F9-499.24 to *P. denitrificans* cytochrome *c*) had more than a slight inhibitory effect (a decrease in the first-order rate constant). Similar observations were made with both the purified cytochrome aa_3 and with the submitochondrial particle preparation.

Possible synergistic effects. No synergistic effects were observed when combinations of antibodies were tested on the oxidase reactions; all inhibitory effects were additive. Also, no stimulatory effects were observed with any antibodies tested in the oxidase assays, including those testing bovine oxidase with *P. denitrificans* cytochrome *c*, which proceed at low rates under the conditions used [11].

NADH: cytochrome c reductase of P. denitrificans

In the reaction of *P. denitrificans* cytochrome *c* with the reductase, addition of purified antibody F3-29.4 resulted in both nonreducible cytochrome *c* and in a decrease in the rate constant of the reaction of the remaining cytochrome *c*, as with the oxidase (Fig. 2). Also, further reduction of the cytochrome *c* by ascorbate was partially inhibited (Fig. 2). All of these effects varied with the ratio of antibody to cytochrome *c* (Table V). However, with this antibody, a molar ratio of IgG to cytochrome *c* of 2 gave complete inhibition, as compared with a molar ratio of 0.5 for the oxidase reaction. Thus, 4 mol binding site/mol cytochrome *c* gave complete inhibition of the reduction of cytochrome *c* by the reductase. Comparison of effects of antibody F3-29.4 on oxidase and reductase reactions is plotted in Figs. 3 and 4.

Another purified antibody specific to *P. denitrificans* cytochrome *c*, F3-10.2, has effects on the reductase reaction qualitatively similar to those of F3-29.4 (data not shown). Some others were less inhibitory to the reductase. However, one antibody, F10-253.10, which had a small inhibitory

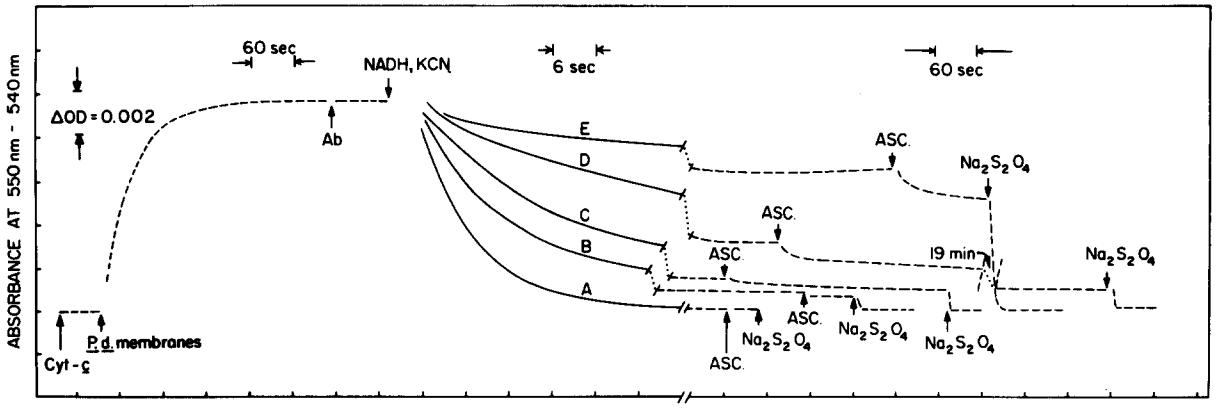

Fig. 2. Recording of change in absorbance at 550–540 nm as 0.464 μM *P. denitrificans* ferrocytochrome *c* is oxidized, and then reduced by the oxidase and reductase segments of *P. denitrificans* (P.d.) membrane fragments in the presence of F3-29.4 antibody (IgG). At the second arrow, 10 μl of the diluted *P. denitrificans* membrane fragments containing 0.0345 mg of protein were added to the ferrocytochrome *c* in 0.05 M Tris-maleate buffer (pH 7.0). Antibody was added at the arrow marked Ab after cytochrome *c* was completely oxidized. At the arrow marked NADH and KCN, these were added to the reaction mixtures to give final concentrations of 0.36 mM NADH and 0.4 mM KCN. At arrow marked ASC., $Na_2S_2O_4$, approx. 1 mg solid sodium ascorbate and dithionite were added. Curve A, no antibody; curve B, 0.130 mol antibody/mol cytochrome *c*; curve C, 0.260 mol antibody/mol cytochrome *c*; curve D, 0.520 mol antibody/mol cytochrome *c*; curve E, 1.039 mol antibody/mol cytochrome *c*. (———) running time at 6 s/unit; (– – –) running time at 60 s/unit.

effect on the rate constant of the oxidase reaction, had almost no effect on the activity of the reductase.

Succinate cytochrome c reductase of bovine heart

None of the antibodies tested had any effect on the succinate cytochrome *c* reductase activity of bovine heart, even some anti-bovine cytochrome *c* antibodies which inhibited the reduction of *P. denitrificans* cytochrome *c* by the *P. denitrificans* reductase.

Cytochrome c peroxidase.

The first-order rate constants of the yeast peroxidase assays with yeast cytochrome *c* were 4-fold greater than those with bovine cytochrome *c* and 14.4-fold greater than the values with the *P. denitrificans* cytochrome *c*. Two antibodies to *P.*

TABLE V

RATIO OF F3-29.4 ANTIBODY ADDED TO NONREDUCIBLE *P. DENITRIFICANS* CYTOCHROME *c* FORMED

Antibody added/mol cytochrome *c* (mol)	Nonreducible cytochrome *c* (%)	Antibody added/mol nonreducible cytochrome *c* (mol)	Percent of initial rate constant remaining (%)
0.104	5.1	2.04	75
0.130	8.4	1.55	53
0.2081	9.4	2.21	48
0.2082	10.0	2.08	40
0.260	14.4	1.80	36
0.416	20.8	2.00	27
0.520	29.8	1.74	25
1.039	66.7	1.56	14
2.083	100.0	↑	0
4.157	100.0	Complete inhibition	0
4.170	100.0		0
8.339	100.0	↓	0

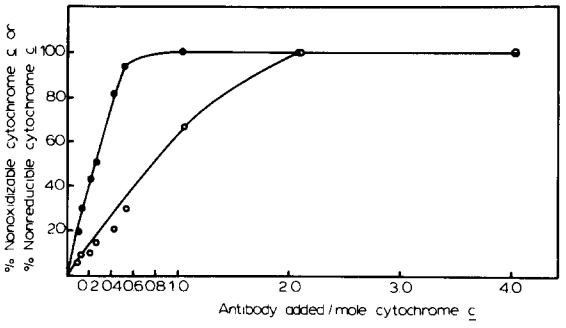

Fig. 3. Effect of F3-29.4 IgG antibody added on the formation of nonoxidizable and nonreducible *P. denitrificans* cytochrome *c* in the reaction with *P. denitrificans* cytochrome *c* oxidase and reductase. (●———●) The percent of nonoxidizable cytochrome *c*; (●———●) the percent of nonreducible cytochrome *c*.

denitrificans cytochrome *c* (F3-10.2 and F3-29.4) decreased the rate constant with *P. denitrificans* cytochrome *c* significantly, but there was no complete blocking of the reaction, even at a ratio of one antibody per cytochrome *c*. Also, the reaction was no longer first-order throughout, but showed an initial linear rate. The effects of the different antibodies on the rate constants for the first-order reactions are summarized in Table VI.

None of the antibodies had any effect on the peroxidase reaction with bovine cytochrome *c* as the substrate, even that antibody (F9-499.24) which inhibited the oxidase.

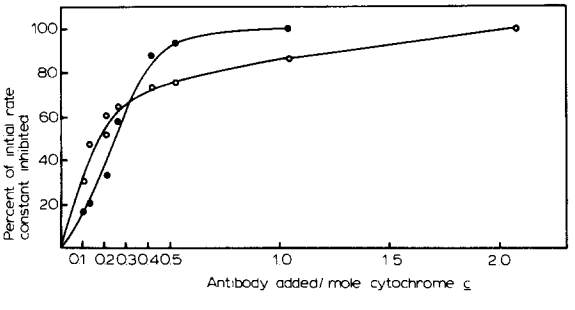

Fig. 4. Effect of F3-29.4 IgG antibody added on the inhibition of initial rate of the reaction of *P. denitrificans* cytochrome *c* with *P. denitrificans* cytochrome *c* oxidase and reductase. (●———●) The percent of initial rate constant of oxidase inhibited; (○———○) the percent of initial rate constant of reductase inhibited.

TABLE VI

EFFECTS OF ANTIBODIES ON THE OXIDATION OF *P. DENITRIFICANS* CYTOCHROME *c* WITH YEAST CYTOCHROME *c* PEROXIDASE

Antibody cell line	Antibody added/ mole cytochrome *c* (mol)	Percent of initial rate constant remaining (%)
F5-162.38	10	66.8
F9-499.24	5	51.4
F10-253.10	5	74.1
	10	76.7
F12-443.4	5	53.6
F12-505.6	10	57.0
F12-505.15	10	52.8
F12-29.23 F12-266.20	Did not bind to *P. denitrificans* cytochrome *c*	
F3-10.2	1	20.3
F3-29.4	1	16.5

Discussion

The reductase and oxidase activities reported here were measured with a range (0.058–2.3 μM) of concentrations of cytochrome *c* [3,31] under conditions where binding of cytochrome *c* to the enzyme systems appears to be rate-limiting [11,13,32]. Only the first-order portions of the reductase reactions were studied [10]. Also, both oxidase and reductase reactions were run in the same buffer to eliminate effects due to variations of pH and ionic strength. These data cannot be compared with those obtained using the polarographic method of assay, which measures a different aspect of the oxidase reaction [3].

Of the ten monoclonal antibodies obtained from ten representative cell lines, two to bovine cytochrome *c* bound only to the bovine cytochrome, two to *P. denitrificans* cytochrome *c* bound only to it, while the other six bound to both species of cytochrome, as demonstrated by ELISA and radioimmunoassay measurements [1,2]. These monoclonal antibodies should be specific in their binding to a unique determinant on the immunogen molecule. Our data show that the different binding

antibodies had different effects on the reactions of the cytochromes *c* with the oxidase, reductases and peroxidase. The antibody preparations contained no nonspecific inhibitory substances, since no inhibitory effects were found with nonbinding antibodies.

A specific antibody to cytochrome *c* could affect the interaction of the cytochrome with the oxidase or reductases in different ways:

(1) It could bind strongly to completely cover the reaction site so that cytochrome *c* could not bind to the enzyme. Or it could bind to cover part of the site in such a way that the cytochrome in the complex cannot bind in a proper orientation to the enzyme for electron transport to proceed. In either case, a ratio of one antibody site per molecule of cytochrome *c* should yield complete inhibition. An example of complete covering of the reaction site by antibody was seen with an antibody to human cytochrome *c* isolated from polyclonal rabbit serum [9]. In those experiments, an amount of cytochrome *c* stoichiometric with antibody site was rendered non-oxidizable, while any unbound cytochrome *c* retained its original activity.

(2) The antibody could bind at a position removed from the reaction site so that the reaction of the cytochrome *c* in the antibody complex with the enzyme is decreased as a result of change in the size, shape, conformation or charge distribution. A decrease in reaction rate with the oxidase without complete blocking was seen with an antibody fraction which bound to human cytochrome *c* at the area including isoleucine 58 (on the back surface of the molecule looking at the heme crevice as the front) [9]. These data with antibodies to human cytochrome *c* were obtained in experiments using the spectrophotometric method of assay.

(3) In addition, the antibody-cytochrome *c* complexes might compete with free unbound cytochrome *c* for binding with the oxidase, reductases or peroxidase.

The antibodies specific to *P. denitrificans* cytochrome *c* had dramatic effects on its reaction with the oxidase and reductase of *P. denitrificans* membranes. Antibody F3-29.4 rendered an equivalent amount of cytochrome *c* non-oxidizable by the oxidase, and the rate constant with the remaining cytochrome *c* was decreased. Thus, the reaction site may be covered by the antibody to the extent that a proper combination of cytochrome *c* with the oxidase for electron transport is not possible. However, the antibody-cytochrome *c* combination may then compete with the unbound cytochrome *c* for binding to the oxidase, as shown by the decreased rate constant of the remaining cytochrome *c*. As an alternate explanation, if the antibody-cytochrome *c* complex remains bound to the oxidase, the unbound cytochrome *c* would react with decreased affinity for the partially covered oxidase site. The data show that the antibody can affect both binding (as shown by the effect on the rate constants) and electron transport (complete blocking of the reaction) and can show these effects separately. The blocking might result from altering residues involved in the electron-transport reaction or the distances between the hemes of cytochrome *c* and the oxidase. Work in Chance's laboratory on derivatives of cytochrome *c* at low temperature showed that not only binding, but also the electron-transport process within the cytochrome *c*-oxidase complex might be the determinant of the overall reaction rate [33]. We have previously postulated that complexes between cytochrome *c* and the oxidase of different reactivities might form under different conditions [3,34].

The NADH : cytochrome *c* reductase reaction was also completely blocked in the presence of antibody, but larger ratios of antibody to cytochrome *c* were required to affect complete blockage (Fig. 3). This points to differences in the reaction sites on cytochrome *c* for the oxidase and reductase. The effects of the antibody on the rate constants of the cytochrome *c* that remained reducible were similar to those seen on the oxidizable cytochrome *c* in the oxidase reactions at ratios of antibody to cytochrome *c* up to about 0.3 (Fig. 4). This gives further evidence for differences between effects on electron transport and competitive effects on binding of available cytochrome *c* by comparison of blocking with decreases in rate constants. These suggestions agree with the observation that the extent of inhibition depends upon the ratio of antibody to cytochrome *c* rather than upon the total concentration of cytochrome *c*.

None of the antibodies tested had much effect on the reaction of the bovine oxidase with bovine cytochrome *c*. The only one which had more than

a slight inhibitory effect was an antibody to *P. denitrificans* cytochrome *c* (F9-499.24), which caused a decrease in the rate constant without complete blocking. This one apparently did not bind strongly near the reaction site. We found no differences in the effects of the antibodies on reactions of cytochrome *c* with purified oxidase as compared with those on submitochondrial particles treated with deoxycholate.

No antibody tested had any effect on the reaction of bovine cytochrome *c* with the bovine succinate-cytochrome *c* reductase, even the one which was inhibitory to the bovine oxidase reaction. Since the two assays were run simultaneously under identical conditions, this is further evidence that cytochrome *c* reacts differently with the oxidase and reductase.

Using a number of different approaches, considerable investigation has sought the nature of the sites on cytochrome *c* for reaction with the oxidase and reductase, although it was sometimes assumed that only association constants were being tested without regard for effects on electron transport. Some data led to the suggestion that the sites for the oxidase and reductase were different [9,11], others suggested that they were 'very similar' [4–6]. Different kinds of methodology were used in the various experiments, with sometimes different reaction conditions for the assays of oxidase and reductase. Recently Osherhoff et al. [7] tested the effects of several antibody populations isolated from rabbit antisera to horse cytochrome *c* on the reactions of the cytochrome *c* with bovine oxidase and reductase. The polarographic method was used with the oxidase and mostly the spectrophotometric method with the reductase, utilizing different conditions of pH and ionic strength for the two kinds of assay. They interpreted changes in K_m and V_{max} as defining the 'interaction domains' on cytochrome *c*. The conclusion is made that "the interaction domains on the surface of cytochrome *c* for the two enzyme systems must be very similar". In fact, they observed considerable quantitative differences as well as some qualitative ones. Also, some of the effects of antibodies on the oxidase were reported to be due to a decreased reduction of the cytochrome *c*-cytochrome oxidase complex by TMPD, which is not involved in the reductase reaction. The 'relative effects' seen with some of the antibody populations do not seem to correlate the proposed site with its proximity to the region of cytochrome *c* claimed to interact with both oxidase and reductase. Different inhibitory effects were actually found with one antibody population in the polarographic as compared with the spectrophotometric method for the reductase. These workers do claim to investigate 'interaction domains' on the cytochrome, rather than binding sites and suggest that the inhibitions they observe could result from several possible mechanisms.

Putting together all of the previous and the present data, and recognizing that both binding and electron-transfer reactions are involved, it seems that they are all saying the same thing: the interaction areas on cytochrome *c* for the oxidase and reductase are not identical, but are nearby or even overlapping and are characterized by subtle reaction differences.

Our data with the monoclonal antibodies also point to some differences in their effects on the peroxidase reaction as compared with those on the oxidase. No antibody had any effect on the reaction of the yeast peroxidase with bovine cytochrome *c* as substrate, even the one which inhibited the oxidase. Also, in reaction with *P. denitrificans* cytochrome *c* different effects were observed on the oxidase and peroxidase with two antibodies to *P. denitrificans* cytochrome *c*. Thus the reaction sites on cytochrome *c* for the peroxidase are not the same as for the oxidase.

It has been proposed that cytochrome *c* binds to the oxidase by two points of attachment in a cleft formed by different subunits [35,36]. To look for evidence for two binding sites, we sought synergistic effects with combinations of different antibodies to cytochrome *c* in reactions with the bovine oxidase, but found none. In all combinations tested, the inhibition with the two antibodies was additive.

We also looked for evidence that an antibody to *P. denitrificans* cytochrome *c* might bind on the 'back side' of the molecule and stimulate the oxidase reaction, since we had found increased activity on binding of poly(L-lysine) to a negatively charged area on the cytochrome [11]. However, no antibodies were found to stimulate the oxidase reaction.

The data described give a measure of the anti-

genic determinants on cytochrome *c*. At least three on both bovine and *P. denitrificans* cytochromes were indicated by measurements of binding affinity, radioimmunoassay, isoelectric focusing and fluorescence quenching [1]. From the effects on the enzymatic reactions at least five different sites are indicated.

Of the ten different monoclonal antibodies we have obtained, two (to *P. denitrificans* cytochrome *c*) appear to bind strongly in the area of the enzymatic reaction sites and can be used to investigate further the organization of the membrane-bound electron-transport systems. Recently, we have obtained additional monoclonal antibodies to bovine and human cytochrome *c* [12], which are providing us with further insights.

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