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Monoclonal antibody to human cytochrome *c*: effect on electron-transfer reactions

Li-Mei Kuo [a], Helen C. Davies [a,*] and Lucile Smith [b]

[a] *Department of Microbiology, University of Pennsylvania, School of Medicine, Philadelphia, PA 19104 and* [b] *Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755 (U.S.A.)*

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A monoclonal antibody has been produced to an antigenic site on human cytochrome *c* which includes amino acid number 58 (isoleucine). This area is on the bottom back of the cytochrome, removed from the postulated binding/reaction sites for oxidase and reductase, but in the area of the molecule where an appreciable change in conformation is seen on oxidation-reduction. In spectrophotometric assays, where binding of cytochrome *c* to the oxidase or reductase is rate-limiting, the antibody gave stimulation of the reductase reaction under some conditions, where the oxidase reaction was inhibited. Also variation of the pH of the reaction medium resulted in differential effects on the oxidase and reductase reactions. Different effects of the antibody were seen when the oxidase was assayed polarographically, as compared to the spectrophotometric measurements. The data show that the binding/reaction sites on cytochrome *c* for the oxidase and reductase must be different. They suggest that binding of antibody may affect conformational changes in the whole molecule, distorting the binding/reaction sites. Conformational changes may be involved as a control mechanism in cytochrome *c*-mediated electron-transfer reactions.

Introduction

We have prepared several monoclonal antibodies to human and bovine cytochromes *c*, using methodology described previously [1]. Most of these antibodies showed considerable cross-reactivity (by ELISA methodology) with cytochromes *c* from other eukaryotic species, as expected, since the tertiary structure of all mammalian cytochromes *c* that have been determined are very similar. However, one of the antibodies made to human cytochrome *c* did not cross-react strongly with any of 20 different species of cytochrome *c*, including that from *Macacca mulatta*. Since the monkey cytochrome *c* differs from the human pigment only in replacement of isoleucine-58 by threonine, the antibody must bind in the area around isoleucine-58, which is at the bottom back of horse and tuna cytochromes *c* when looking into the heme crevice as the front of the molecule [2]. Recent work has indicated that it is in this part of the molecule where an appreciable change in conformation is seen on oxidation and reduction of horse cytochrome *c* [2–4]. However, the postulated binding/reaction sites for the oxidase and reductase are on the front at the top of the heme crevice [5].

* To whom correspondence should be sent.

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

We tested the effect of this antibody on the oxidation and reduction of human cytochrome *c* by the cytochrome *c* oxidase and the NADH cytochrome *c* reductase of bovine heart mitochondrial membranes. We saw no complete blocking of the reactions as we have found when an antibody completely occludes the binding/reaction site [6,7]. Spectrophotometric assays were made, where binding of cytochrome *c* to the enzymes is rate-limiting [8,9]. Here some antibody-to-cytochrome *c* ratios resulted in stimulation of the reductase, but resulted in inhibition of the oxidase reaction. Also variations in pH had different effects on the rates of the oxidase and reductase reactions. In polarographic assays of the oxidase (where a cytochrome oxidase-cytochrome *c* complex turns over repeatedly), addition of antibody gave stimulation with ratios that were inhibitory in the spectrophotometric measurements.

The data suggest that binding of the antibody to the area around isoleucine-58 in human cytochrome *c*, possibly by preventing a conformational change (or interconversion of two forms), affects the binding/reaction of cytochrome *c* at the reaction sites, decreasing the binding at the reductase, while increasing that at the oxidase site. The binding/reaction sites on cytochrome *c* for the oxidase and reductase are not identical.

Methods

Preparation and characterization of antibodies

Monoclonal antibodies to bovine and human cytochromes *c* were prepared following our previously described method. Some preparations were used as the total IgG pool; others were those isolated on Affigel columns, as with other antibodies to *c*-type cytochromes [1].

Cross-reactivity of the antibodies with cytochromes *c* from numerous species was tested with ELISA methodology [1].

The affinity constant for binding of the antibody (F16-464.1) to human cytochrome *c* was measured using a fluorescence quenching method described previously [1]. The cytochrome *c* was reduced with $Na_2S_2O_4$ or oxidized with $K_3Fe(CN)_6$ and chromatographed on a G-25 sephadex column.

Cytochromes c

The different cytochromes *c* were obtained from the following sources: those from horse, cattle, chicken, pig, sheep, pigeon, rabbit, rat, tuna, dog heart and yeast were purchased from Sigma Chemical Co.; Dr. M. Reichlin kindly supplied cytochrome *c* from *Macacca mulatta* and cytochromes from fly, guanaco, tobacco moth, snapping turtle, *Samia cynthia*, Pacific lamprey and Pekin duck were gifts from Dr. E. Margoliash and had been stored in the frozen state for several years. We prepared cytochromes from bovine and human heart and from *Paracoccus denitrificans* by the methods of Margoliash and Walasek [10] and Scholes et al. [11], respectively, followed by isoelectric focusing [12].

The concentrations of bovine and human cytochrome *c* were assessed spectrophotometrically, using $A_{550} = 27.6\ mM^{-1}$.

Cytochrome c oxidase and reductase

The source of oxidase and reductase was phosphate-washed submitochondrial particles [13], kindly furnished by Dr. B. Trumpower. These were treated with deoxycholate to expose reaction sites maximally (1 mg deoxycholate per mg protein added to a concentrated suspension, as described in Ref. 14).

Enzymatic assays

Cytochrome *c* oxidase and NADH cytochrome *c* reductase activities were followed spectrophotometrically with deoxycholate-treated bovine submitochondrial particles and human cytochrome *c*, using methods we have described previously [9,15]. The kinetics were followed at 550–540 nm using a turbine-driven, time-sharing multichannel spectrophotometer built by the Johnson foundation, School of Medicine, University of Pennsylvania, Philadelphia, PA. The activities are expressed as the first-order rate constants [9,15]. Except when testing the effects of pH variation, the assays were run in 0.05 M Tris-maleate buffer (pH 7.67) to eliminate any effects which might result from variations of pH or ionic strength. The reductase reactions were followed at concentrations of cytochrome *c* where the reaction was first order in ferri-cytochrome *c* [9]. Under the conditions used,

the binding of cytochrome *c* appears to be rate limiting in assays of both oxidase and reductase [8,9]. When the effect of antibody was tested, it was added to the cytochrome *c* in buffer in the cuvette before addition of the submitochondrial particles.

The oxidase activity was also assayed polarographically in the same buffer, 0.05 M Tris-maleate buffer (pH 7.67) in the presence of 0.75mM TMPD and 10 mM ascorbate, as described previously [16].

Results

Reaction of the antibodies with different cytochromes c

Table I summarizes observations on the reactions of a number of monoclonal antibodies generated by bovine and human cytochromes *c* with cytochromes *c* from many eukaryotic species, as evidenced by ELISA methodology. Several of the antibodies could bind to most of the cytochromes; others showed cross-reactivity with a few, while one made to human cytochrome *c* (F16-464.1) could bind strongly only to this cytochrome. It also showed a weak affinity toward tuna cytochrome *c*. Thus this antibody is unusual among this group in its specificity. Since cytochrome *c* from *Macacca mulatta* differs from the human pigment only at amino acid number 58 (where isoleucine is replaced by threonine), and since this antibody (F16-464.1) does not bind to *Macacca mulatta* cytochrome *c*, the binding site of the antibody must be in the area of this amino acid. In tuna cytochrome *c*, amino acid 58 is valine, which is more similar to isoleucine than is threonine.

Binding of antibody to oxidized and reduced cytochrome c

Fig. 1 plots data from an experiment testing the binding of antibody F16-464.1 to both oxidized and reduced forms of human cytochrome *c*, using the fluorescence quenching methodology. To correct for dilution and attenuation of the fluorescence signal, a normal rabbit IgG was used in control titrations. The plots with the two forms of cytochrome *c* run in 0.05 M Tris-maleate buffer (pH 7.0) appear to be superimposable. The K_a

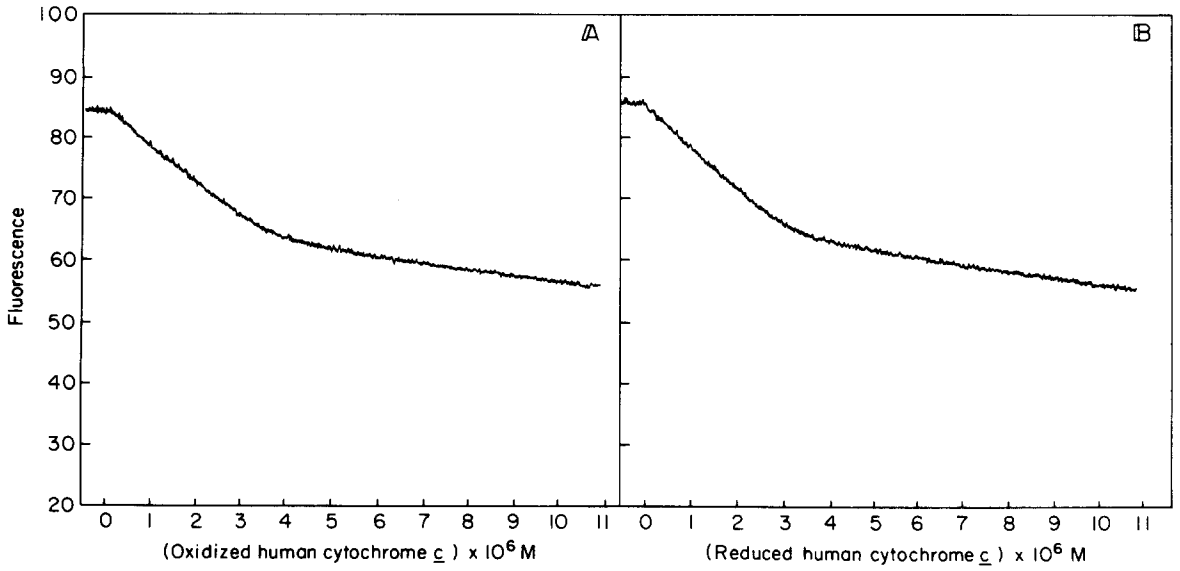

Fig. 1. Fluorescence quenching curves for F16-464.1 monoclonal IgG_1 purified antibody with oxidized (A) and reduced (B) human cytochrome *c*. The excitation wavelength was 295 nm and emission wavelength was 345 nm. Initial antibody site concentration, $1.14 \cdot 10^{-6}$ M; initial cytochrome *c* concentration, $6 \cdot 10^{-5}$ M; buffer 0.05 M Tris-maleate (pH 7.0) with 2 mM EDTA. Fluorescence in arbitrary units.

TABLE I

MONOCLONAL ANTIBODIES

ELISA assays were run as previously described [1]. + + +, $1.5 \leqslant A$; + +, $0.5 \leqslant A < 1.5$; +, $0.1 \leqslant A < 0.5$; −, $A < 0.1$. Species of cytochrome *c* are used as antigen.

Cytochrome *c*	F15-3.5 (Cattle) IGM	F15-62.1 (Cattle) IGM	F15-193.10 (Cattle) IGG	F15-201.95 (Cattle) IGM	F15-383.5 (Cattle) IGM	F15-517.6 (Cattle) IGM	F15-523.7 (Cattle) IGM	F15-589.58 (Cattle) IGM	F16-140.11 (Man) IGC_3	F16-187.2 (Man) IGM	F16-464.1 (Man) IGG_1
Cattle	+ + +	+ + +	+	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	−
Man	+ +	+ + +	−	+ + +	−	−	+ + +	−	+ + +	+ + +	+ + +
Sheep	+ + +	+ + +	+	+ + +	+ + +	+ + +	+ + +	+	+ + +	+ + +	−
Horse	+ + +	+ + +	−	+ + +	−	−	+ + +	−	+ + +	+ + +	−
Guanaco	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	−
Dog	+ + +	−	+ +	+ + +	+ + +	+ + +	−	+ +	+ + +	+ + +	−
Rabbit	+ +	+ + +	−	+ + +	−	−	+ + +	−	+ + +	+ + +	−
Rat	+ + +	+ + +	−	+ + +	−	−	+ + +	−	+ + +	+ + +	−
Chicken	+ + +	−	−	+ + +	−	−	−	−	+ + +	+ + +	−
Pekin duck	+ + +	−	−	+ + +	−	−	−	−	+ + +	+ + +	−
Pigeon	+ + +	−	−	+ + +	−	−	−	−	+ + +	+ + +	−
Snapping turtle	+ + +	−	+ +	+ + +	+ + +	+ + +	−	+ +	+ + +	+ + +	−
Tuna	+ + +	−	−	+ + +	−	−	−	−	−	+ + +	+
Lamprey	+ + +	−	−	+ + +	−	−	−	−	+ + +	+	−
Fly	+ +	−	−	+ + +	−	−	−	−	+	+ + +	−
Moth	+ + +	−	−	−	−	−	−	−	+ +	+ + +	−
Samia cynthia	+ + +	−	−	−	−	−	−	−	+ + +	+ + +	−
Yeast	+ + +	−	−	+	−	−	−	−	+ +	−	−
Pig	+ + +	+ + +	+	+ + +	+ + +	+ + +	+ + +	+ +	+ + +	+ + +	−
Monkey (*M. mulatta*)	+	+ + +	−	+ + +	−		+ + +		+ + +	+ + +	−

values calculated from these plots using the computer program described in Ref. 1 were $1.04 \cdot 10^6$ for the oxidized form and $1.49 \cdot 10^6$ for the reduced form. Thus, there appears to be little or no difference in the binding affinity to the two forms. Similarly, Reichlin et al. [17] observed only a small difference in the binding of horse cytochrome *c* in oxidized and reduced forms to polyclonal antibody made in rabbit to horse cytochrome *c* with the complement fixation technique. Smith et al. [7], however, found no difference in binding of the two forms of horse cytochrome *c* to antihorse antibodies in goat antiserum, measured by fluorescence quenching assays.

Spectrophotometric assays

When the oxidase and reductase were assayed under the same conditions in the presence of increasing antibody-to-cytochrome *c* ratios, there was no complete blocking, but the first-order rate constants of both oxidase and reductase reactions were affected (Fig. 2). In this experiment specific antibody was used. Molar ratios of antibody to cytochrome *c* up to around 0.125 (0.25 antibody sites/cytochrome *c* *) gave appreciable stimulation of the reductase reaction, while inhibiting the oxidase reaction. At a ratio of about one antibody site per cytochrome *c*, 34% of the oxidase activity remained, but 75% of the reductase activity was still present. At all ratios tested there was inhibition of the oxidase, but there was stimulation of the reductase at low ratios, then inhibition at higher ratios.

At a fixed ratio of 2 antibody sites per cytochrome *c*, both oxidase and reductase are inhibited. The extent of inhibition of the oxidase

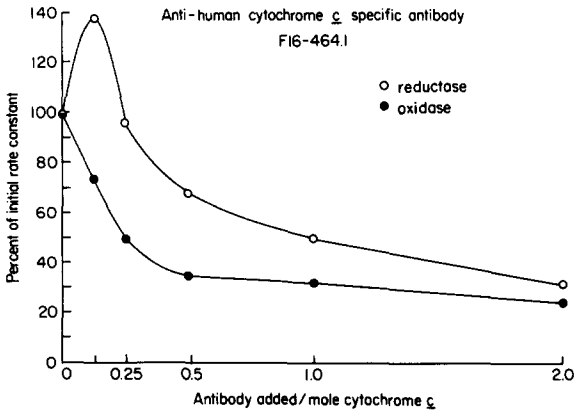

Fig. 2. Effect of antibody to human cytochrome *c* on the oxidase and reductase activity measured spectrophotometrically. The assays were run in 2.8 ml 0.05 M Tris-maleate buffer (pH 7.67)+2 mM EDTA with 0.0533 mg submitochondrial-particle protein and 0.46 μM cytochrome *c*. For the reductase assays 0.36 mM NADH and 1 mM KCN were used. Antibody was a purified sample of F16-464.1.

* The antibodies are divalent.

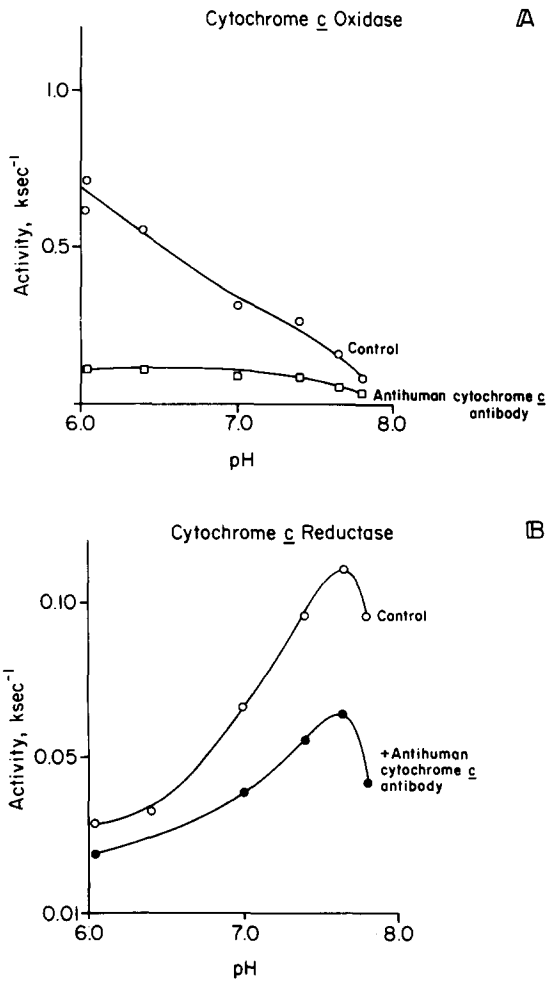

Fig. 3. Effect of pH on the oxidase (A) and reductase (B) activities measured spectrophotometrically in the presence of antibody. The assays were run in 0.05 M Tris-maleate of varying pH with 0.0533 mg of submitochondrial-particle protein and 0.46 μM cytochrome *c* at an antibody concentration of 0.46 μM. In the reductase assays, 0.36 mM NADH and 1 mM KCN were added.

TABLE II

EFFECT OF ANTIBODY TO HUMAN CYTOCHROME *c* ON THE ACTIVITY OF CYTOCHROME *c* OXIDASE ASSAYED POLAROGRAPHICALLY

A deoxycholate-treated submitochondrial-particle preparation containing 1.2 mg protein was assayed in the presence of 0.75 mM TMPD plus 10 mM ascorbate in 0.05 mM Tris-maleate buffer (pH 7.60) containing 2 mM EDTA. Antihuman cytochrome *c* (F16-464.1) antibody from IγG pool was added in two aliquots.

Additions	O_2 uptake ($\mu M \cdot s^{-1}$)
TMPD + ascorbate	0.08
+ Submitochondrial particles with deoxycholate	0.12
+ Human cytochrome *c* (0.7 μM)	0.33
+ Antibody 0.23 Ab/Cyt *c*	0.43
+ Antibody 0.46 Ab/Cyt *c*	0.33

reaction by antibody decreases with increasing pH between 6 and 8, while the inhibitory effect on the reductase increases over this pH range. Actually the effect on the reductase reaction is to decrease the rate constant at all pH values, but to a greater extent at pH 7.67 (Fig. 3A). In contrast, the effect on the oxidase is to decrease the activity at all pH values to a low similar value (Fig. 3B).

Polarographic assays

Oxidase activity was tested polarographically with the same buffer, oxidase preparation, human cytochrome *c* and antibody (F16-464.1) used in the spectrophotometric assays depicted in Fig. 2. In contrast with the observations made spectrophotometrically, a ratio of 0.46 antibody sites per cytochrome *c* gave stimulation of the reaction measured with the O_2 electrode (Table II). Finally, increase of the ratio to 0.92 sites per cytochrome *c* resulted in a decrease of activity back to the control level.

Discussion

Most of the monoclonal antibodies to bovine and human cytochromes *c* showed considerable cross-reactivity with cytochromes *c* from different eukaryotic species, as expected from the observed similarity in structure. However, one antibody to human cytochrome *c* was highly specific in binding strongly only to human cytochrome *c* among a large number tested. The binding area includes amino acid 58, since the antibody does not bind to cytochrome *c* from *Macacca mulatta*, which differs from human cytochrome *c* only in replacement of isoleucine-58 with threonine. Tuna cytochrome *c*, which has another hydrophobic amino acid, valine, in position 58, binds weakly to the antibody. The structural requirements of the molecule in this area appear to be quite specific, and a hydrophobic amino acid at position 58 is important. The adjacent amino acid at position 57 is also isoleucine in human cytochrome *c*. We had recognized this site as being antigenic when we isolated an antibody population to the same area on human cytochrome *c* from rabbit immune serum previously [7].

Experiments with X-ray crystallography [2], chemical modification [3] and ^{1}H-NMR spectroscopic measurements [4] indicate that the area of horse cytochrome *c* around isoleucine residue-57 is an area most affected by conformational changes on oxidation-reduction. The observations with ^{1}H-NMR showed this region of the molecule to be relatively flexible in both oxidized and reduced forms [18], and there was a small difference in the structure in the two states [4]. There may actually be small cooperative conformational changes over large distances in the molecule. Or, different conformations of cytochrome *c* may exist in rapid interconversion in solution. Even larger conformational changes may occur in solution than those revealed by methods such as X-ray crystallography of the oxidized and reduced crystals.

The area around isoleucine-58 is at the bottom back of the molecule in horse and tuna cytochromes *c*, looking into the heme crevice as the 'front'. (The 3-dimensional structures of mammalian-type cytochromes *c* are similar). This area is displaced from the postulated binding/reaction site with the oxidase and reductase, on the front at the top of the heme crevice [5], about as far as possible. Thus it is surprising that the antibody (F16-464.1) to the antigenic site at the bottom back had such large effects on the reaction of the cytochrome with bovine heart oxidase and reductase.

The direction and extent of the effects of the antibody depended upon the ratio of antibody to cytochrome *c*, but also upon the assay method utilized. Both oxidase and reductase were assayed with the spectrophotometric method under the same reaction conditions, where the binding of the cytochrome to the oxidase or reductase is rate-limiting [8,9]. The antibody was inhibitory to the oxidase reaction at all ratios of antibody to cytochrome *c* tested. There was no complete blocking, as seen with antibodies covering the reaction site [6], but there was a decrease in rate constant, suggesting that the binding of antibody distorts the oxidase binding site, possibly by preventing a conformation change (or interconversion of two forms) so that the release of the oxidized cytochrome *c* is retarded. Addition of antibody had both stimulatory and inhibitory effects on the reductase reaction. At ratios up to about 0.25 antibody sites per cytochrome *c*, the reductase reaction was stimulated. This could result from more rapid release of reduced cytochrome *c* from the reductase. Higher ratios produced decreased rate constants, following a course similar to that of the oxidase inhibition, as if the predominant effect is that at the oxidase sites. Our previous data suggests that the two sites are nearby [6,19]. Oxidized cytochrome *c* adhering to the oxidase site could inhibit getting more cytochrome *c* to the reductase reaction site.

It could also be argued that the effects of this antibody which binds at the bottom back of the molecule is evidence for the suggestion that cytochrome *c* binds to the oxidase by two points of attachment in a cleft in the oxidase molecule [20,21]. However, this would not readily explain why we observed no complete blocking or why the reductase activity was increased in spectrophotometric assays. There was little or no difference in the binding affinity of the antibody to the bottom back area of the oxidized and reduced forms of the cytochrome *c* (Fig. 1). The conformational change on oxidation-reduction does not have a large effect in this respect. This means that the different effects of the antibody on the oxidase and reductase reactivities do not result simply from a greater extent of binding of antibody to one of the forms of the cytochrome.

When tested with the spectrophotometric assays, variations of the pH of the reaction medium yielded differential response of the oxidase and reductase reactions in the presence of antibody. The inhibitory effects on the oxidase were greatest around pH 6, while those on the reductase were manifested rather similarly between pH 6 and pH 8. It is known that electrostatic interactions play a part in the reaction of the oxidase and cytochrome *c* [22,23]. The rate of productive encounter at a reaction site may actually depend upon the spatial orientation of all of the charged groups on the cytochrome with respect to the electron-transfer site. In any case, the situation is different with the oxidase and reductase. If the binding/reaction sites on cytochrome *c* for the oxidase and reductase were identical, no differences in pH effect should be apparent. This observation plus those of the preceding paragraph add weight to our previous conclusion that the oxidase and reductase sites on cytochrome *c* are not identical [6,19].

We studied the effects of antibody on the oxidase reaction also with polarographic assays and found some differences from the effects seen using spectrophotometric assays. With ratios of antibody to cytochrome *c* where spectrophotometrically inhibition was observed, polarographically we found stimulation. This would fit the suggestion made above that changes resulting from binding of antibody inhibit release of cytochrome *c* from the oxidase site, since in the polarographic method an oxidase-cytochrome *c* combination appears to turn over repeatedly [5,16]. We have shown that the two kinds of methodology measure different aspects of the oxidase reaction [16]. However, the data of Osheroff et al. [24] show that the reaction of human cytochrome *c* with the bovine oxidase, measured polarographically, may be limited by a low reduction rate by TMPD of the cytochrome *c* bound to the oxidase. It is not clear how this would affect our observations.

We previously worked with an antibody population from rabbit serum (as Fab) to an antigenic site of human cytochrome *c* including isoleucine-58 [7]. This antibody was also found to inhibit the reaction of human cytochrome *c* with bovine oxidase at Fab-to-cytochrome *c* ratios from 0.022 to 0.44, when tested with the spectrophotometric assays. The effect on the reductase was not tested over a range of Fab-to-cytochrome *c* ratios.

Several groups have obtained from rabbit serum an antibody fraction to an antigenic determinant of horse cytochrome *c* around amino acid number 60 (lysine), near the center of the back surface of the molecule [25–27]. With a 20% excess of this antibody (Fab) over cytochrome *c*, Osheroff et al. [28] found a slight inhibition of the oxidase reaction, tested with polarographic assays. (The V_{max} of the high-affinity phase was about 70% of that for free cytochrome *c* and the K_M was unchanged). Actually, the small inhibitory effect could have resulted from a decreased rate of reduction of bound cytochrome *c* by TMPD. Thus the effects of antibody to amino acid-60 of horse and to amino acid-58 of human cytochrome *c* are quite different, emphasizing the significance of the latter area. The effect of antibody to amino acid-60 of horse cytochrome *c* was found to have little effect on the reductase reaction tested spectrophotometrically [28]. Polarographic assays of the reductase under different experimental conditions gave different results from the spectrophotometric measurements; a 2-fold decrease in K_M and a 60% decrease in V_{max} were seen in the presence of antibody.

In summary, a monoclonal antibody to human cytochrome *c* has been produced which binds to the cytochrome in an area at the rear bottom of the molecule, removed from the postulated binding site for the oxidase and reductase, but in the area showing the most significant conformational change on oxidation and reduction. When assayed spectrophotometrically, where binding of the cytochrome to the enzymes is rate-limiting, the antibody inhibits the reaction with the oxidase (decreases the rate constant), possibly by inhibiting the release of the oxidized cytochrome from the oxidase. At certain ratios of antibody to cytochrome *c* the reductase reaction is stimulated, as if the cytochrome *c* is released more readily from the reductase site. It may be that a freezing of the conformational change on binding of the antibody affects the binding/reaction of the cytochrome *c* with the oxidase and reductase. Local changes in conformation on oxidation-reduction may be involved as a control mechanism in the cytochrome *c*-mediated electron shuttle between reductase and oxidase.

Acknowledgements

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