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STUDIES ON A CYTOCHROME OXIDASE ANTIBODY

II. INHIBITION KINETICS

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SUMMARY

1. The effects of a cytochrome oxidase antibody on the reactivity of a solubilized and particulate (*i.e.* Keilin–Hartree heart muscle particles) preparation of cytochrome oxidase have been examined.

2. With both the soluble and particulate preparations of the oxidase, the antibody was shown to be an effective inhibitor of the oxidase reaction with cytochrome *c*.

3. The antibody was found to be a competitive inhibitor with respect to cytochrome *c* of the soluble oxidase whereas with the oxidase of the Keilin–Hartree particles, non-competitive kinetics were observed.

4. The influence of the antibody on the reactivity of the soluble and particulate oxidase towards N_3^- and cytochrome *c* has also been examined.

5. The results are considered in terms of the reactivity of the oxidase *in situ*.

INTRODUCTION

In the preceding paper[1] we presented evidence showing that a highly purified solubilized cytochrome oxidase is capable of eliciting an immunological response in rabbits.

The present paper shows that the antibody so prepared, is an effective inhibitor of the reaction of cytochrome *c* with both a solubilized preparation of the oxidase as well as the oxidase present in Keilin–Hartree heart muscle particles. We have therefore examined the inhibition of both oxidase preparations in an attempt to better understand the reactivity of the oxidase in a biological membrane system.

MATERIALS AND METHODS

The antibody to soluble cytochrome oxidase was prepared and characterized as described in the preceding paper[1]. The turnover number (molecular activity) at

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylene diamine.

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infinite cytochrome *c* concentration of the oxidase preparation at the time of injection into the rabbits was 81 sec^{-1}. Normal rabbit serum (Pel Freez, Type II or preinjection bleedings) and the anti-cytochrome oxidase sera were decomplemented and clarified as described in the preceding paper. Oxyhemoglobin present in the serum was oxidized to methemoglobin by the addition of $K_3Fe(CN)_6$ and the serum exhaustively dialyzed for 24 h against several changes of 0.06 M sodium–potassium phosphate buffer, pH 7.4. Soluble cytochrome oxidase (Prep. 2 of preceding paper) was prepared by the method of YONETANI[2]. Keilin–Hartree heart muscle particles were prepared by the blender modification[3] of the original preparation of KEILIN AND HARTREE[4]. Cytochrome *c* (Sigma Chemical Co., Type III) was reduced by the anaerobic gel filtration method of YONETANI[2].

Cytochrome oxidase was assayed spectrophotometrically using a recording spectrophotometer (Cary 11, Cary Instruments, Varian Industries) by following the rate of aerobic oxidation of ferrocytochrome *c* by cytochrome oxidase at 550 mμ according to SMITH AND CONRAD[5]. The reactions were initiated by rapidly mixing 5 μl of a 1–2 μM (heme *a*) solution of cytochrome oxidase into a 1.0-ml cuvette containing varying quantities of ferrocytochrome *c*. The apparent first-order rate constants were calculated from the slope of a line extrapolated to zero time on the plot of the logarithm of the change in absorbance *versus* time. The initial velocities were either calculated from the product of the first-order rate constant and cytochrome *c* concentrations or directly determined from a tangent drawn on the extended time-course curve of the reaction at zero time.

Kinetic parameters, v_{max} and K_m, were determined according to the procedures of LINEWEAVER AND BURK[6]. The turnover number was obtained by dividing the v_{max} at infinite ferrocytochrome *c* concentration by the enzyme concentration.

The initial velocities in the inhibitor studies, were obtained in the same manner as above with the inclusion of various concentrations of inhibitor in the reaction vessel prior to the initiation of the reaction with cytochrome oxidase. The K_i was either calculated from the Lineweaver–Burk plots or directly estimated from DIXON plots[7] of the reciprocal of the initial velocity *versus* concentration of inhibitor.

Cytochrome oxidase was also measured polarographically by following the rate of utilization of O_2. The general procedure followed for the assay, was injection of the substrate and enzyme into a closed reaction vessel (2.5-ml capacity) containing a Clark oxygen electrode (Yellow Springs Instruments Co.) and constantly stirred with a micro magnetic stirring bar and apparatus. The rate of autoxidation of cytochrome oxidase in the presence of the electron donors, sodium ascorbate and TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine·2 HCl) was measured and the reaction was then initiated by injection of cytochrome *c*. The velocity was calculated from the slope of the polarographic trace recording the time-course of O_2 consumption. The rate of oxidation in the absence of cytochrome *c* was subtracted from the final rate. Activities were expressed as turnover number (molecular activity) which represents the number of electron equivalents utilized per mole of cytochrome oxidase per second. Cytochrome oxidase concentration was represented in terms of heme *a*, employing a difference extinction coefficient at 605–630 mμ of 11 $mM^{-1} \cdot cm^{-1}$ (ref. 8). The temperature was controlled by a constant temperature bath at 26°.

The turnover number, K_m and K_i were obtained as described above. When

inhibitors were used, the inhibitor was injected into the reaction vessel prior to addition of any of the reactants.

Cytochrome oxidase activity was also assayed spectrophotometrically using a Cary 11 spectrophotometer by measuring the time of the aerobic steady state[9] with 20–30 μM cytochrome *c* at 550 mμ in the presence of 10 mM sodium ascorbate. The activity of the endogenous components in the respiratory chain was followed in a similar fashion using 15 mM sodium succinate at various wavelength pairs, as described by CHANCE[10], in a dual wavelength spectrophotometer (Aminco-Chance). In either case the baseline was the oxidized preparation, and calculations of percentage reduction were determined using 100% as the difference between the baseline and the final anaerobic state.

Complement fixation by the serial dilution technique was performed qualitatively according to the procedure of KABAT AND MAYER[11], and quantitative complement fixation by the method of WASSERMAN AND LEVINE[12]. Anti-sheep hemolysin and guinea-pig complement were obtained from Grand Island Biological Co.

RESULTS

Antibody inhibition of soluble cytochrome oxidase

Fig. 1 illustrates the time-course curves for the oxidation of ferrocytochrome *c* by cytochrome oxidase in the presence of an antibody specific for cytochrome oxidase. It can be seen that cytochrome *c* oxidation in the presence and absence of antibody

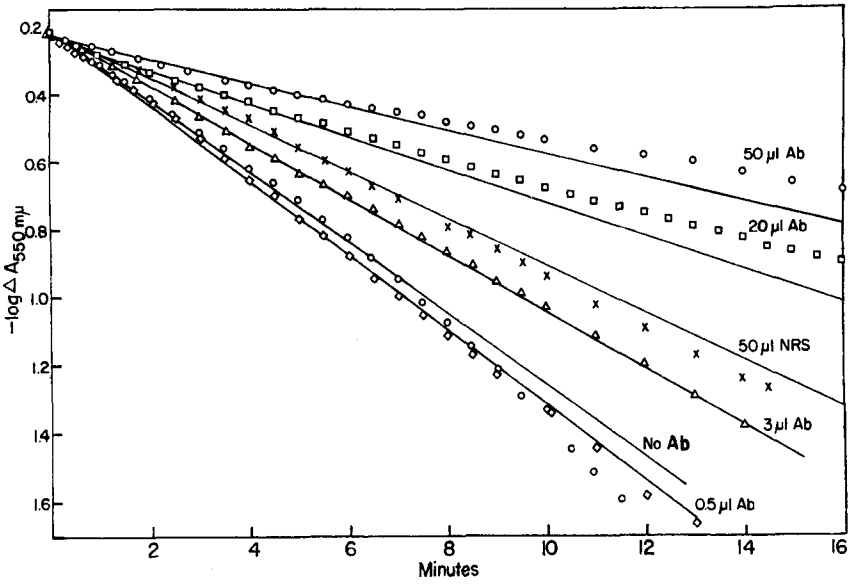

Fig. 1. Time-course for the oxidation of cytochrome *c* by soluble cytochrome oxidase in the presence of anti-cytochrome oxidase serum. The oxidation of ferrocytochrome *c* was followed by recording the change in absorbance at 550 mμ upon the addition of 7.4 nM (heme *a*) cytochrome oxidase to a solution containing the indicated volume of antiserum (Ab), or normal rabbit serum (NRS), and 30 μM ferrocytochrome *c* in 0.05 M potassium phosphate buffer + 1 mM EDTA + 0.1% Tween-80 (pH 7.4); 25°. The reaction volume was 1 ml. The antiserum (No. 127-i) was obtained from a single bleeding. Both sera were decomplemented and clarified. 1 μl of antiserum corresponds to approx. 1–10 pmoles antibody (*cf.* ref. 1).

follows first-order kinetics for the major portion of the reaction, and that the apparent first-order rate constant decreases with increasing antibody concentration. The slight upward deviations in the time-courses observed at high antibody concentrations, possibly reflect secondary interactions between antibody–oxidase complexes. Normal rabbit serum, used as a control, showed only a slight inhibition at high concentrations.

Dixon and Lineweaver–Burk plots of the initial velocities of cytochrome *c* oxidation plotted against antibody concentration are illustrated in Figs. 2A and 2B, respectively. Lines drawn through the points in Fig. 2A (Dixon plot) intersect above the X-axis at a distance on the Y-axis equal to the $1/v_{max}$ of the uninhibited reaction. This pattern clearly indicates a competitive type of inhibition with an apparent K_i

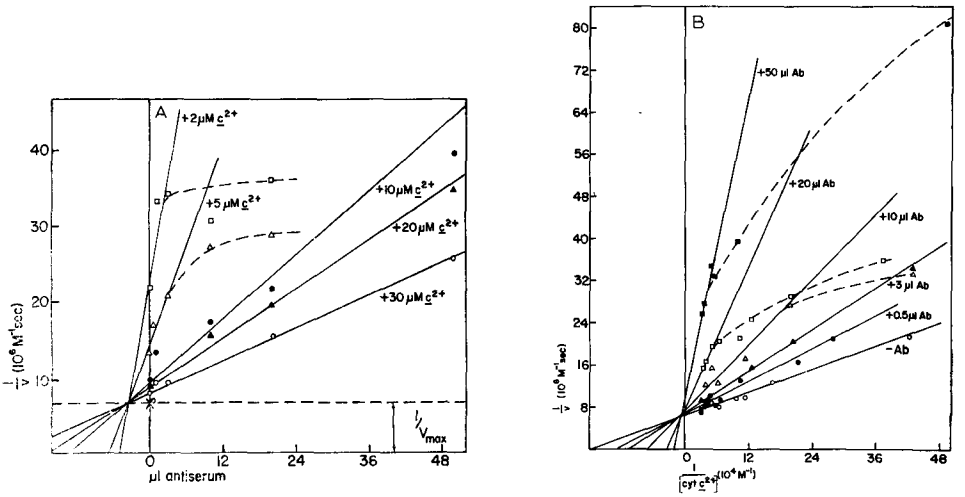

Fig. 2. Velocity plots of the oxidation of cytochrome *c* by cytochrome oxidase in the presence of anti-cytochrome oxidase serum. A. Dixon plot. Initial velocities (v) were determined by multiplying the observed first-order rate constant (obtained from slopes of plots as in Fig. 1) by the initial concentration of ferrocytochrome *c*. The conditions were the same as described in Fig. 1. B. Lineweaver–Burk plot. The conditions were the same as in A and Fig. 1.

of 3.6 μl per ml antiserum (1 μl antiserum approximates 1–10 pmoles antibody[1]). In similar experiments using normal rabbit serum, an apparent K_i of 37.5 μl per ml serum was obtained. This shows that the control serum is a weak inhibitor in comparison with the antiserum. The inhibition appears to reach a maximum level of approx. 50–60 % at high antibody and low cytochrome *c* concentrations. The deviations observed are not due to a general serum effect because a similar type of inhibition pattern is obtained when the initial velocities are corrected for serum inhibition. Neither are the deviations caused by antibody-induced precipitation during the initial course of the reaction, for linear time-courses of cytochrome *c* oxidation are observed (*cf.* Fig. 1). The deviations then appear to reflect a true effect of the antibody on the oxidase. The reason that total inhibition is not reached may be that a portion of the oxidase population is in a high aggregation state and therefore is unaffected by the inhibiting antibody.

Lineweaver–Burk plots of initial velocities at varying antibody concentrations are illustrated in Fig. 2B. When data are represented in this fashion, the deviations from linearity described in Fig. 2A at low concentrations of ferrocytochrome *c* in the

presence of high antibody concentrations become more evident. At high concentrations of ferrocytochrome *c*, the points were linear in the Lineweaver–Burk plots as well as in the Dixon plots. When lines were drawn through the experimental points, favoring the higher concentrations of ferrocytochrome *c*, they were found to intersect in a fashion indicative of a competitive-type inhibition.

Antibody inhibition of cytochrome oxidase was also followed polarographically employing ascorbate and TMPD, under conditions of constant mixing. A competitive inhibition pattern with respect to cytochrome *c* was once again observed, as well as deviations from linearity in both the Dixon and Lineweaver–Burk plots at high antibody and low cytochrome *c* concentrations. The apparent K_i value was 25 μl per ml antiserum. Thus both the polarographic and spectrophotometric data are in good agreement (*cf.* Table IV and DISCUSSION).

Antibody reaction with particulate cytochrome oxidase

In addition to reacting with the solubilized oxidase, the antibody is also capable of reacting with a particulate preparation of the oxidase. This is demonstrated in the qualitative complement fixation experiment using Keilin–Hartree heart muscle particles and solubilized oxidase listed in Table I. When antigen (heart muscle particles or soluble oxidase) was added in a constant amount to various dilutions of antiserum, the end-point of the titration (1/64 dilution) which gave total hemolysis was the same for both heart muscle particles and soluble oxidase (Table IA). When the concentrations of antigens were varied and the antiserum was held constant (Table IB), the end point with the soluble oxidase (0.057 μM heme *a*) was in close agreement with the end point for the particulate system (0.05 μM heme *a*). These results indicate

TABLE I

COMPARISON OF THE REACTIONS OF HEART MUSCLE PARTICLES AND SOLUBLE OXIDASE BY SERIAL DILUTION COMPLEMENT FIXATION TEST

All dilutions were in triethanolamine buffer (pH 7.3) containing 5 mM $MgCl_2$, $I = 0.15$. Readings were taken at 30 min. Soluble oxidase: cytochrome oxidase, beef Prep. 2, 5.9 μM (heme *a*). Beef heart muscle particles (HMP), 6.0 μM (heme *a*). Normal rabbit serum (NRS); preinoculation-bleeding of rabbit 127. 127-i: (A) trial bleeding i of rabbit 127; (B) trial bleeding i of rabbit 127 diluted 1:20. H, hemolysis; NH, no hemolysis; PH, partial hemolysis.

Dilution of serum	(*A*) *Constant antigen*				(*B*) *Constant antiserum*					
	Soluble oxidase		*HMP*		*Soluble oxidase*			*HMP*		
	NRS	*127-i*	*NRS*	*127-i*	μM (*heme a*)	*NRS*	*127-i*	μM (*heme a*)	*NRS*	*127-i*
1:4	H	NH	PH	NH	1.84	H	NH	—	—	—
1:8	H	NH	PH	NH	0.92	H	NH	0.81	H	NH
1:16	H	NH	H	NH	0.46	H	NH	0.405	H	NH
1:32	H	NH	H	NH	0.23	H	NH	0.202	H	NH
1:64	H	PH	H	PH	0.115	H	NH	0.101	H	NH
1:128	H	H	H	H	0.0575	H	PH	0.0505	H	PH
1:256	H	H	H	H	0.0288	H	H	0.0252	H	H
1:512	H	H	H	H	0.0144	H	H	0.0126	H	H
1:1024	H	H	H	H	0.0072	H	H	0.0063	H	H
1:2048	H	H	H	H	0.0036	H	H	0.0031	H	H
1:4096	H	H	H	H	0.0018	H	H	0.00155	H	H
					0.0009	H	H	0.00077	H	H
					—	—	—	0.00039	H	H

that the antibody is reacting with the same antigen. Further, it would appear that the antibody binding site on the oxidase in the Keilin–Hartree particles is as accessible as in the soluble preparation.

Antibody inhibition of particulate cytochrome oxidase

As with the solubilized oxidase, the antibody can also inhibit the particulate oxidase in its reaction with cytochrome *c*. Fig. 3 illustrates the antibody inhibition effect on cytochrome oxidase activity of Keilin–Hartree particles measured polarographically using the ascorbate–TMPD system in the presence and absence of added cytochrome *c*. In the absence of antibody, the turnover number and K_m (obtained from Lineweaver–Burk plots at varying cytochrome *c* concentrations) for the particles were 91 sec^{-1} and 6.7 μM cytochrome *c*, respectively. This figure shows that the antibody can inhibit the oxidase reaction with both endogenous (*i.e.* that present in the Keilin–Hartree particles) and exogenous (*i.e.* added) cytochrome *c*; the inhibition being most marked with the latter. It can also be seen here that normal rabbit serum shows a slight stimulation of the exogenous cytochrome *c* oxidase activity followed by inhibition which levels off after approx. 70 μl of control serum are added. The normal rabbit serum had a similar effect on the reaction of endogenous cytochrome *c* with the oxidase.

A Dixon plot of the antibody inhibition at two concentrations of cytochrome *c* and varying concentrations of serum is present in Fig. 4. In contrast to the competitive-type inhibition pattern observed with soluble cytochrome oxidase, the particulate oxidase appeared to show a non-competitive-type inhibition. The apparent

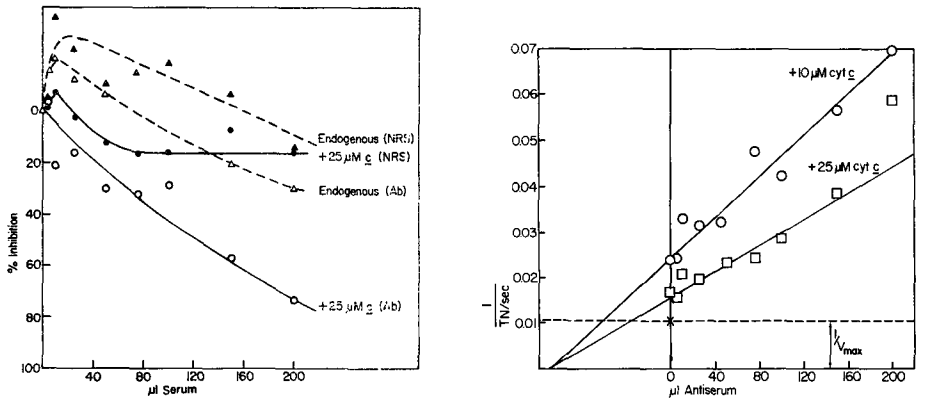

Fig. 3. Oxidation by particulate cytochrome oxidase in the presence of antibody. The rate of O_2 uptake was measured polarographically in the presence of 10 mM sodium ascorbate and 1 mM TMPD in 0.06 M sodium–potassium phosphate buffer + 1 mM EDTA (pH 7.4) at 26°. The heart muscle particles (50 nM heme *a*) were added to the system of antiserum (No. 127-final bleeding) containing in 1 μl serum approximates 1–10 pmoles antibody (*cf.* ref. 1), or normal rabbit serum and ascorbate *plus* TMPD. The rate of oxidation with endogenous cytochrome *c* was measured for approx. 1 min prior to the addition of cytochrome *c* (25 μM). The velocities with exogenous cytochrome *c* were corrected for the contribution of endogenous cytochrome *c* by subtracting the rate in the absence of exogenous cytochrome *c*. The endogenous rate was corrected for the autoxidation of the ascorbate + TMPD system. The sera were treated as described in MATERIALS AND METHODS. NRS, normal rabbit serum.

Fig. 4. Dixon plot for the inhibition of particulate cytochrome oxidase by antibody to soluble cytochrome oxidase. The conditions were the same as in Fig. 3 with the exception that exogenous cytochrome *c* concentrations were 10 and 25 μM. TN, turnover number.

K_i from the Dixon plot of 43 μl per ml antiserum is in close agreement with that obtained from the concentration of antiserum required to give 50 % inhibition (Fig. 3). The antibody appears to be a less effective inhibitor with the particulate oxidase. The soluble oxidase gave a K_i apparent of approx. 10 μl per ml antiserum (*cf.* Fig. 2 and Table IV) when assayed under similar conditions.

TABLE II

COMPARISON OF EFFECTS OF ANTIBODY ON FRESHLY PREPARED AND AGED HEART MUSCLE PARTICLES

Antiserum (R127-i; Ab) and normal rabbit serum (NRS) were incubated with heart muscle particles (1:1 (v/v) of undiluted serum with the original preparation diluted to 20 μM (heme *a*)). The particulate–serum mixture (0.63 μM heme *a*, final concn.) was added to a cuvette containing 34.5 μM cytochrome *c* in 0.06 M sodium–potassium phosphate buffer + 1 mM EDTA (pH 7.4) at 25°. The reaction was initiated with 10 mM sodium ascorbate.

Conditions	*Anaerobiosis time (sec)*	*Cytochrome c^{2+} in steady state (%)*
New particles		
− Ab	63	75.7
+ NRS	68	75
+ Ab	85	80.2
Aged particles (about 10 weeks)		
− Ab	40	66.6
+ NRS	43	69.2
+ Ab	76	81.7

TABLE III

EFFECT OF ANTIBODY ON THE STEADY STATE OF ENDOGENOUS CYTOCHROME *c* IN HEART MUSCLE PARTICLES

The duration and percentage reduction of endogenous cytochrome *c* was measured in the steady state with a dual-beam spectrophotometer at the wavelength pair 550 and 540 mμ. Cytochrome oxidase was measured at 605 and 630 mμ. Sera were decomplemented and clarified. R127-i (Ab), normal rabbit serum (NRS) or reaction buffer was incubated (1 μl serum: 1 μl of 20 μM (heme *a*) heart muscle particles) for 1 h prior to measurement of absorbance changes. The serum–particulate oxidase (0.33 μM heme *a*) was placed in a cuvette containing 0.06 M sodium–potassium phosphate buffer (pH 7.4) + 1 mM EDTA at 25°. The reaction was initiated with 15 mM sodium succinate.

Conditions	*Anaerobiosis time (sec)*	*Reduced (%)*
Expt. 1: 605–630 mμ		
Buffer	144	37
+ NRS	150	43
+ Ab	480	43
+ Ab	472	43
Expt. 2: 550–540 mμ		
Buffer	175	23
+ NRS	202	20
+ Ab	503	15

Steady state analysis of antibody inhibition of particulate oxidase

The antibody–oxidase interaction also appears, under certain conditions, to be dependent upon the age of particles. This is shown in Table II where the anaerobiosis times and steady-state reduction levels for the reaction of exogenous cytochrome *c* with freshly prepared and aged Keilin–Hartree particles are compared. It can be seen that the antibody increases both the anaerobiosis time of the particles and the percent steady-state reduction of cytochrome *c* with both particles, although the inhibition is more marked in the aged particles. This implies that the oxidase may be more accessible in aged particles and is in agreement with similar conclusions made by SMITH AND CAMERINO[15] from their detergent studies with Keilin–Hartree particles.

Table III lists the results of an experiment where the effect of antibody on the reaction of the oxidase with endogenous cytochrome *c* was examined in a dual-wavelength spectrophotometer at the wavelength pairs 550–540 mμ (cytochrome *c*) and 605–630 mμ (cytochrome $a + a_3$) with succinate as substrate. In agreement with above polarographic studies, an inhibition of the endogenous cytochrome *c* oxidase reaction was observed as indicated by the increased anaerobiosis time when the particles were preincubated with antibody. However, no change in the steady-state reduction level of endogenous cytochrome *c* was evident and which possibly suggests multiple reactive antibody sites on the Keilin–Hartree particles (see DISCUSSION).

Influence of the antibody on the reactivity of soluble and particulate oxidase

Reaction of soluble and particulate oxidase with N_3^-. In the previous paper[1], it was noted that when the antibody combined with cytochrome oxidase, there was little effect on the spectrum of the hemoprotein. This suggested that the antibody site was distinct from the heme or heme-linked groups. It was therefore, of interest to determine whether the antibody influenced the reactivity of the oxidase with ligands such as N_3^- or could inhibit N_3^--induced shifts, in the steady-state spectrum of the oxidase. This study was of further value in assessing the similarities between the soluble and particulate oxidase in their reactivities towards N_3^-.

Fig. 5 illustrates the spectral results of adding N_3^- to the antibody–soluble

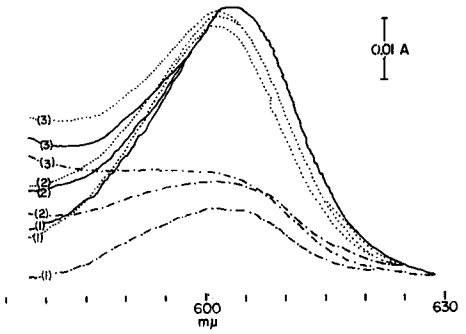

Fig. 5. Absolute absorption spectra of the peak of the antibody–cytochrome oxidase complex in the presence of N_3^-. Cytochrome oxidase and antibody were incubated for 1 h in 3.0 ml of 0.06 M sodium–potassium phosphate buffer + 1 mM EDTA (pH 7.4) at 25°. NaN_3 (5 mM) was added prior to the addition of sodium ascorbate (10 mM) and TMPD (1 mM). The region 630–575 mμ was scanned starting approx. 15 sec after the addition of TMPD. –·—·–, oxidized; ·····, steady state; ———, anaerobic. (1) no antiserum; (2) + 30 μl antiserum (R127-final bleeding); and (3) + 75 μl antiserum. The serum was treated as described in MATERIALS AND METHODS.

TABLE IV

SUMMARY OF KINETIC PARAMETERS FOR ANTIBODY INHIBITION OF SOLUBLE AND PARTICULATE CYTOCHROME OXIDASE

Spectrophotometric assays were performed as described in Figs. 1 and 2 using 7.4 nM (heme *a*) cytochrome oxidase. Polarographic measurements for soluble cytochrome oxidase (100 nM heme *a*) were identical to those used for particulate cytochrome oxidase (50 nM heme *a*) and described in Figs. 3 and 4. The apparent K_i values are represented in units of μl antibody serum per ml of reaction mixture, where 1 μl of undiluted antiserum is approximately equal to 1–10 pmoles antibody (*cf.* ref. 1). K_i values were obtained from Dixon plots. K_m and TN_{max} (turnover number at infinite cytochrome *c* concentration) values were obtained from Lineweaver–Burk plots.

	K_m (μM cytochrome *c*)	TN_{max} (sec^{-1})	*Apparent* K_i ($\mu l/ml$)	*Type of inhibition*
Soluble oxidase				
Spectrophotometric assay	5.5	20.4	3.6	Competitive
Polarographic assay	4.8	16.7	10.0	Competitive
Keilin–Hartree particles				
Polarographic assay	6.7	91	43	Non-competitive

oxidase complex in the steady state with ascorbate and TMPD. The antibody and oxidase were incubated for 1 h prior to addition of N_3^-, ascorbate or TMPD. The observed scattering may be the result of either aggregation of the antibody–cytochrome oxidase complexes or the presence of a slight amount of methemoglobin in the antiserum. In agreement with the observation of others[16,17], a spectral shift with N_3^- was observed from 602 mμ in the steady state to 604 mμ in the anaerobic state. In the presence of antibody, the shift was not altered, although there appeared to be a slight increase in the steady-state reduction level. The latter observation is consistent with the finding (see below) that the antibody increases the affinity of the oxidase for N_3^-.

Similarly the antibody did not prevent the N_3^--induced shift in the Keilin–Hartree particles. In this case, however, no change in the steady-state reduction level of the oxidase was noted (see below). It should also be noted that the antibody alone had no appreciable effect on the steady-state spectrum of either the soluble or particulate oxidase.

A more sensitive method of detecting an effect of antibody on the oxidase reactivity with N_3^- was to measure the apparent affinity of the oxidase for N_3^-. In this experiment, illustrated in Fig. 6, soluble cytochrome oxidase was preincubated for 1 h with the antibody and the oxidase–antibody mixture was injected into a reaction vessel containing N_3^- *plus* ascorbate (10 mM) and TMPD (1 mM). The reaction was initiated with cytochrome *c*. A Dixon plot of the initial velocities at several concentrations of cytochrome *c* indicated that in the presence of antibody, N_3^- still acted as a non competitive inhibitor with respect to cytochrome *c* concentration. Fig. 6 compares the activities of the oxidase in the presence of N_3^- alone with that in the presence of antibody or normal rabbit serum. The apparent K_i was the value giving 50% inhibition of the oxidase, and for N_3^- in the absence of antibody was approx. 27 μM which is in agreement with the value reported by NICHOLLS AND KIMELBERG[17] (26 μM). The apparent K_i value in the presence of normal rabbit serum

was approx. 35 μM. In the presence of antibody, the affinity for N_3^- appears to be increased since an apparent K_i of approx. 21 μM was obtained. Compared with the normal rabbit serum control, the affinity of the oxidase, in the presence of antibody, for N_3^- has been increased by about 50 %.

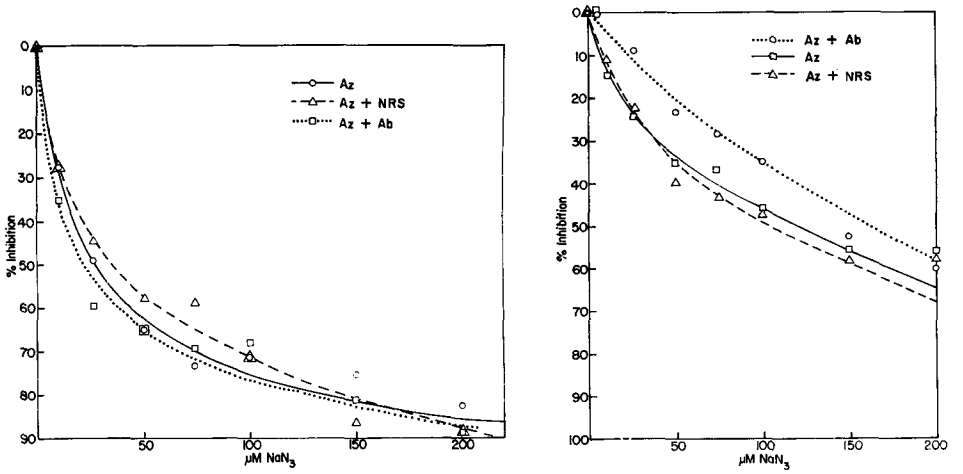

Fig. 6. Inhibition of cytochrome oxidase–antibody complex by NaN_3. Cytochrome oxidase and antiserum (R127-final bleeding) were incubated for 1 h at a ratio of 1 μl antibody (1 μl = 1–10 pmoles of antibody, *cf.* ref. 1) to 1 μl of 25 μM (heme *a*) oxidase. The oxidase–antibody complex (150 nM heme *a*) was added to a 2.5-ml reaction vessel containing 10 mM sodium ascorbate, 1 mM TMPD and NaN_3 in 0.06 M sodium–potassium phosphate buffer *plus* 1 mM EDTA (pH 7.4) at 26°. The reaction was initiated by the addition of cytochrome *c* (25 μM). The rate of autoxidation in the absence of substrate was subtracted from the final rate.

$$\text{Percent inhibition} = 100\ \frac{v\ (\text{Ab}) - v\ (\text{Ab} + \text{Az})}{v\ (\text{Ab})}$$

where v (Ab) = velocity in presence of antibody alone and v (Ab + Az) = velocity in presence of antibody and N_3^-. The percent inhibition for normal rabbit serum (NRS) was calculated in the same manner. The apparent K_i was determined from the N_3^- concentration at 50 % inhibition.

Fig. 7. Inhibition of the particulate oxidase–antibody complex by NaN_3. A mixture of heart muscle particles and antiserum (Ab) or normal rabbit serum (NRS) (1:1 (v/v) with original preparation (25 μM heme *a*)) was incubated for 1 h prior to measurement of activity. In the absence of N_3^- the antibody–particulate oxidase complex had an activity of 54 %. The incubated particulate oxidase (0.1 μM heme *a*) was added to a 2.5-ml reaction vessel containing N_3^- and sodium ascorbate (10 μM) + 1 mM TMPD, in 0.06 M sodium–potassium phosphate buffer + 1 mM EDTA; pH 7.4 at 26°. The concentration of cytochrome *c* added was 25.3 μM. The percent inhibition was calculated as in Fig. 6. Sera were treated as described in MATERIALS AND METHODS.

Similar N_3^- experiments with the particulate heart muscle oxidase preincubated with antiserum are illustrated in Fig. 7. Dixon plots varying N_3^- at several cytochrome *c* concentrations indicated no change in the non-competitive kinetics displayed by N_3^-. As can be seen the particulate oxidase like the soluble oxidase is slightly inhibited by normal rabbit serum. The apparent K_i for the control was 120 μM N_3^- compared to 105 μM N_3^- in the presence of normal rabbit serum. The apparent K_i in the presence of antibody was increased to 162 μM N_3^-, an increase in K_i value of approx. 57 μM N_3^- from normal rabbit serum and thereby indicating a decrease in apparent affinity. This result is in contrast to that of the soluble oxidase in which

the affinity for N_3^- was increased from 35 μM for normal rabbit serum to 21 μM N_3^- in the presence of antibody.

Influence of cytochrome c on the antibody–cytochrome oxidase interaction. Both kinetic[18,19] and physical[20] evidence have indicated that cytochrome *c* forms a complex with cytochrome oxidase. The antibody was therefore employed as a possible alternative method for detecting the existence of such a complex.

A slight modification of the Wasserman–Levine quantitative complement fixation reaction for the antibody in the presence of cytochrome *c* was employed. Cytochrome *c* was incorporated into the various antigen dilutions and allowed to reach equilibrium (after 30 min), prior to the addition of antibody and complement. Assuming that the K_m (5 μM ferrocytochrome *c* under these conditions) of cytochrome *c* for cytochrome oxidase is approximately equal to the dissociation constant for the cytochrome *c*–cytochrome oxidase complex, approx. 90 % of the oxidase would be in the complexed form at this concentration of cytochrome *c*. Since fixation of complement is dependent upon antigen–antibody complex formation, at constant antigen and complement concentration, the amount of complement fixed is proportional to the concentration of antibody forming such complexes. Any change in the concentration of antibody–antigen complex present would be reflected by a difference in the amount of complement fixed. As can be seen in Fig. 8, inclusion of the substrate into the complement fixation experiment does alter the response curves for both

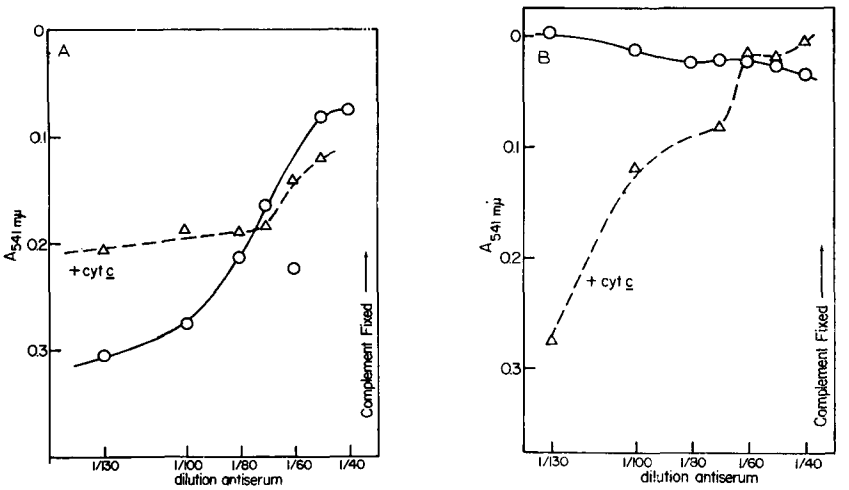

Fig. 8. Influence of cytochrome *c* on the complemented fixation reaction with soluble and particulate cytochrome oxidase. Cytochrome *c* and cytochrome oxidase, both soluble and particulate, were equilibrated 0.5 h prior to addition of 0.1 ml of antiserum dilution, R127-i (or normal rabbit serum) and 0.1 ml of guinea-pig complement (1:20 dilution) to a final volume of 0.3 ml. A volume of 75 μl was removed from each sample and assayed in a reaction mixture containing 100 μl of sensitized red blood cells and 0.525 ml of triethanolamine buffer–5 mM $MgCl_2$ (pH 7.3). The tubes were incubated at 37° for 1 h and the absorbance at 541 mμ was read after sedimentation of the intact red cells at 3000 × *g* for 10 min. An absorbance of 0.35 approximates 50 % hemolysis. Absorbance values for the antisera were corrected for the effect of normal rabbit serum. A. Complement fixation reaction with soluble cytochrome oxidase (0.39 μM heme *a*). ○—○, no cytochrome *c*; △--△, + 140 μM cytochrome *c*. B. Heart muscle particles were approx. 0.4 μM with respect to heme *a*. ○—○, no cytochrome *c*; △--△, + 140 μM cytochrome *c*.

soluble and particulate oxidase. This suggests that cytochrome *c* can change the binding of the antibody to the oxidase, and this is particularly evident with the oxidase of heart muscle particles (Fig. 8B). Cytochrome *c* is having a dual effect on the binding of antibody to soluble oxidase (Fig. 8A). At antiserum dilutions greater than 1/80, cytochrome *c* causes a marked increase in complement fixation, but at higher concentrations of antibody, significant inhibition is observed compared to the corresponding control. The effect may be explained by the presence of various populations of antibodies with different specificities. The reaction of some antibodies with soluble oxidase is enchanced by conformational changes induced by enzyme–substrate complex, whereas others, present in lower concentrations are inhibited from binding by the presence of cytochrome *c*.

In the case of particulate oxidase, the high titer antibodies (1/130 antiserum) are readily reactive and result in complete fixation of available complement. This most likely accounts for the observed horizontal response curve for the particulate oxidase in contrast to the normal sigmoidal curve seen with the soluble oxidase. These antibodies are markedly inhibited by cytochrome *c* complex with oxidase. At higher concentrations of antiserum, antibodies of other specificities are reactive and show less inhibition by cytochrome *c*.

Taking into consideration the more preciseness and sensitivity of the quantitative test, these results are consistent with the findings presented in Table I. At a 1/20 dilution of antiserum and 0.4 μM of oxidase (heme *a*), 100 % of complement was fixed (visual end point, Table IB), and similar results were observed in the quantitative test (Figs. 8A and 8B, 1/40 dilution of antiserum). The apparent difference in the antiserum end point (Table IA), may be explained by the difference in the concentration of oxidase (6.0 μM heme *a vs.* 0.4 μM) creating antigen inhibition at high antibody dilutions and the difference in the sensitivity of the two tests.

DISCUSSION

From both the spectrophotometric and polarographic studies presented above, it is clear that the cytochrome oxidase antibody can inhibit the oxidation of ferrocytochrome *c* catalyzed by the oxidase. Complexities obviously involved in studies using whole serum were minimized by measuring the initial reaction velocities without preincubation, comparing the effect of control serum, and clarifying and decomplementing the serum. In addition measurements were performed in the antibody excess region where only soluble complexes exist[21]. To eliminate heterogeneity in the antibody that occurs over the course of immunization, serum was employed from a single bleeding of the same rabbit. It should be noted, however, that chemical heterogeneity probably exists even in this single antibody fraction.

Antibody interaction with soluble oxidase

Employing the spectrophotometric assay[5], it was observed that at high antibody concentration deviations from the normal first-order kinetics of cytochrome *c* oxidation were observed after about 40 % of the reaction had proceeded. Normal rabbit serum showed the opposite effect; the oxidase becoming slightly more active (time-course deviates downward). This indicates that the upward deviations in the time-courses are due to the antibody and not to a non-specific serum effect. The observation

that the antibody becomes more inhibitory as the reaction continues, then, perhaps reflects a progressive increase in secondary interactions between antibody molecules. The initial complex may be (antibody–oxidase) with secondary complexes of the form of $(\text{antibody}_x\text{–oxidase}_y)_z$. These types of interactions, as indicated from the quantitative precipitation study are probably "lattice" formation leading to precipitation.

The Lineweaver–Burk and Dixon plots of initial velocities indicate that the antibody is acting as a competitive inhibitor towards cytochrome *c*. This inhibition pattern can be explained by a combination of the antibody with the catalytic site for cytochrome *c*, or by a block of the catalytic site by overlapping and thus preventing the entry of cytochrome *c*. The latter view of the competitive kinetics found for antibody reactions in general, has been discussed in detail by BRANSTER AND CINADER[22]. They prefer to view the inhibition as a form of steric hindrance of the enzyme in which access to the catalytic site is blocked by the proximity of the antibody or by the antigen–antibody aggregate, as a whole, blocking the accessibility of the site. The "lattice blockage" hypothesis does not seem plausible as an explanation of the competitive kinetics in the present study since the assays were performed without any preincubation of the oxidase and antibody, and only initial velocities were measured when monitoring the antibody inhibition reaction. Although the inhibition pattern has not been examined with the preincubated enzyme, it most likely would give rise to a more complex pattern.

To summarize the inhibition kinetics of the antibody in the simplest fashion, the present data are consistent with the inhibiting antibody either binding at the same site as cytochrome *c* or overlapping the catalytic site and inhibiting the reactions by steric interference. It is not impossible that an antibody could be produced to the catalytic site. However, in view of the fact that several large antibody molecules (mol. wt. 160000, ref. 11) are apparently capable of binding the oxidase[1], inhibition by "steric interference" is favored in the present case. In addition this theory receives indirect support from the phenomenon of "substrate protection" of the enzyme against the reaction with antibody (see review by CINADER[21]). CINADER points out that in general there may be a correlation between the size of the substrate and the inhibitory capacity of the antibody. For example, BRANSTER AND CINADER[22] and CINADER AND LAFFERTY[23] have found that the degree of inhibition of ribonuclease is greater in the presence of large substrates. Observations of this nature might indicate that antibodies in general may inhibit sterically. Therefore, it would not be surprising if cytochrome *c*, a large substrate, could "protect" the oxidase from its antibody reaction as, indeed, the complement fixation study Fig. 8 appears to indicate. An alternative explanation for the substrate protection effect of cytochrome *c*, as well as for the competitive kinetics could be that cytochrome *c* produces a conformational change in the oxidase such that the affinity for the antibody is decreased. Of course a combination of both steric hindrance and conformational change in the oxidase is also possible. Indeed, KING *et al.*[20], on the basis of a study of an isolated cytochrome *c*–cytochrome oxidase complex, have suggested the possibility of such conformational changes occurring.

Some interesting features of the nature of the antibody–oxidase reaction were also observed during the present study. For example, there was no change in the visible absorption spectrum of the precipitated antibody–oxidase complex. In addition, no spectral shifts occurred in the position of the 605-mμ steady-state ab-

sorption peak of the antibody–oxidase complex in the ascorbate–TMPD system. These observations indicate that the antibody neither reacts directly with heme *a* nor produces any dramatic conformational change in the oxidase that would be reflected in the visible absorption spectrum. The idea that there is no interaction between heme *a* and the antibody is supported from a negative qualitative passive hemagglutination reaction with isolated heme *a* (B. S. MOCHAN, unpublished observations). In addition the antibody does not apparently interfere with the N_3^--induced steady-state spectral shift of the oxidase[16,17]. This shift has been interpreted by WILSON[16] as due to a combination of cytochrome a^{2+} with N_3^- whereas NICHOLLS AND KIMELBERG[17] prefer viewing the effect as due to heme–heme interaction *via* a protein conformational change. The lack of prevention of this shift by the antibody cannot be used to distinguish these two theories, but it does suggest that conformational changes, if present, are not very large.

Although the above results indicate that the antibody is not causing a dramatic conformational change in the oxidase, kinetic studies of the N_3^- sensitivity of the antibody–oxidase complex have indicated that there may be small changes. Thus the amount of N_3^- required for 50% inhibition of the complex was slightly lower than that required for the same inhibition of the free enzyme while non-competitive kinetics toward N_3^- were observed in both cases. The simplest interpretation of this finding is that the antibody may be inducing a change in the oxidase necessary for reaction with N_3^-. A similar explanation may be offered for the lowering of the normal steady-state reduction and increase in the N_3^--induced steady-state reduction produced by the antibody in the ascorbate–TMPD system.

Antibody interaction with particulate oxidase

The complement fixation data in Table I have shown that the antibody prepared to a highly purified cytochrome oxidase also reacts with the oxidase present in the Keilin–Hartree submitochondrial particles. On a semi-quantitative basis, both the soluble and particulate oxidase had similar end point titrations with the antibody.

As with the soluble oxidase, the antibody inhibits the reaction with exogenous cytochrome *c* as measured polarographically using ascorbate and TMPD. DIJESO *et al.*[24] and MAHLER *et al.*[25] have reached similar conclusions in their study utilizing beef and yeast cytochrome oxidase antibody, respectively. Further evidence for antibody inhibition in the present study was obtained from the demonstration that both the steady-state level of exogenous cytochrome *c* reduction with ascorbate and the anaerobiosis time were increased in the presence of antibody. Measurements of endogenous cytochrome *c* reduction in the particles with succinate, employing the dual-beam spectrophotometer indicated a lengthened anaerobiosis time when the particles were observed with antibody. No steady-state increase was observed, however, perhaps indicating a more complex inhibition pattern than that observed with exogenous cytochrome *c*. The polarographic measurements of endogenous cytochrome *c* reactivity toward ascorbate–TMPD further support the idea that the antibody may inhibit the reaction of the particulate oxidase with endogenous cytochrome *c*.

Studies on the antibody kinetics have revealed information on the "state" of the oxidase in the particulate system. When the polarographic assay with varying amounts of cytochrome *c* was employed, the antibody exhibited non-competitive kinetics in the particulate system, which contrasts with the competitive kinetics with

cytochrome *c* in the soluble system. Table IV summarizes the inhibition patterns and apparent K_i values for the soluble and particulate oxidase with anti-cytochrome oxidase serum. Thus the antibody is apparently not able to compete with exogenous cytochrome *c* or block the site on the particulate oxidase, suggesting that the site for exogenous cytochrome *c* may be more protected in the particles than in the soluble system. An alternate explanation for the kinetic differences between the soluble and particulate cytochrome oxidase may be that cytochrome *c* is unable to release the antibody inhibition in the particles. In terms of the location of cytochrome oxidase on the inner mitochondrial membrane[26–29], the above kinetic findings are in accord with a model[30,31] favoring an antibody reaction with the oxidase on the outside (*i.e.* M side) of the Keilin–Hartree particles (*i.e.* the inside of the inner mitochondrial membrane). Similar results, favoring accessibility of at least part of the oxidase from either side of the membrane have been reported by RACKER[32].

Besides having an effect on the v_{max} for the oxidation of exogenous cytochrome *c*, the binding of the antibody to the particulate oxidase apparently has other consequences. This was illustrated by the observation that the antibody produced a marked decrease in the apparent affinity of the oxidase for N_3^-. A possible explanation for the effect on v_{max} is that the rate constants comprising this parameter are intimately associated with an intramolecular electron transfer reaction within the oxidase molecule. Since cytochrome oxidase in the particulate system is membrane bound and therefore in a restricted position the binding of a divalent antibody may be envisaged as further restricting any conformational changes in regions of the oxidase molecule linked to the electron transfer involved in cytochrome *c* oxidation. This may be achieved either by adding a "protein halo" around the oxidase or by forming "lattice-type" cross networks between the oxidase molecules within the membrane. Such an inhibition of the intramolecular electron transfer reaction could also explain the antibody inhibition of endogenous cytochrome *c* reactions cited above.

This suggestion concerning the mode of antibody inhibition of the exogenous cytochrome *c* reaction with particulate oxidase perhaps receives more convincing support from studies of the effects of antibody on the affinity for azide. These studies indicated that the binding of the particulate oxidase produced a marked decrease in the apparent affinity of the oxidase for N_3^-. Thus it would appear that the antibody by combining with the oxidase has produced a "conformational state" of the oxidase which has a decreased affinity for N_3^-. In this regard, it is also interesting to note that exogenous cytochrome *c* may be capable of producing a similar conformational change in the oxidase. For complement fixation studies have indicated a change in the affinity of the antibody for particulate oxidase in the presence of cytochrome *c*. It should be noted, however, in this case that it is possible that cytochrome *c* is simply blocking the antigenic site.

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