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Modulation of cytochrome oxidase kinetics by indirect antibody action

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Polyclonal antibodies raised against isolated subunit V from beef heart cytochrome oxidase or against the intact enzyme increase its apparent affinity for the substrate cytochrome *c* at the high-affinity site while diminishing the turnover at that site. At the low-affinity site the major action of both types of antibody is to reduce the apparent affinity for cytochrome *c*. At high ionic strengths the kinetic effect of anti-subunit V is very small although it still binds to the enzyme. The results are interpreted in terms of a model for the enzyme in which antibodies can modulate cytochrome oxidase kinetics by affecting the binding of cytochrome *c*, even if the antibody-binding site is on a subunit not directly involved in substrate binding.

Cytochrome oxidase; Subunit V; Cytochrome *c*; Substrate binding; Antibody; Enzyme inhibition

1. INTRODUCTION

Eukaryotic cytochrome oxidase comprises both mitochondrially synthesized and cytoplasmically synthesized polypeptide subunits (see, e.g. [1]). The mitochondrially encoded subunits appear to contain all the catalytic centres of the enzyme. The nuclear-encoded subunits have not yet been assigned functions, although it is speculated that they may be involved in control of the enzyme by intramitochondrial or cytoplasmic factors [2,3].

Antibodies directed against either the whole enzyme [4,5] or specific subunits [6,8] can, if they modify the catalytic activity of the oxidase, be used to obtain information about the location and functional interrelationships of the several subunits. In 1970 Mochan et al. [4] were able to show that anti-oxidase could bind at a site that interfered with the reaction of the substrate cytochrome *c*. The antibody behaved as a competitive inhibitor towards cytochrome *c* at high concentrations of *c*, but acted as a mixed inhibitor at lower *c* concentrations, resulting in nonlinear Lineweaver-Burk plots. However, the maximal inhibition was only 50–60%. More recently anti-oxidase preparations have been obtained which show increased inhibitory effects [5,8]. Thus, we were able to show that a preparation of anti-oxidase blocked not only the reaction with cytochrome *c*, probably by interaction with the putative cytochrome *c*-binding site on the enzyme's subunit II, but also that with the non-physiological reductant TMPD [9]. This suggested that antibody binding could affect intramolecular electron-transfer events, including those which are presumed to occur between redox centres in subunit I [9,10].

Indirect effects of antibody binding have also been observed using monoclonal antibodies prepared against subunit IV of the oxidase [11]. These monoclonals react specifically with epitopes on the fourth subunit that extend into the matrix phase of the mitochondrion. One monoclonal increases the turnover of the enzyme, while the other decreases turnover. In neither case, however, can the observed effects be interpreted as a simple kinetic effect on one of the enzyme parameters.

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Antibody prepared against the nuclear-encoded subunit V also modulates the kinetics in a characteristic way [12–14]. Not only is the binding dependent upon the presence of reduced cytochrome *c* [12,13], and specific for an epitope exposed on the N (matrix) face of the membrane, but the antibody also affects the catalytic behaviour of the enzyme in an unusual manner [14]. It induces the appearance of biphasicity in the Hofstee plot of $v/[c]$ vs v, mimicking the behaviour usually seen only at low ionic strengths [14]. This effect can therefore be analysed as involving the high-affinity phase of the oxidase reaction; whether it is necessary to postulate other influences of the antibody is uncertain.

To enable these and similar results to be interpreted satisfactorily, it was therefore necessary to extend the previous study to include a range of ionic strengths and to examine the influence of ionic strength on antibody binding and on the effect of anti-subunit V on the enzyme kinetics. At the same time we have compared the action of the specific anti-V antibody with that of the anti-oxidase. We show that antibody binding to a nuclear-encoded subunit which is not directly involved in the formation of the enzyme-substrate complex can inhibit activity by decreasing the rate of product release. Such antibody-induced conformational changes may mimic genuine physiological control mechanisms in cytochrome oxidase acting via the nuclear subunits.

2. MATERIALS AND METHODS

Cytochrome oxidase was prepared from beef heart according to Kuboyama et al. [15]. Anti-subunit V from rabbits was a kind gift from Dr Jo A. Freedman, and had been prepared as described [13]. Its specificity was checked by Western Blot analysis as in [9,14]. Anti-oxidase was prepared from rabbit serum as described [14]. Cytochrome oxidase activity was measured either spectrophotometrically according to Smith and Conrad [16], using an Aminco DW-2 spectrophotometer at the dual-wavelength pair 550–540 nm or polarographically in the presence of ascorbate and TMPD as in [14] using a Clark electrode (Yellow Springs Instrument). Preincubation of enzyme with the indicated amount of antibodies was carried out as described in [14] and the figure legends.

Cytochrome *c* was Sigma type VI from horse heart. It was reduced when necessary with sodium ascorbate, removing the excess reductant on a Sephadex G-25 column. TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride) and sodium ascorbate were also Sigma products. Lauryl-β-D-maltoside was obtained from Calbiochem.

3. RESULTS

Incubation of cytochrome *c* oxidase with antibody prepared against subunit V induces inhibition of the catalytic activity in the isolated enzyme, as previously reported [14]. However, the action is not complete. Fig.1 shows the inhibitory effect obtained using a spectrophotometric assay at varying antibody-antigen ratios and a fixed level of substrate at a low pH to optimize the turnover according to Wilms et al. [17]. An antibody-antigen ratio of 100 achieves 50% inhibition of the reaction (with an apparent K_i equivalent to an Ab/Ag ratio of about 40) and increasing this ratio to 900 has no further effect. The antibody-enzyme complex is therefore catalytically active. However, the degree of inhibition is significantly greater than that of other polyclonal anti-subunit V preparations assayed under identical conditions [12]. As different preparations also show distinctly different antigen binding characteristics [13] it is clear that subunit V must expose more than one antigenic determinant in the holoenzyme.

The titration with antibody illustrated in fig.1 was carried out at a single substrate concentration. However, we have previously shown that at pH 7.4

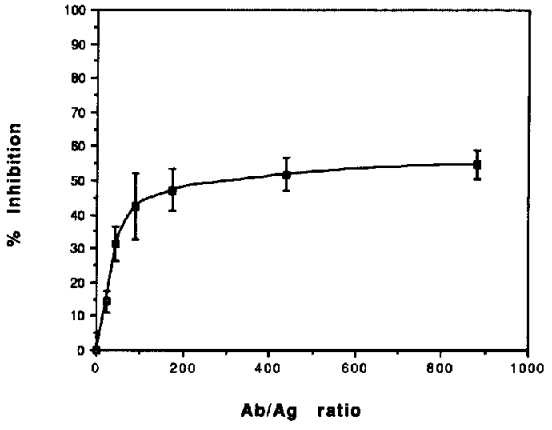

Fig.1. Anti-subunit V γ-globulin as a partial inhibitor of cytochrome oxidase: spectrophotometric asay. The response of the enzyme to varying amounts of antibody was monitored spectrophotometrically by following cytochrome *c* oxidation at the indicated Ab/Ag ratios. The medium contained 27 μM ferrocytochrome *c* in 100 mM potassium phosphate buffer (pH 6.0) containing 0.5 mM EDTA and 0.05% Tween-80. The reaction induced by addition of 5.4 nM cytochrome aa_3 was monitored at 550–540 nm. Error bars are ± 1 SD ($n=3$).

increasing the Ab/Ag ratio from 250:1 to 500:1 has no effect at any cytochrome *c* concentration [14]. At saturating concentrations of cytochrome *c* the system presumably contains a triple complex of antibody-enzyme-cyt.*c*, which is turning over at half the rate of the usual enzyme-cyt.*c* complex. This may reflect the rate of either a decrease in product dissociation or an internal electron-transfer step, or a combination of both. Whichever process is modified, it is seen both at high [14] and low (fig.1) pH, and thus over a substantial range of activities [17].

When the ionic strength is reduced to 5 mM phosphate and the assay is carried out polarographically the control enzyme shows the characteristic biphasic behaviour towards cytochrome *c* concentration (fig.2A). This behaviour has been interpreted in terms of 'high- & low-affinity' binding sites for the substrate [18,19] but it may be noted the phenomenon is also capable of several other types of interpretation [20,21]. Fig.2A shows that upon addition of anti-subunit V, the enzyme is always inhibited, but that the pattern of inhibition differs in the two phases of the plot. In the 'high-affinity' region the action of the antibody appears to be non-competitive, reducing V_{max} without affecting K_m; in the 'low-affinity' region its action is competitive (or 'pseudo-competitive'; cf. [14,22]).

Fig.2B shows the corresponding Hofstee plots for the inhibition both by anti-subunit V and by an anti-oxidase preparation obtained at much higher ionic strengths. Under these conditions using the polarographic assay the anti-V antibody has almost no inhibitory action (fig.2B: note coincident points for the upper curve). Anti-oxidase, on the other hand, remains an effective inhibitor at these high ionic strengths, and also shows a tendency to induce biphasic patterns of response to cytochrome *c*. The results shown in fig.2A,B for anti-subunit V are consistent with the previous report [14] that the inhibitor induced the appearance of biphasicity in the Hofstee plots under conditions of intermediate ionic strength (50 mM phosphate) in which the control enzyme behaves monophasically (see fig.5 of [14]).

The failure of the anti-subunit V antibody to secure appreciable inhibition at ionic strengths of 150 mM phosphate or above is not due to a failure of this antibody to bind at those ionic strengths.

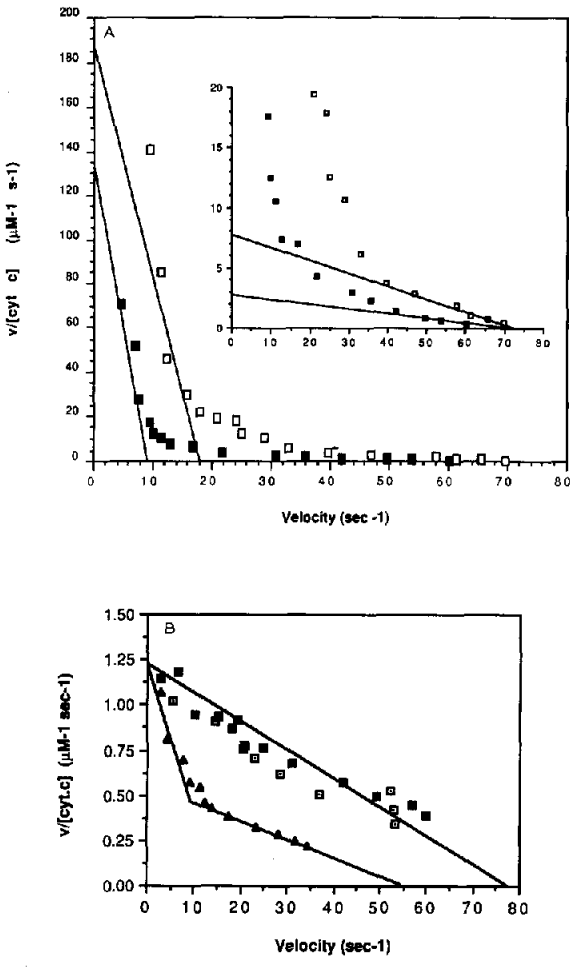

Fig.2. Anti-subunit V γ-globulin as an inhibitor of cytochrome oxidase at low and high ionic strengths: polarographic assay. (A) Eadie-Hofstee plot of the activity of cytochrome oxidase in the presence and absence of anti-subunit V: low ionic strength conditions. Cytochrome *c* concentrations were varied from 67 nM to 0.09 mM in a medium containing 5 mM potassium phosphate (pH 7.4), 0.1% lauryl maltoside, 5 mM sodium ascorbate, 0.2 mM TMPD and 25 nM cytochrome aa_3, preincubated in the presence (■) and absence (□) of anti-subunit V antibody (Ab/Ag ratio = 200:1). 30°C (Inset) Expanded plot of the low-affinity region. The lines drawn represent approximate asymptotes for the low- and high-affinity regions of activity. (B) Eadie-Hofstee plot of the activity of cytochrome oxidase in the presence of anti-subunit V and anti-oxidase: high ionic strength conditions. The reaction medium was 150 mM potassium phosphate (pH 7.4) containing 0.1% lauryl maltoside and cytochrome *c* concentrations up to 0.15 mM. Other conditions as in A. Control preincubations were carried out in the absence of antibody (□). The Ab/Ag ratio for anti-subunit V antibody was 200:1 (■); and for anti-oxidase, 75:1 (▲).

This can be seen by comparing the inhibitions seen when the enzyme is incubated with antibody at different ionic strengths but assayed at a common (50 mM) ionic strength (table 1). Such a method can be employed because the binding and release of antibody take time [14] and in consequence the initial level of inhibition seen in the assay reflects the incubation rather than the assay conditions. Approx. 20% inhibition is obtained in each case. ELISA assays carried out at comparatively high ionic strengths (cf.[13]) also do not show evidence of appreciable salt-induced dissociation of the enzyme-antibody complex (Freedman, personal communication).

This behaviour towards the enzyme of the antibody raised against subunit V contrasts with the behaviour of the anti-oxidase. The latter affects both V_{max} and K_m when tested at high (fig.2B) and at low ionic strengths (not shown). This polyclonal preparation of anti-oxidase, like our current anti-subunit V preparation, increases the biphasicity seen at intermediate ionic strengths. The anti-oxidase also induces a biphasicity in the high ionic strength medium where the anti-subunit V is without effect. It is clear that the anti-oxidase contains components capable of modulating the kinetics of the enzyme like the anti-subunit V, but in addition contains components that react directly with the catalytic subunits I and II, directly modulating the binding of cytochrome *c* and the internal electron transfer processes.

Table 1

Effect of preincubation at different ionic strengths upon the inhibition of cytochrome oxidase by anti-subunit V

Ionic strength of preincubation	Turnover (electrons/aa_3 per s)	
	Control	+ Anti-V
5 mM phosphate	58.0 ± 0.6	43.6 ± 4.6
50 mM phosphate	59.0 ± 0.9	45.7 ± 2.7
150 mM phosphate	57.6 ± 2.2	49.4 ± 1.6

Enzyme was preincubated with anti-subunit V γ-globulin at a ratio of 275:1 Ab/Ag in the indicated phosphate buffer concentrations for 15 min. It was then assayed spectrophotometrically in 50 mM potassium phosphate buffer (pH 7.4) containing 0.075% lauryl maltoside at 30°C. 9 μM ferrocytochrome *c* was used as substrate with 4 nM (final concentration) cytochrome aa_3 as enzyme. Rates are shown as means ± 1 standard deviation ($n = 3$)

4. DISCUSSION

As pointed out by Gai et al. [11], the differential action of antibodies on the two kinetic phases of cytochrome *c* oxidation supports the idea of separate binding sites or at least separate reaction types for cytochrome *c* in the high- and low-affinity regions, as outlined in the model of Brooks and Nicholls [23]. Anti-subunit V apparently binds to subunit V at the N (matrix) face of the enzyme [12,13] and in so doing it reduces the off constant for cytochrome *c* bound at the high affinity site. It consequently renders the kinetic profile at intermediate ionic strength similar to that seen for the control enzyme at a lower ionic strength. We propose that the bound cytochrome *c* is less readily dissociated following oxidation; the entry of further reducing equivalents into the system is slowed.

At low ionic strength two phases are seen in both the control and the inhibited enzyme systems. The effects of anti-subunit V can be explained using either one of two possible models. The first such model assumes that the low-affinity cytochrome *c* binding site increases the flux through the enzyme by enhancing the dissociation rate of the catalytic cytochrome *c* bound at the high-affinity site [20,24]. The alternative assumes that cytochrome *c* at the low-affinity site increases the reduction rate of tightly bound cytochrome *c* [23]. In both models product dissociation limits V_{max} at low ionic strengths and low concentrations of cytochrome *c* [19,23]. However, the turnover at high levels of *c* (V_{max} at the low-affinity site) is largely limited by the speed of internal electron transfer, as it is independent of ionic strength (see [23,24].

The main effect of anti-subunit V in the high-affinity region is to decrease V_{max}. However, there is no effect on V_{max} in the low-affinity region as the increase in product dissociation or reduction rate caused by the binding of the second molecule of cytochrome *c* offsets the decrease caused by the antibody. In order to overcome the antibody effect, however, it is necessary to have a higher concentration of cytochrome *c* to interact with the low-affinity site (or a higher 'occupancy' at the site). This accounts for the increase in low-affinity K_m observed after antibody binding. Thus the complex kinetic effects of anti-subunit V binding can all be explained by assuming that it induces a conformational change that decreases the dissociation rate of

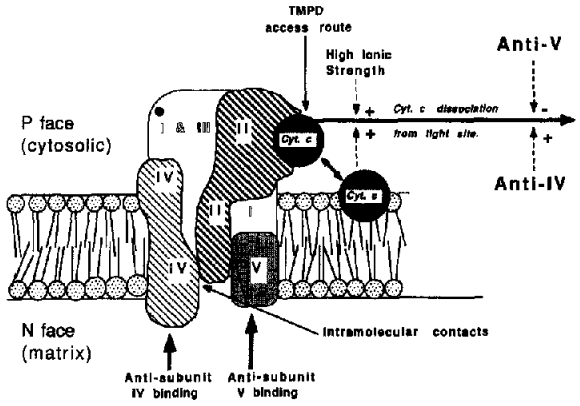

Fig.3. A schematic representation of the arrangement of the subunits of the oxidase in the mitochondrial membrane and the sites of action of the effective antibodies.

ferricytochrome *c* from the high-affinity site on subunit II. Such a long range effect could be communicated directly from subunit V to subunit II as cross-linking experiments show the two subunits to be within 11 Å of each other [25].

A very similar explanation can be advanced to account for the kinetic action of the two types of monoclonal antibodies prepared against subunit IV [11]. Monoclonal QA4 inhibits the enzyme by increasing the K_m seen in the high-affinity region. It binds on the 'N' (matrix) side, as does anti-subunit V; its action therefore also reflects a transmembranous effect. Such an effect can possibly be exerted through the subunit IV-subunit II interface, with a consequent destabilization of the tight complex. Monoclonal QA4 also increases the K_m of the low-affinity reaction; this could be a result of a change in the accessibility of cyt. *c* at the high-affinity site towards the second molecule of cyt. *c*. The monoclonal QA4/C4 actually increases the apparent V_{max} at the high-affinity site by a factor of more than two. It does not significantly alter the high-affinity K_m nor does it have any effect on V_{max} in the low-affinity region (though there is a slight increase of K_m). In an analogous way to the effect postulated for anti-subunit V, QA4/C4 could increase the high affinity V_{max} by increasing the rate of product dissociation. Alternatively its action could be envisaged as increasing the accessibility of bound cyt. *c* to the reductant TMPD, one of the usual rate-limiting events that determines the magnitude of V_{max} at the high-affinity site [23].

Fig.3 summarizes our present picture of the relationships between the various subunits involved in catalysing and controlling the turnover of this enzyme. Events at the matrix (N) face of the oxidase can modify its behaviour towards the substrate cytochrome *c* at the cytosolic (P) face. Such modifications may include changes in the reactivity and dissociability of cytochrome *c* at the high-affinity site on subunit II. The antibodies thus mimic the action of some of the more physiological influences, such as the levels of metabolites and nucleotides on the two sides of the inner mitochondrial membrane, that have been postulated [2,3] to control cytochrome oxidase.

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