The effect of specific antibodies on oxygen uptake and H^+ pumping by cytochrome *c* oxidase vesicles

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Received 29 July 1983; revised version received 25 August 1983

Antibodies to solubilized cytochrome *c* oxidse and to subunit III were incubated with liposomal oxidase. In oxygen uptake experiments, the inhibiting effects on RCI of anti-oxidase (primarily anti- subunits II and IV) and anti-III were by different mechanisms: the former, by inhibiting the uncoupled rate; the latter, by stimulating the coupled rate. In experiments with H^+ translocation, anti-oxidase was without effect, while anti-III was a potent inhibitor of proton pumping. These results are conclusive evidence for redox-linked proton extrusion from the vesicles by the oxidase (and its subunit III).

Cytochrome c oxidase *Reconstituted vesicle* *Proton pumping* *Chemiosmosis* *Antibody*

1. INTRODUCTION

There are two possible mechanisms of energy transduction by cytochrome *c* oxidase vesicles (COV). The first is that the electrochemical gradient is created as oxygen is reduced and cytochrome *c* oxidized on opposite sides of the membrane. The second mechanism is that the oxidase per se acts as a proton pump. There is no dispute about the former [1,2]; but as reviewed [3], there is much controversy as to whether the observations of a transient acidification of the medium-suspended COV, when these are pulsed with oxidants or reductants [5–10], are evidence for the latter. If so, the inhibition of this proton translocation by DCCD [11], which binds to a glutamyl residue of subunit III [9], and the loss of proton pumping by COV prepared from enzyme lacking in the subunit [12] would not only constitute additional evidence, but actually localize the pumping to one part of the oxidase.

Abbreviations: COV, cytochrome oxidase vesicles; DCCD, *N,N'*-dicyclohexylcarbodiimide; MOPS, morpholinopropane sulfonic acid; *RCI*, respiratory control index

However, alternative explanations have been proposed [13]:

(i) Transient acidification could result artifactually from oxidation of a cytochrome *c*–phospholipid complex;
(ii) DCCD might inhibit by interacting with the phospholipids of the complex, particularly phosphatidyl serine;
(iii) The absence of acidification on pulsing COV prepared from the III-less enzyme might stem from residual detergent or changes in the quaterary structure of the remaining enzyme and their effect on the nature of the nearby cytochrome *c*–phospholipid complexes.

Two of these explanations have been partially refuted [14]:

(i) Azide inhibition of acidification is not reversed by ferricyanide oxidation of cytochrome *c* (however, the soluble reagent may have oxidized the cytochrome before it entered into a complex;
(ii) DCCD inhibition did not correlate with the phosphatidyl serine content of the COV (however, other phospholipids might be susceptible elements of a complex). A clear resolution paper is needed.

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00145793/83/$3.00

In this laboratory, potent anti-oxidase and anti-subunit III rabbit sera are available. The former (predominantly anti- subuntis II and IV, but not III, as determined by Western blotting [15]) shows high titer in inhibiting (competitively with reduced cytochrome *c*) electron transport by the solubilized enzyme, and the latter affects electron transport only slightly and shows precipitin activity against subunit III alone [16]. Thus there is a way to delicately, specifically, and unambiguously probe energy transduction by oxidase in general and subunit III in particular.

2. MATERIALS AND METHODS

2.1. *Vesicular oxidase*

Published methods were used to prepare oxidase [15,17,18], and to incorporate it into vesicles [6] of partially purified [19] soybean phospholipids. Respiratory control indices varied from 3.75 to 6.75. Oxygen uptake assays used conditions similar to [20]; uncoupling for respiratory control measurements was as in [7]. Proton translocation was at 3°C using phenol red [6] as modified by [7] except that the reduced cytochrome *c* was chromatographed and stored in 50 mM K_2SO_4/1 mM MOPS (pH 7.2). Protein determination was as in [21], compensating for phospholipid interference.

2.2. *Immunological procedures*

Anti-III and anti-oxidase were raised and gamma globulin isolated as in [16], except that the gamma globulin preparations were used as stock solutions of 40–50 mg/ml in 50 mM K_2SO_4 (pH 7.5) and the data were recalculated to 20 mg gammaglobulin/ml. Incubation of these antibodies or non-immune gammaglobulin with the COV prior to oxygen uptake assay was for 15–20 min at 4°C in 50 mM K_2SO_4/1 mM MOPS/pH 7.2 to a total volume of 200 μl of which 20 μl were COV. Oxygen uptake was initiated with 180 μl mixture. For each day's assays, rates, percent inhibition or activation, and *RCI*-values were corrected for the first-order decay of oxidase activity in the coupled and uncoupled states.

Prior to proton translocation (in a 2.5 ml assay volume), the antibodies were incubated 15–20 min. in a total bolume of 2.0 ml 50 mM K_2SO_4, of which 200 μl were COV. The pH was brought to 7.2 (using an electrode) immediately after the addition of the COV, and again at the end of the incubation after the entire mixture was added to phenol red, valinomycin and K_2SO_4 in the concentrations of [6] by adjusting the absorbance difference to that of a pH 7.2 standard with additions of 10 mM KOH. The initial rates of proton extrusion were measured, extending the straightline portion of the curve by correcting for the subsequent proton backflow into the vesicles. Any step artifacts were graphically corrected. The buffering effect of the added protein was compensated by comparison with pulses of 5 nequiv. HCl at the end of each assay; however, such buffering imposed practical limits on the range of antibody/antigen ratio.

3. RESULTS AND DISCUSSION

3.1. *Effect of antibodies on oxygen uptake*

The anti-oxidase was shown to inhibit 50% of spectrophotometrically measured activity of solubilized cytochrome *c* oxidase at a gamma globulin to oxidase ratio of < 1.5 μl/μg [23]. The titer for the inhibition of oxygen uptake catalyzed by the solubilized enzyme was similar (about 1 μl/μg), and increased to about 3.5 μl/μg in uncoupled vesicles (see fig.1). This loss of effectiveness is to be expected from the sequestration of part of the oxidase in the vesicles. Unexpected was the additional loss of effectiveness with respect to the coupled COV, for the same determinants are exposed. The simplest explanation is that bound anti-oxidase, in competitive equilibrium with substrate [23], could inhibit electron transport to oxygen only to the extent that a 'leak' relieved the transmembrane potential. If the observed respiratory control ratio represents an average of COV populations coupled to various degrees, the antibodies would be least effective against the best coupled particles, which contribute the most to proton translocation. This bode ill for the planned proton translocation studies.

Anti-III had shown poor ability to inhibit electron transport, requiring 17 μl/μg to produce half its maximum of 22% inhibition [23]. In fig.1 it is shown to be virtually ineffective against uncoupled COV in a practical range of ratios. However, it strongly stimulates the coupled particles with

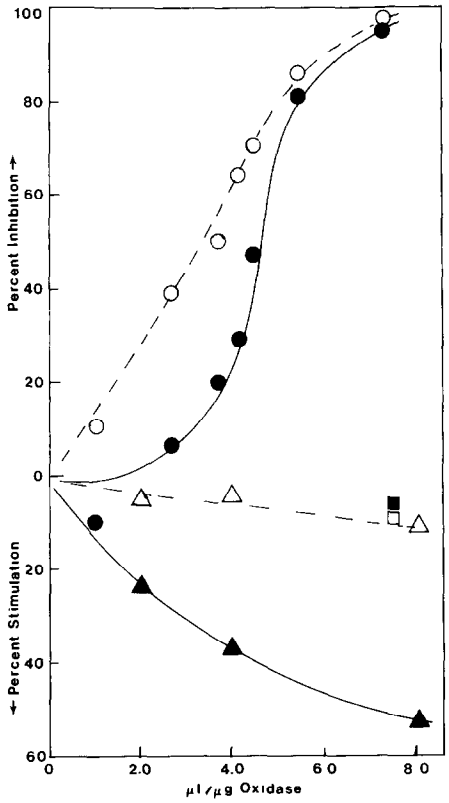


Fig.1. Quantitation of the effect of antibodies on oxygen uptake catalyzed by COV. Upward direction indicates inhibition: open symbols, effect in the presence of 1 μM valinomycin and 4 μM carbonyl cyanide *m*-chlorophenylhydrazone (uncoupled); closed symbols, effect on coupled rates; circles, anti-oxidase; squares, non-immune globulin, triangles, anti-III.

hyperbolic kinetics, approaching a maximum of 90%; i.e., at infinite ratio, half the coupling would remain. Since, in this preparation, half the coupling would still leave $RCI > 3$, our results are consistent with the observation of *RCI* in vesicles prepared form oxidase free of subunit III [22].

Inspection of fig.1 shows that both antibody →

Fig.2. Proton translocation in the presence and absence of anti-III. For conditions, see section 2. At the arrow, 0.24 mg oxidase in vesicles were pulsed with 20 μl 1.25 mM reduced cytochrome *c*. The COV had been incubated with: (A) no gamma globulin; (B) 0.58 μl anti-III/μg oxidase; (C) 1.44 μl/μg; (D) 2.5 μl/μg. The horizontal bar is 10 s; vertical bars (downward), the effect of 5 nequiv. of HCl on the assay mix.

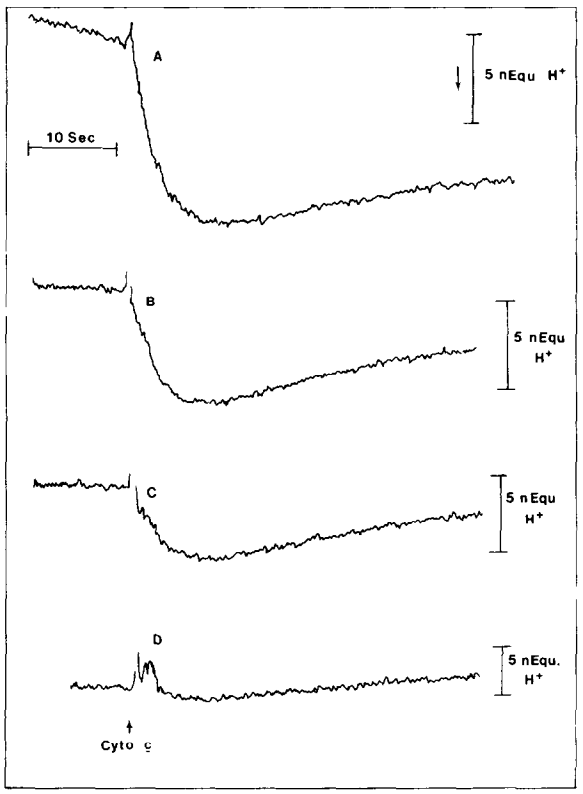
Table 1

The effect of gammaglobulin on respiratory control index

Preparation of gamma-globulin	Ratio of gamma-globulin to antigen (μl/μg)	*RCI*[a]	% of control[b]
None	0	6.75	100
Non-immune	7.5	6.97	104
Anti-oxidase	4.0	4.40	59
Anti-III	2.0	5.56	79
	4.0	5.14	72
	8.1	4.90	68
	Infinite	3.55	44

[a] Calculated from rates compensated for first order decay of oxidase activity in coupled and uncoupled vesicles

[b] $\frac{100 \times (\text{Experimental } RCI - 1)}{\text{Control } RCI - 1}$; Control was without globulin



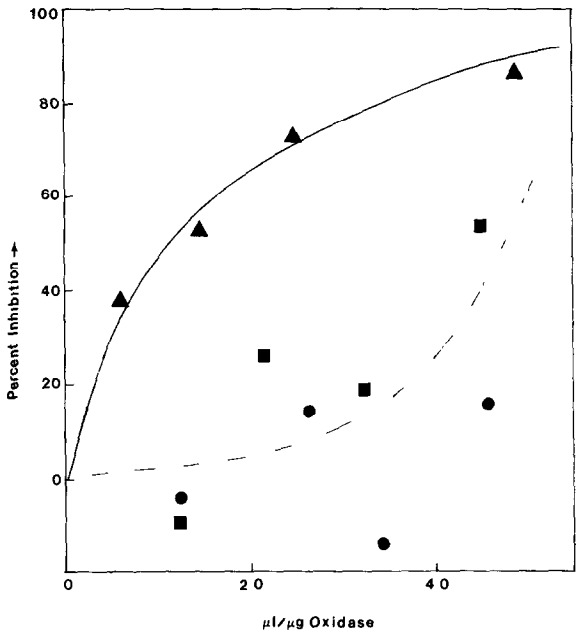


Fig.3. Quantitation of antibodies on proton translocation. The points are based on initial slopes, calculated as in section 2 from traces such as those of fig.2. For symbols see fig.1.

preparations reduce the RCI. Anti-oxidase is maximally effective in the central region, and anti-III as the antibody/antigen ratio is increased (see table 1). Yet, clearly the mechanisms for these reductions are different. In the absence of demonstrable anti-III content [15], that by anti-oxidase appears to be the result of a loss of its ability to inhibit an activity which has been reduced by coupling. However the effect of anti-III is to interfere with coupling to inhibit energy transduction but only half the energy is transduced. It seems to block one of two transduction mechanisms. This result immediately calls to mind work on vesicular III-less oxidase [23] in which *RCI* was halved by the removal of subunit III. Here a similar decrease in *RCI* was observed, but from the same COV preparation after exposure to anti-III (and not to non-immune gamma globulin, see fig.1) Since the III-less material had previously been shown unable to translocate protons [12], we were much encouraged to try the effect of anti-III on proton translocation.

3.2. *Effect of antibodies on proton translocation*

The result is shown in fig.2. Even compensating for the increased buffering capacity due to the added gamma globulin, it is clear that increasing ratios of anti-III/oxidase leads to increased inhibition. Fig.3 quantitates this relationship, showing also the effect of anti-oxidase and non-immune globulin. As anticipated, the former is without effect, supporting our interpretation of fig.1. Fortunately, the non-specific effects, resulting from difficulty in measuring slopes in the face of buffering supplied by the globulin, do not show until outside the range wherein more than 3/4 of proton translocation would be abolished by specific anti-III activity. Recall this is a range where electron transport is unimpeded; electrons can still be removed from a cytochrome *c*-phospholipid complex. We have shown that, for this preparation (which showed an $RCI > 6$) the contribution of the artifact in [13] is not present. Non-specific interference with a cytochrome *c*-phospholipid complex by anti-III would be anticipated from non-immune globulin; yet it does not appear in this range. Nor can this effect be explained by residual detergent or drastic treatment to remove one subunit, thus disrupting the quaternary structure of the others. We are left only with an intimate involvement, possibly through the subtle conformational dynamics demonstrated in [15], of subunit III in proton pumping by cytochrome *c* oxidase. Moreover, the specificity of the subunit antibodies has enabled us to split energy translocation into its component mechanisms. It is our hope in the future to use the higher specificity of the monoclonal antibodies we are raising in this laboratory to probe structure-function relationships in the enzyme in still greater detail.

ACKNOWLEDGEMENTS

We thank Dr R.P. Tracy for preparing the anti-III used in this study. Funds for the research were from grants from NIH (CA20454) and the American Heart Association.

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