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The Mechanism of Antibody Inhibition of Proton Pumping by Cytochrome Oxidase Vesicles

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Summary: Antibodies previously shown to inhibit vectorial proton translocation through cytochrome oxidase vesicles were converted to $F(ab')_2$ and Fab'. Neither fragment inhibited proton pumping, although binding capacity was present. However, when a surrogate Fc was added to $F(ab')_2$, inhibition of proton translocation was restored; indicating that the inhibition is due to steric hindrance. These results provide insight into mechanisms of energy transduction by oxidase.

We have shown (1) that antibodies against subunit III of cytochrome c oxidase (E.C.1.9.3.1) inhibit the vectorial translocation of protons through phospholipid vesicles inlaid with that enzyme. Wikström et al. (2,3) have indicated that they believe that oxidase's proton pumping stems from the enzyme's ability to act as a reciprocating dimer whose monomers articulate via subunit III. But the fact that binding of DCCD** to subunit III inhibits the translocation (4), and the obvious analogy to the proton trans- locating ATPase (5) suggest a possible proton channel through the subunit.

It occurred to us that antibody fragments to subunit III could be used to explore the degree to which the inhibition we observed (1) was due to a steric effect by the antibodies, perhaps interfering with the mechanism postulated by Wikström by locking together the subunits III of a dimer. If such were the case, the divalent $F(ab')_2$ would be much more effective than the monovalent Fab'. But if the antibodies acted by binding to the channel-bearing portion of the subunit, they would disrupt intrasubunit amino acid interactions required for the translocation, in which case Fab' would be at least as strong an

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** Abbreviations: DCCD, N, N'-dicyclohexylcarbodiimide; Anti-III, antiserum to subunit III; COV, cytochrome oxidase vesicles; SDS, sodium dodecyl sulfate.

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inhibitor as $F(ab')_2$. So we set out to prepare mono- and divalent anti-III fragments and compare their effects, one with the other, and each with that of the IgG. This communication describes the unexpected results, together with further experimentation that answered our original question.

Experimental

Materials

Antibodies to sununit III were raised in this laboratory (6). Goat IgG against rabbit $F(ab')_2$ conjugated to peroxidase was purchased from Cappell Laboratories. Pepsin was from Millipore, and trypsin from Sigma. Chemical reagents were of the highest grade available.

Preparation of Other Immunological Materials

For experiments with inhibition of proton translocation, $F(ab')_2$ was prepared from nonimmune and anti-III rabbit serum by the procedure of Madsen and Rodkey (7), increasing the concentration of pepsin and the length of incubation as required by the quality of the protease for complete digestion. The $F(ab')_2$ was reduced by dithiothreitol and alkylated with iodoacetamide (8). Affinity-purified goat fragments were prepared from the serum of a goat immunized against purified rabbit IgG (9). The goat $F(ab')_2$ was prepared using trypsin digestion by the combined procedures of Madsen and Rodkey (7) and Davies et al., (11). The column was saturated with goat $F(ab)_2$' washing out any nonimmune or anti-Fc material. The goat $F(ab')_2$ recognizing anti-rabbit Fab' was eluted (pH 2.5), neutralized and dialyzed. It was then reduced, alkylated (8), and repeatedly concentrated in 50 mM K_2SO_4 by ultrafiltration.

Proton Translocation

COV were prepared from beef heart oxidase and partially purified soybean phospholipids by published procedures (1,12-15). Respiratory control, determined by oxygen uptake (16) in the presence and absence of uncoupler (17) varied from 4.5 to 6.4. The procedure for measurement of proton translocation (1) was miniaturized. In order to save materials, the assay volume was reduced to 0.75 ml by lining a 3 ml cuvette with a 75 mm (i.d.) test tube. Cytochrome c pulses were delivered by means of capilary tubing into the stirred cell.

Antibody Inhibition of Proton Pumping

The conditions described (1) for measurement of inhibition by whole IgG molecules were found suitable for use of antibody fragments, except that quantities were reduced by miniaturization. A double incubation in 50 mM K_2SO_4, pH 7.2 was used in experiments with goat IgG fragments. The first incubation was in 250 ul containing 45 ul COV (at 1.5 mg cytochrome oxidase per ml) and 2 mg rabbit $F(ab')_2$, equivalent to a ratio of 2.5 ul (20 mg IgG/ml) per ug oxidase. After 20 minutes, 350 ul containing either 0 or 2 mg goat Fab' antibodies to rabbit $F(ab')_2$ was added to the COV-rabbit protein mixture, and the incubation was continued 15 minutes longer before completing the assay mixture and initiating the assay as described (1).

Western Blots

The methods were as described (13), except that the second antibody-peroxidase conjugate was directed against rabbit $F(ab')_2$, rather than whole rabbit IgG, and the peroxidase staining procedure was an adaptation of that of De Blas and Cherwinski (18). We found excellent sensitivity, requiring second antibody dilutions 1/16000. The background was reduced by using 5% bovine serum albumin to saturate nonspecific sites, once we omitted the post-fixation step with OsO_4.

Analytical Procedures

Polyacrylamide gel electrophoresis in the presence of SDS and urea was by the method of Swank and Munkres (19), as modified in this laboratory (13). Protein determination was according to Lowry et al. (20), compensating for interference by phospholipids.

Results

Fragmentation of Anti-III IgG Destroys its Ability to Inhibit Proton Pumping

The monovalent and divalent fragments of nonimmune and anti-III rabbit IgG were incubated with the COV in a ratio to oxidase content equivalent to 2.8 ul (20 mg IgG/ml) per ug oxidase. We had previously observed (1) that incubation of the COV with anti-III gamma globulin in this ratio inhibited 75% of proton translocation. But the fragmented anti-III had no more effect than the nonspecific binding of nonimmune material, regardless of the valence of the antibody fragments (Table 1). There were only two possible explanations, both unusual immunochemical events: (a) the procedures used for fragmentation had destroyed binding capacity; or (b) the loss of the Fc portion of the IgG had destroyed its capacity to exert inhibiting steric effects. We looked at binding first.

Fragmented Anti-III Binds to Oxidase Subunits

Using the "Western" (21) blot technique, we transferred subunits of oxidase to nitrocellulose paper and examined the capacity of the fragmented anti-III to bind to them. We used anti-oxidase gamma globulin (see 13) as a positive control. All types of anti-III (whole IgG, $F(ab')_2$ and Fab') bound; Figure 1 shows the most fragmented, the Fab'. "Anti-III" reveals also some anti-II

Table 1. Effect of Fragmentation of Rabbit IgG on its Ability to Inhibit the Rate of Proton Pumping by Cytochrome Oxidase Vesicles

Globulin	Whole Molecule	Fragments $F(ab')_2$	Fragments Fab'
Nonimmune	22%	- 9%	-9%
Anti-III	75%	-18%	8%

Rabbit protein was preincubated with 50 ul cytochrome oxidase vesicles in amounts equivalent to 2.8 ul (20 mg IgG/ml) per ug oxidase. For other conditions, see Experimental and references therein. Positive values are percent inhibition, and negative ones are percent stimulation relative to the rate of proton pumping observed in the absence of rabbit protein.

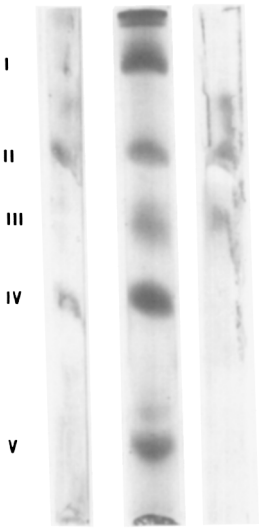

Fig. 1. Western Blots of Cytochrome Oxidase Subunits Resolved by Polyacrylamide Gel Electrophoresis in the Presence of SDS and Urea

Center: The original gel. Period of electrophoresis was long enough run peptides smaller than subunit V off the gel. Left: Nitrocellulose blot of a gel half from the same run. The blot was saturated with 5% bovine serum albumin prior to incubation 90 minutes with 0.2 mg rabbit anti-oxidase gamma globulin/ml. Right: Nitrocellulose blot of another gel half in which the rabbit antibody solution was 0.2 mg anti-III Fab'/ml. Second antibody-peroxidase conjugate incubation and staining was as described in Experimental and the references therein.

binding in addition to its anticipated ability to bind to subunit III, as reported previously (6). It will henceforth be called "anti- (II+III)". The common recognition of subunit II by the two antibody preparations together with the inability of anti-oxidase to affect proton translocation (1) makes anti-III as the source of anti-pump activity, but confirmation of this awaits the greater specificity of monoclonal antibodies.

The important point is that the loss of anti-pump activity did not result from an inability to bind. Furthermore, by elimination, this result suggested that anti-pumping activity was due to a steric effect conferred by the bulky Fc, which was removed by pepsin in the first step of fragmentation. One could not restore the Fc. But such restoration could be approximated.

Goat Fab' Anti-Rabbit $F(ab')_2$ Restores Anti-Pump Capacity to $F(ab')_2$ Anti-(II+III)

Consider the Y-shaped IgG molecule. Treatment by pepsin or trypsin results in the loss of the Y-stem, creating a divalent "V", the $F(ab')_2$. Reduction of

this yields the monovalent, I-shaped Fab'. By allowing a monovalent goat anti-rabbit "I" to bind to the rabbit "V", one could bring about the attachment of a surrogate Fc to the rabbit $F(ab')_2$. In order to avoid destruction of the rabbit combining valences by goat antibodies which recognize determinants near the ends of the "V", this attempt to restore the Fc was made after a preliminary incubation of rabbit antibodies with the COV (see Experimental). Large increases in buffering capacity due to the presence of considerable amounts of fragments of nonimmune goat IgG were avoided by means of affinity-purification. The result is shown by the traces in Figure 2. The alteration in slope is well beyond the approximately 25% variation exhibited by the nonimmune IgG or fragments (see Table 1 and ref. 1), with or without inclusion of goat fragments.

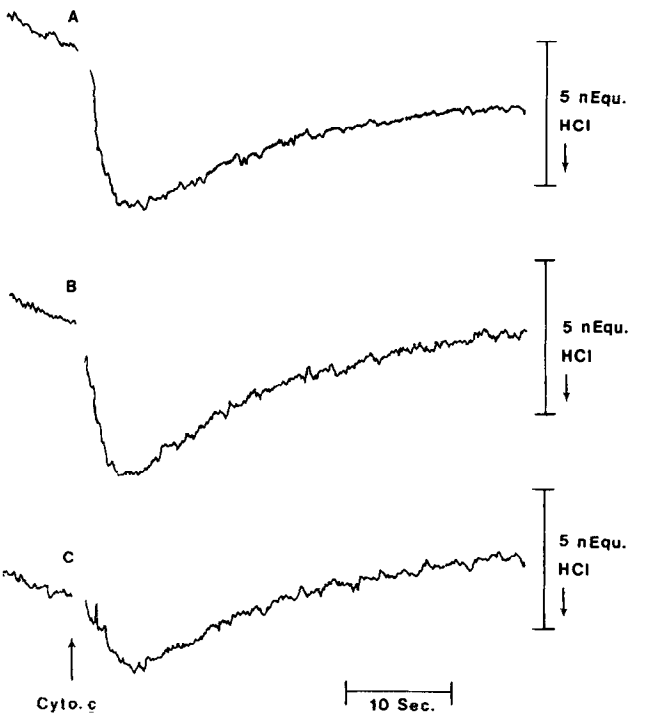

Fig. 2. Proton Translocation Through COV in the Presence and Absence of Rabbit and Goat IgG Fragments

2 mg rabbit $F(ab')_2$ was preincubated 20 minutes on ice with 45 ul cytochrome oxidase vesicles in amounts equivalent to 2.5 ul (20 mg IgG/ml) per ug oxidase. This incubation was followed by a second one of 15 minutes, after which the COV were assayed for proton pumping capacity. See Experimental for further details. A: The rabbit fragments were prepared from nonimmune gamma globulin. The second incubation contained no goat IgG fragments. B: Rabbit fragments were prepared from anti-(II+III) gamma globulin. The second incubation contained no goat IgG fragments. C: Rabbit fragments were prepared from ant-(II+III) gamma globulin. The second incubation contained 2 mg goat IgG fragments.

In fact, the 57% inhibition shown by trace C is 78% of the inhibition seen with the original IgG.

Discussion

These results establish that proton translocation through oxidase requires precise articulation between protein surfaces. Probably, since the divalent fragment unites identical determinants which would then be distanced by the Fc, the requirement is for interaction between monomers of an oxidase dimer. And the point of interaction is either between subunits II or subunits III, more likely, as we have pointed out, the latter. Thus, the findings are supportive of the model of Wikström *et al.* (2,3).

There are, of course, broader implications. The sense has been growing that mammalian oxidase probably exists as a dimer (22). But the function of this structural happenstance was unclear, since it has long been known that monomerization of the enzyme does not impede electron transport (23). We had previously dissected energy transduction through oxidase into its two components (1). One is its charge translocation from cytochrome *c* to oxygen, and this is obligately coupled to electron transport (24). But the other, the proton translocation, is more loosely coupled, since it is blocked by the removal of subunit III (25,26) or treatment with anti-III (1) or DCCD (27) without inhibition of electron transport. This looseness may ultimately be a source of control of oxidative phosphorylation, and the present work offers insight into possible mechanisms; for any phenomenon which disrupts intermolecular interactions between proteins in their membrane environment weakens the efficiency of oxidative phosphorylation. It is even possible that this dependence of proton translocation through cytochrome oxidase on correct interprotein contact has caused problems in the study of energy transduction through mitochondrial membranes. Isolation of the contributing sources of proton pumping requires selective inhibitors, some of which may be hydrophobic enough to disrupt such contacts, leading some investigators to believe that the proton pumping seen in COV "is not related to the capacity displayed by the enzyme in its native environment" (28).

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