

CHARACTERIZATION OF MONOCLONAL ANTIBODIES DIRECTED
AGAINST PYRUVATE OXIDASE FROM ESCHERICHIA COLI:
MODULATION OF ANTIBODY-INDUCED INHIBITION BY
ENZYME CONFORMATION

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Monoclonal antibodies have been prepared against pyruvate oxidase, a flavoprotein dehydrogenase isolated from Escherichia coli. Six monoclonals were obtained, but only one was found to bind to the native form of the enzyme. This monoclonal, 1I1, was a potent inhibitor. Although this antibody inhibited the unactivated and lipid-activated forms of the enzyme, it had much less of an inhibitory effect on the protease-activated form of the enzyme, although the antibody still bound to this form. Hence, the coupling between antibody binding and the conformation at the active site can itself be modulated by the conformation of the protein. © 1986 Academic Press, Inc.

Pyruvate oxidase is a flavoprotein dehydrogenase associated with the aerobic respiratory chain of Escherichia coli (1,2). The enzyme catalyzes the oxidative decarboxylation of pyruvate to acetate plus CO₂ and reduces ubiquinone-8 in the cytoplasmic membrane (3). The enzyme is

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Abbreviations used: PIPES, piperazine-N,N'-bis (2-ethanesulfonic acid), mAb, monoclonal antibody; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; MES, 2-(N-morpholine) ethanesulfonic acid.

a peripheral membrane protein which is easily dislodged from the membrane and purified in a water-soluble form without the aid of detergents (1,2). The purified enzyme has been extensively studied, primarily because the protein binds to lipids and this lipid binding has dramatic effects on the kinetic properties of the enzyme (4-7). The so-called "lipid-activated" form of the flavoprotein has a turnover number 20- to 30-fold higher than the "unactivated" form of the enzyme in assays using ferricyanide or other water-soluble dyes as electron acceptors (7).

Pyruvate oxidase can also be "protease-activated" when exposed to chymotrypsin in the presence of the substrate and cofactor, pyruvate and Mg^{2+} -thiamin pyrophosphate (6,8,9). The protease-modified enzyme no longer binds to or is activated by lipids, and the presence of lipids prior to the addition of the protease protects against proteolysis. Furthermore, the protease-modified form of the enzyme is activated to the same extent as can be achieved by lipid binding. Lipid-binding and proteolytic cleavage, both probably in the same region, each result in altering the active site of the enzyme in a similar manner (10).

MATERIALS AND METHODS

Materials: Unless otherwise specified, all materials were reagent grade, obtained from commercial sources. The protein A-Sepharose CL-4B resin was obtained from Pharmacia. ^{125}I iodide was from Amersham. Pansorbin and thiamin pyrophosphate were purchased from Cal Biochem-Behring, and chymotrypsin, PIPES, phenylmethylsulfonylfluoride, Triton X-100 and MES were from Sigma. The X-ray film used for autoradiography was Kodak XS-1.

Preparation of monoclonal antibodies (mAbs): Procedures used have been previously described (11). Briefly, BALB/c mice were immunized and boosted with purified pyruvate oxidase. Spleen lymphocytes were fused with Sp2/0-Ag14 myeloma cells and hybridomas tested for antibody production using a solid-phase radioimmunoassay using 1 μ g of dried

pyruvate oxidase in Dynatech immulon wells. Following subcloning, ascites fluid was induced by injection of approximately 10^7 hybridoma cells of each subclone into pristane-primed BALB/c mice. Immunoglobulins were all purified using a protein A-Sepharose CL-4B column. All mAbs were isotyped using affinity purified rabbit anti-mouse immunoglobins in Ouchterlony immunodiffusion tests (12). Fab fragments were prepared by proteolysis (13) followed by removal of the F_c portion by chromatography on protein A-Sepharose (14). Immunoblotting, SDS-PAGE, and protein determinations have been previously described (15).

Preparation of Pyruvate Oxidase: Purification of the pyruvate oxidase flavoprotein was as described previously (1,2). Activation of the pyruvate oxidase flavoprotein by chymotrypsin was performed by a modification of the previously described procedures (9). The activation was performed at room temperature in 10 mM sodium pyruvate, 30 mM thiamin pyrophosphate, 30 mM $MgCl_2$, 50 mM sodium PIPES, pH 7. The flavoprotein (350 μ g) was added to the solution and incubated for five minutes. After that, freshly dissolved chymotrypsin (14 μ g) in 1 mM HCl was added, and the mixture incubated for 30 additional minutes. The reaction was terminated by addition of concentrated phenylmethylsulfonylfluoride in isopropanol, up to 5 mM final concentration.

Assays of Enzymatic Activity: The pyruvate oxidase activity was measured in vitro in the absence and presence of detergents (7,16), using a ferricyanide assay (9). Incubations of monoclonal antibodies with the pyruvate oxidase were performed in TBS, pH 7.2, overnight at 4°C. When binding assays were done, Pansorbin was suspended in TBS, pH 8, and incubated with the mAb-Antigen complexes for 1 hr at 37°C. Enzymatic assays were done on the supernatants following centrifugation. In all cases, the pH of solution was adjusted with 0.1 M phosphate buffer, pH 6, immediately before measuring pyruvate oxidase activity.

RESULTS

Six positive hybridomas were obtained. Solid phase radio-immunoassay results (not shown) indicated the relative binding

affinities to be as follows, with the isotype in parentheses: 1I1(IgG 3)>B3-5(IgG 2b)>B6-11(IgM)>1H1(IgG 2a)>2K6-15(IgG 1)>2K6-18(IgG 1). All six monoclonals immunoblotted the pyruvate oxidase polypeptide following SDS-PAGE (Western blotting). Following proteolytic activation of pyruvate oxidase (6,8,9), all six monoclonals immunoblotted the large, 58,000 Da fragment. Incubation of pyruvate oxidase with chymotrypsin in the absence of the substrate produces fragments of 51,000 Da and 9,000 Da, with the cleavage site near the C-terminus (9). Western blotting

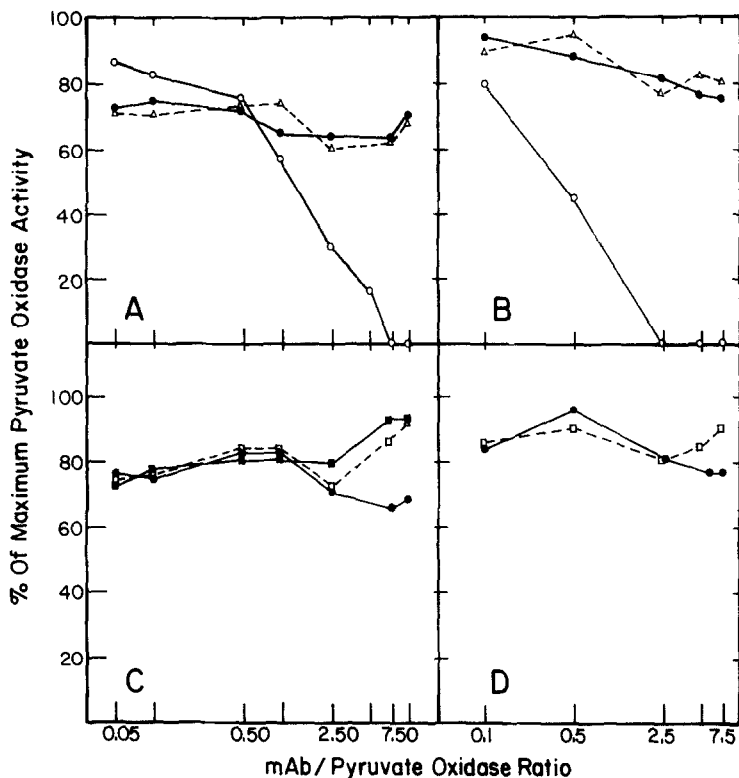


Figure 1: Inhibition of the pyruvate oxidase activities using purified monoclonal antibodies. Panels A and C: Seven μ g of pure pyruvate oxidase in TBS, pH 7.2, was incubated with different amounts of mAbs overnight, at 4°C. Pyruvate oxidase activity was determined as described in the text, in the presence of 0.02 mM SDS. Panels B and D: mAb binding was measured by precipitation with Pansorbin followed by measuring the pyruvate oxidase activity in the supernatant. Panels A and B: (○--○) 1I1; (△--△) B6-11; (●--●) B3-5. Panels C and D: (□--□) 2K6-15; (■--■) 2K6-18; (●--●) 1H1. Non-specific effects of mAb on pyruvate oxidase activity were determined using a mAb directed against an unrelated enzyme from *R. sphaeroides* as control. The small effects observed were subtracted from data obtained with anti-pyruvate oxidase mAbs.

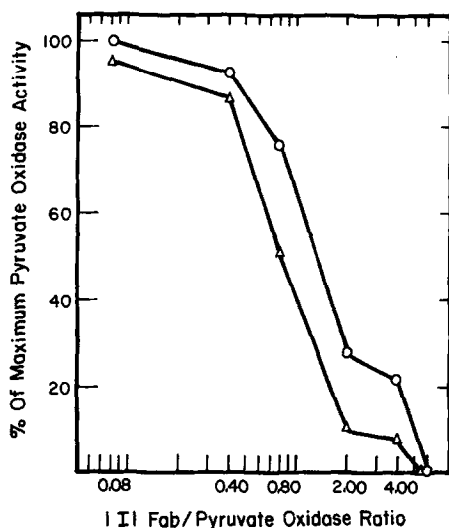


Figure 2: Inhibition of the pyruvate oxidase activities using Fab fragments from purified 1I1 mAb. The experimental conditions were the same as described in Figure 1. Pyruvate oxidase activity (o-o) was measured with increasing Fab concentrations. Fab binding to pyruvate oxidase (Δ - Δ) was assayed by precipitation with anti-mouse polyclonal antiserum with Pansorbin and measuring pyruvate oxidase activity in the supernatant, as described in Materials and Methods.

after this proteolytic treatment of the enzyme showed that the epitope for monoclonal B3-5 is on the small fragment, whereas those for 1I1,2K6-15 and 2K6-18 are on the large fragment. Monoclonals B6-11 and 1H1 did not immunoblot after this proteolytic treatment, suggesting that the epitopes of these two antibodies are destroyed by cleavage at this site.

Antibody 1I1 was the only one to inhibit pyruvate oxidase activity. This antibody fully inhibited the enzyme (Figure 1). The Fab fragment prepared from this antibody inhibited the oxidase equally well (Figure 2), thus ruling out unusual effects which could result from bivalent antibody binding to two different subunits within the tetrameric oxidase. Only the 1I1-pyruvate oxidase complex could be precipitated with Pansorbin, indicating that monoclonal 1I1 is the only one to bind to the enzyme in solution. Apparently the use of dried protein in the screening procedure exposed epitopes that are not antigenic when the enzyme is in solution.

Table 1: Effect of Monoclonal Antibody 1I1 on Pyruvate Oxidase Activity, Measured in the Absence or Presence of Detergents^a

	mAb/pyruvate oxidase ratio (w/w)	
	1.0	5.0
Detergent Activator:		
(a) None	22	64
(b) 0.02 mM SDS	28	84
(c) 0.1% Triton X-100	36	84

^aEnzyme aliquots (7 μ g) were incubated either with 7 μ g or 35 μ g of purified 1I1 mAb overnight, at 4°C, in TBS buffer, pH 7.0. Pyruvate oxidase activity was determined as described in the text in the presence of indicated detergent activators, and is expressed as percent of inhibition of maximum pyruvate oxidase activity.

Table 1 shows that the inhibition of pyruvate oxidase by 1I1 is similar regardless of whether the enzyme is assayed in the absence of detergent or whether the enzyme is activated by either SDS or by Triton X-100. In addition, inclusion of detergent in the presence or absence of the substrate and cofactor of the oxidase does not alter the results.

Table 2 compares the effects of 1I1 on pyruvate oxidase on the native and chymotrypsin-activated forms. The results show a striking difference. Under comparable conditions, the antibody binds to the protease-activated form almost as well as to the native enzyme. However, the antibody has very little effect on the activity of the protease-activated form of the enzyme under conditions where the native enzyme is substantially inhibited. Table 2 also shows that this is true regardless of the buffer conditions used during the incubation of the antibody with the enzyme.

It is clear that antibody 1I1 binds to all three conformational states of pyruvate oxidase: unactivated, lipid-activated, and protease-activated. However, whereas antibody binding is inhibitory for the unactivated and lipid-activated forms, the protease-activated form is not substantially affected by antibody binding. Since antibody binding and enzyme activity are compatible, it is clear that the epitope recognized by 1I1 is not directly at the active site. It appears,

Table 2: Pyruvate Oxidase Inhibition by 1I1 Monoclonal Antibody After Incubation of Native and Chymotrypsin-Activated Pyruvate Oxidase^a

Incubation Buffer ^b	Native pyruvate oxidase ^c		Chymotrypsin-activated pyruvate oxidase ^d	
	inhibition	binding	inhibition	binding
(1) Buffer alone	83	100	24	91
(2) Pyruvate	71	100	--	--
(3) TPP, Mg ²⁺	77	100	--	--
(4) Pyruvate, TPP, Mg ²⁺	72	100	10	81

^aData is expressed as percent of inhibition of maximum pyruvate oxidase activity, prior to ("inhibition") or following ("binding") precipitation with Pansorbin.

^bIncubations with substrate and cofactors were done in:

(1) 0.15 M MES buffer, pH 6.0; (2) 200 mM pyruvate in 0.15 M MES buffer, pH 6.0, (3) 1.0 mM TPP, 10 mM MgCl₂ in 0.15 M MES buffer, pH 6.0, (4) 200 mM Pyruvate, 1 mM TPP, 10 mM MgCl₂ in 0.15 M MES, pH 6.0. Preincubations were carried out for 1 hr, at 4°C. After incubation, 1I1 mAb was added at a 5.0 mAb/ pyruvate oxidase mass ratio, and incubated for two additional hours, at 4°C.

^cPyruvate oxidase activity was determined in the presence of 0.1% Triton X-100 as the detergent activator.

^dChymotrypsin-activated pyruvate oxidase activity was determined in the absence of detergents.

rather, that the effects of antibody binding are dependent on the conformational state of the protein.

The conformational dependence on the effect of antibody binding, rather than binding per se, is a potentially important phenomenon, clearly demonstrated by this enzyme and monoclonal 1I1. This may prove to be a useful tool in studying other allosteric enzymes which exist in different ligand-induced conformational states.

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