pyruvate dehydrogenase complexes of mitochondria (Reed, 1974) or *Bacillus stearothermophilus* (Henderson et al., 1979) and *Bacillus subtilis* (Hodgson et al., 1983), in all of which the E1 component is divided into E1α and E1β chains, it should prove especially helpful in localizing the pyruvate binding site within the structure.

Registry No. Pyruvate decarboxylase, 9001-04-1; lipoate acetyltransferase, 9032-29-5; pyruvate dehydrogenase, 9014-20-4; bromopyruvate, 1113-59-3.

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Structural and Functional Influence of Enzyme–Antibody Interactions: Effects of Eight Different Monoclonal Antibodies on the Enzymatic Activity of *Escherichia coli* Tryptophan Synthase†

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ABSTRACT: Twelve monoclonal antibodies directed against the β_2 subunit of *Escherichia coli* tryptophan synthase (EC 4.2.1.20) were produced from hybridoma clones. These monoclonal antibodies are found to recognize at least eight different epitopes on β_2, and eight classes of monoclonal antibodies are thus defined. The effects of these monoclonal antibodies on the enzymatic activities of β_2 are studied. The monoclonal antibodies from three classes rapidly inhibit the serine deaminase activity catalyzed by the β_2 subunit alone; two of them lead to an inhibition plateau under stoichiometric conditions, and their inhibitory effects are cumulative. With the antibodies from two of these three classes, the tryptophan synthase activity of the $\alpha_2\beta_2$ complex is recovered, through a competition between the α subunit and the monoclonal antibody. On the contrary, the antibody from the third class is inhibitory even in the presence of an excess of α subunit. The antibodies from the five other classes, though binding easily to the coated antigen in the enzyme-linked immunosorbent assay, react only very slowly with β_2 in solution and, only after a long time of incubation, inhibit the enzymatic activity at different levels.

The tryptophan synthase from *Escherichia coli* has been shown to contain two types of subunits, α and β_2,[1] normally associated in an $\alpha_2\beta_2$ active complex (Yanofsky & Crawford, 1972). The formation of the complex, as well as the folding of both the α and β_2 subunits, has been the subject of extensive

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[1] Abbreviations: β_2, holo-β_2 (the β_2 subunit of tryptophan synthase saturated with pyridoxal 5'-phosphate); EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; PMSF, phenylmethanesulfonyl fluoride.

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studies. The α chain is made of two independently folding domains (Miles et al., 1982). The refolding of the denatured β chain has been analyzed in some detail in our laboratory and was also found to proceed through the independent folding of two domains. This was shown by a variety of experimental approaches involving the preparation and analysis of two complementary fragments, F_1 and F_2, which can be isolated after a mild proteolytic treatment of β_2 by trypsin. It was first observed that each denatured fragment is able to spontaneously refold into a "native-like" structure which, by several physical criteria, cannot be distinguished from the corresponding domain within the native protein (Högberg-Raibaud & Goldberg, 1977). By investigating the kinetics of renaturation of intact β_2 or of nicked β_2 (i.e., β_2 cleaved by trypsin), we identified several steps in the folding process. In particular, it was shown that the rate-limiting step in the renaturation of both β_2 and nicked β_2 is the same and involves an isomerization (change in conformation) which occurs after the initial refolding of F_1 and F_2 and after their association (Zetina & Goldberg, 1982).

From all the methods used, only immunochemistry was able to directly detect these conformational changes: the "average affinity" of F_1 and F_2 for specific anti-β_2 antibodies is much higher when these fragments are associated (Zakin et al., 1980). However, though specific, the antiserum used consisted of a complex mixture of immunoglobulin molecules, from a large variety of classes, each exhibiting a different epitope specificity and a different affinity for the antigen. This precluded the use of the total immunoglobulin fraction of the antiserum for a careful investigation of the protein–antibody interaction. Therefore, for studying in detail the conformational changes associated with the last steps in the renaturation of β_2, we decided to turn to monoclonal antibodies and to examine in detail the interactions between each monoclonal antibody and the domains of β_2.

It seemed necessary, however, to first ascertain that the monoclonal antibodies, to be chosen as probes of the native conformation, would not alter too much the structure of β_2. Indeed, it had been reported (Rocha et al., 1972) that β_2 is strongly inactivated by the antibodies from rabbits immunized with β_2. Furthermore, β_2 appears as a rather flexible protein: it is known to undergo an allosteric transition upon binding pyridoxal 5′-phosphate (Tschopp & Kirschner, 1980) and to change its conformation when interacting with the α subunit (Kirschner et al., 1975; Wilhelm et al., 1982).

This led us to ask the following questions about the mechanism of the inactivation of β_2 by specific antibodies: Is the precipitation of β_2 by antibodies the basis of the inactivation? Do the antibodies inactivate β_2 simply by preventing its association with α? Does the inactivation result from a change in the conformation, or in the conformational flexibility, of the protein when binding to antibody molecules? Such conformation-mediated effects of antibodies on the function of a protein have been reported in several instances and can lead either to the loss or to the appearance (Rotman & Celada, 1968; Choe et al., 1982) of the functional properties of the antigen.

The present paper reports the characterization of eight classes of monoclonal anti-β_2 antibodies (the first of a series to be extended) and describes their effects on the two enzymatic activities of β_2: the tryptophan synthase activity of the $\alpha_2\beta_2$ complex and the serine deaminase activity catalyzed, in the absence of the α subunit, by β_2 (Crawford & Ito, 1964). The mechanisms of inactivation of β_2 by antibodies are discussed in light of the results which also allow the selection of several classes of monoclonal antibodies as conformational probes of the native structure of β_2.

Materials and Methods

Preparation of β_2, Nicked β_2, and the F_1 and F_2 Fragments. The β_2 subunit, nicked β_2, and the proteolytic fragments F_1 and F_2 were prepared as described by Zakin et al. (1980) with minor modifications. The Sephadex G-100 column equilibrated with 6 M urea in 100 mM potassium phosphate (pH 7.8) containing 2 mM EDTA and 100 mM 2-mercaptoethanol was replaced by a Sephacryl S-200 column equilibrated with the same buffer. The β chain and the F_1 and F_2 fragments were found to migrate as a single band on a 12.5% polyacrylamide gel containing sodium dodecyl sulfate, indicating the homogeneity of each preparation.

Monoclonal Antibody Production. (*a*) *Immunizations.* BALB/c mice were immunized with native β_2 as reported previously (Friguet et al., 1983). Serum was collected from these mice and from nonimmunized mice in order to prepare polyclonal immunoglobulins. The spleen was harvested and used as a source of immunocytes.

(*b*) *Cell Fusion and Cloning.* Two hybridization procedures were used: either that reported previously (Friguet et al., 1983) using the technique of Köhler & Milstein (1975) or that of Buttin et al. (1978). The supernatants were screened for the presence of antibodies by the indirect enzyme-linked immunosorbent assay (ELISA) with β_2 as the coated antigen. The cloning was done either by micromanipulation or by limit dilution.

(*c*) *Preparation of the Monoclonal Antibodies.* The cloned hybridoma cells were cultured and injected intraperitoneally into pristane-primed BALB/c mice. The immunoglobulin fraction was precipitated from the ascitic fluids by adding sodium sulfate to 18% (w/v). Immunoglobulins were dissolved in a 100 mM potassium phosphate buffer, pH 7.8, exhaustively dialyzed against the same buffer, and kept at −20 °C.

Enzyme-Linked Immunosorbent Assays (ELISA). For screening the clones and investigation of fragment recognition, the enzyme tracer was peroxidase and the test was conducted as described in Bahr et al. (1980). To determine the epitope specificities of the monoclonal antibodies, an ELISA additivity test was performed as reported previously (Friguet et al., 1983).

Assays of Enzymatic Activity. The activity of the β_2 subunit was measured in two ways: (i) in the indole to tryptophan reaction in the presence of an excess of α subunit to activate β_2 as described by Faeder & Hammes (1970); (ii) in the serine deamination reaction coupled with the lactate dehydrogenase–NADH system as reported previously by Crawford & Ito (1964).

Incubations of monoclonal antibodies with the β_2 subunit were performed in 100 mM potassium phosphate buffer, pH 7.8, containing 2 mM EDTA, 0.1 mM pyridoxal 5′-phosphate, 5 mM 2-mercaptoethanol, and 1% (w/v) bovine serum albumin (Sigma) (buffer A). For 24-h incubations, buffer A was supplemented with 0.5 mM PMSF.

Exclusion Chromatography. The separation of the β_2 subunit and the β_2–antibody complex was run on a column (0.8 cm × 16 cm) of ACA34 (Ultrogel-LKB). The sample (0.5 mL) was layered onto the column and eluted at room temperature in 100 mM potassium phosphate buffer (pH 7.8) containing 2 mM EDTA and 0.1 mM pyridoxal 5′-phosphate. Fractions of 0.25 mL were collected at a flow rate of 0.5 mL/min.

Preparations of Fragments Antigen Binding (Fab). Proteolysis of rabbit polyclonal immunoglobulins was conducted

Table I: Characteristics of Monoclonal Antibodies and Their Effects on the Enzymatic Activities of the β_2 Subunit[a]

monoclonal antibody	Ig isotype	F_1 or F_2 recognition	class	residual activity (%) of serine deaminase 15 min[b]	serine deaminase 24 h	tryptophan synthase 15 min
C_2-A_4			NS[c]	98	84	97
B_3-B_5	IgG1	F_1	1	95	7	97
C_6-C_5	IgG1	F_1	1	98	5	106
30-2	IgG1	F_1	2	103	63	91
172-3	IgG1	F_1	2	112	50	95
46-9	IgG2a	F_1	3	31		51
136-3	IgG2a	F_1	3	28		43
179-2	IgG1	F_1	3	29		35
68-1	IgG1	F_1	4	17		21
69-4	IgG1	F_1	5	98	31	97
169-3	IgG1	F_1	6	102	68	91
93-6	IgG1	F_1	7-a	23		19
D_4-B_6[d]	IgG2b, IgG1	F_2	7-b	89	10	92

[a] A fixed amount of β_2 subunit (6.75 μg) is incubated with 0.6 mg (protein content) of each monoclonal antibody in 0.1 mL (serine deaminase reaction) or 0.15 mL, containing the α subunit in little excess (tryptophan synthase reaction), of incubation buffer (buffer A). [b] After 15 min of incubation at room temperature or 24 h at 4 °C, 0.1 mL of the incubation medium is mixed with 0.9 mL of the assay mixture, and the enzymatic activity is measured. The residual activity is expressed as the ratio between the activity found in the assay and the activity obtained without any antibody. [c] NS = nonspecific. [d] The monoclonal antibody D_4-B_6 (as well as three other antibodies produced by three clones which were isolated from the same positive hybrid population as D_4-B_6) is positive for both IgG1 and IgG2b and is probably a hybrid molecule similar to that described by Accolla et al. (1981).

as in Porter (1959). The nonproteolyzed immunoglobulins were removed by exclusion chromatography on a column (1.2 cm × 68 cm) of Sephacyl S-200 (Pharmacia) in 100 mM potassium phosphate buffer (pH 7.8). Electrophoresis on a polyacrylamide gel containing sodium dodecyl sulfate showed that no detectable heavy chain was present in the Fab pool.

Ouchterlony Immunodiffusion. Immunodiffusion tests were performed by standard methods (Ouchterlony, 1965). Monoclonal antibody solutions (about 600 μg in 20 μL) and the β_2 solution (2 μg in 20 μL) were applied to appropriate wells prepared in 1% (w/v) agarose and 25 mM 5,5-diethylbarbituric acid, sodium salt, pH 8.2.

Reagents. Immunoglobulin G (IgG) fraction rabbit anti-mouse IgG (Institut Pasteur Production, Marnes La Coquette, France) was coupled to either horseradish peroxidase (Boehringer, grade I) or pure *E. coli* β-D-galactosidase kindly supplied by Dr. Agnes Ullmann (Department of Biochemistry and Molecular Genetics, Institut Pasteur, Paris) by the glutaraldehyde one-step procedure of Avrameas et al. (1978).

Class-specific antibodies from Bionetics were used to identify the isotypes of the monoclonal antibodies.

An immunoadsorbent was prepared according to Cambiaso et al. (1975) with Sepharose 4B-AH (Pharmacia, Uppsala, Sweden) and immunoglobulin G (IgG) fraction rabbit anti-mouse IgG (Institut Pasteur Production, Marnes La Coquette, France).

To avoid nonspecific adsorption on the immunoadsorbent, without altering significantly the enzymatic activity of β_2, the incubations were performed in a 10 mM potassium phosphate buffer, pH 7.8, containing 0.4 M potassium chloride, 2 mM EDTA, 0.1 mM pyridoxal 5′-phosphate, 5 mM 2-mercaptoethanol, and 1% (w/v) bovine serum albumin (Sigma) (buffer B). All chemical reagents were of analytical grade.

Protein Measurement. Protein concentrations were established by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Results

Hybridomas producing anti-β_2 antibodies were prepared, 150 positive populations were cultured on a large scale for storage in liquid nitrogen, and 12 of them were cloned. The positive clones were then cultured for storage and production of antibody in mice. One clone producing an antibody with no detectable affinity for β_2 was chosen as a control. The isotype and epitope specificities of the immunoglobulin fraction of each ascitic fluid were characterized, and the effects of these monoclonal antibodies on the enzyme activities of β_2 were studied.

Characterization of the Monoclonal Antibodies. (*a*) *Isotype Determination.* β_2 was coated onto a microtitration plate. Each monoclonal antibody was then added, and after being washed, the isotype of the monoclonal antibody specifically retained was determined with class-specific antibodies linked to horseradish peroxidase. The results are shown in Table I.

(*b*) *Localization of the Antigenic Determinants on* β_2. After cleavage of the β_2 subunit by mild proteolysis (Högberg-Raibaud & Goldberg, 1977), two complementary fragments of the β chain, F_1 (M_r 29 000) and F_2 (M_r 12 000), can be obtained. F_1, the N-terminal extremity, and F_2, the C-terminal extremity, are not overlapping (Crawford et al., 1978). In addition, it has been shown that the two fragments do not cross-react with one another (Zakin et al., 1980). The monoclonal antibodies, which were initially selected by an ELISA test with intact β_2 as the coated antigen, were then studied in an additional indirect ELISA test in which either F_1 or F_2 was coated in the microtitration plate. Ten of the monoclonal antibodies were found to be directed toward F_1 and the two others toward F_2 (Table I).

(*c*) *Epitope Specificity.* To test whether the monoclonal antibodies recognize different epitopes on β_2, the ELISA double antibody binding test (the ELISA additivity test) of Friguet et al. (1983) was used. Briefly, the β_2 subunit (5 ng per well) is coated onto a microtitration plate, and then monoclonal antibodies at saturating concentrations, alone or in pairs, are allowed to bind to the antigen. Finally, rabbit immunoglobulin anti-mouse IgG's coupled to β-galactosidase are used to detect quantitatively the amount of bound antibodies by measuring the enzymatic activity of bound β-galactosidase. The additivity index [AI, previously defined by Friguet et al. (1983)] was calculated from the absorbances obtained in the ELISA test with each pair of monoclonal antibodies analyzed. This index (expressed in percent) allows

Table II: Additivity Index (AI) for 12 Monoclonal Antibodies[a]

monoclonal antibodies	B_3-B_5	C_6-C_5	D_4-B_6	30-2	46-9	68-1	69-4	93-6	136-3	169-3	172-3	179-2
B_3-B_5		7	80	67	77	62	47	91	63	46	51	66
C_6-C_5			84	64	72	63	52	82	62	45	46	65
D_4-B_6				88	106	88	92	22	71	79	69	65
30-2					91	71	66	91	72	49	11	85
46-9						79	76	85	4	80	76	7
68-1							63	85	85	68	69	82
69-4								94	93	57	65	81
93-6									84	93	88	88
136-3										72	61	8
169-3											40	68
172-3												67
179-2												

[a] For each pair of antibodies, an additivity index (AI) is calculated with the equation $AI = 100\{[2A_{1+2}/(A_1 + A_2)] - 1\}$ where A_1, A_2, and A_{1+2} are respectively the absorbances reached in the ELISA test with the first antibody alone, the second antibody alone and the two antibodies together. Low values of AI are underlined in the table.

appreciation of the simultaneous binding of two antibodies on the antigen. A low AI value indicates that the antibodies do not bind simultaneously; in such a case, it is generally concluded that they recognize the same antigenic region and can be gathered in a class of the same epitope specificity. On the contrary, a high AI value indicates simultaneous binding and hence distinct epitopes. The 12 antibodies were studied in pairs, and the corresponding AI values are reported in Table II. Each low AI value underlined in the table defines a pair of antibodies belonging to the same class on the basis of their inability to bind simultaneously on the antigen. For the 12 monoclonal antibodies, 7 classes corresponding to different epitope specificities were thus found (Table I). This is a lower limit since the method used, as with all other methods based on determining simultaneous binding, cannot distinguish whether two antibodies which exclude one another recognize exactly the same antigenic site, overlapping sites, two close sites involving steric hindrance, or conformational-dependent distinct sites. These classes are constituted by the antibodies produced by the following clones: class 1, B_3-B_5, C_6-C_5; class 2, 30-2, 172-3; class 3, 46-9, 136-3, 179-2; class 4, 68-1; class 5, 69-4; class 6, 169-3; class 7, 93-6, D_4-B_6.

Ouchterlony immunodiffusion tests (Ouchterlony, 1965) were performed between the β_2 subunit and various mixtures of the monoclonal antibodies. The antibodies of the different classes failed to precipitate β_2 when taken in pairs; furthermore, among all the combinations of three distinct classes of antibodies, only one was found to give a precipitation line: class 3 + class 4 and antibody 93-6 from class 7. From these results, it appears that the double-immunodiffusion method is not flawless for the determination of epitope specificity since, in our case, there is no immunoprecipitate formation even though simultaneous binding of two monoclonal antibodies to the antigen is possible, according to the ELISA additivity test.

Effects of the Antibody–β_2 Interaction on the Catalytic Mechanism of β_2. As previously described (Rocha et al., 1972), the immunoglobulin fraction of a rabbit antiserum raised against the β_2 subunit was found to inhibit the enzymatic activity of β_2.

The enzymatic activity of the β_2 subunit is also inhibited by specific polyclonal antibodies of an immunized mouse in the case of the indole to tryptophan reaction catalyzed by the $\alpha_2\beta_2$ complex as well as in the serine deamination reaction catalyzed by β_2 alone. A total inhibition was obtained after 10 min of incubation at room temperature of 5 μg of β_2 subunit (with or without an excess of α subunit) with 0.5 mg (protein content) of the immunoglobulin fraction of the serum of an immunized mouse. In order to test whether precipitation of

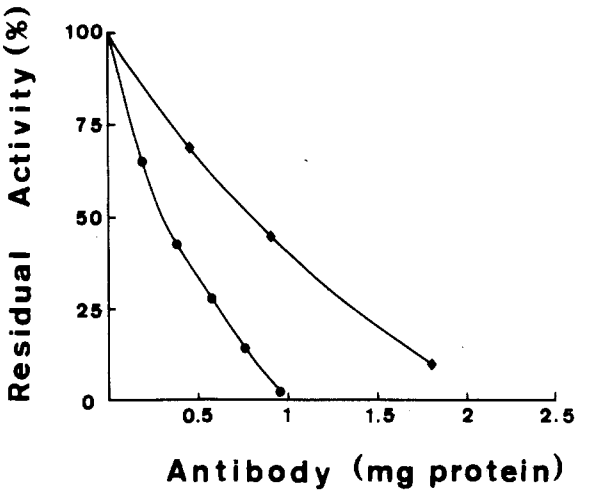

FIGURE 1: Inhibition of the tryptophan synthase activity of β_2 by rabbit polyclonal immunoglobulins and their Fab fragments. A fixed amount of β_2 subunit (4 μg) is incubated with increasing amounts of immunoglobulins or Fab fragments in 0.15 mL of 100 mM potassium phosphate buffer, pH 7.8, containing 2 mM EDTA, 0.5 mM pyridoxal 5′-phosphate, and 5 mM 2-mercaptoethanol. After 10 min of incubation at room temperature, 0.85 mL of assay medium containing the α subunit in small excess is added, and the enzymatic activity is measured. The residual activity is expressed as the ratio between the activity found in the assay and the activity obtained in a control without any antibody. The residual activity (in percent) is plotted as a function of the quantity of antibody added: polyclonal immunoglobulins (♦); polyclonal fragments antigen binding (●).

β_2 by the divalent immunoglobulin is involved in the inhibition phenomenon, we investigated the effect on the tryptophan synthase activity of monovalent Fab fragments prepared from rabbit polyclonal immunoglobulins. Figure 1 shows that the enzymatic activity of β_2 is inhibited by the Fab fraction in a molar concentration range similar to that found for uncleaved IgG molecules. From this result, it can be concluded that the inhibition mechanism does not stem from the precipitation of the β_2 subunit by the specific immunoglobulins.

In an attempt to elucidate the mechanism of inactivation of β_2 by specific antibodies and with the aim of selecting appropriate monoclonal antibodies as conformation probes of the native structure of β_2, the effects of the 12 monoclonal antibodies on the two enzymatic activities of β_2 were studied. A monoclonal antibody (C_2-A_4) which did not recognize the β_2 subunit was used as a control.

(*a*) *Serine Deaminase Activity.* First, holo-β_2 was incubated for 15 min with an excess of each antibody and assayed for its serine deaminase activity. Table I shows that three anti-

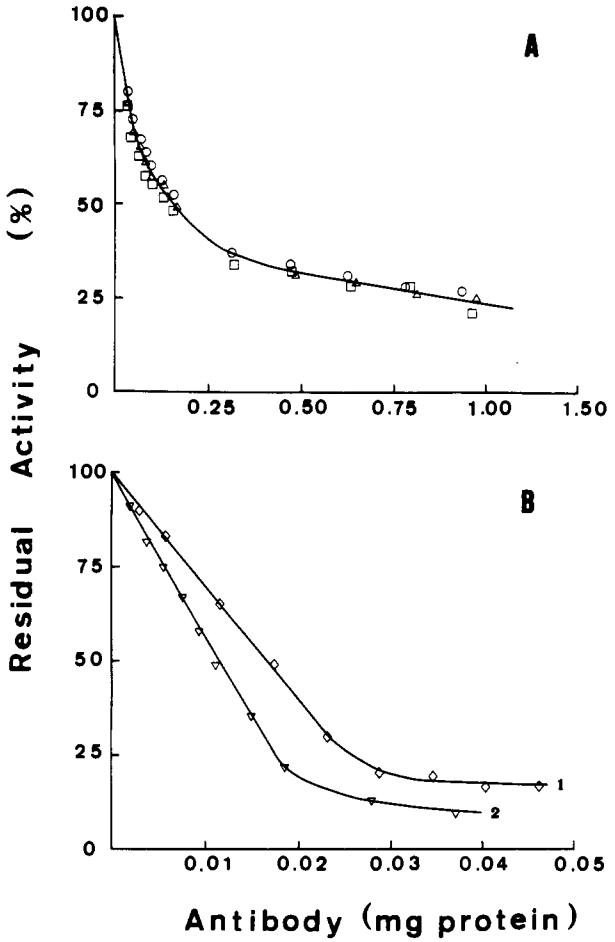

FIGURE 2: Inhibition of the β_2 serine deaminase activity with three classes of monoclonal antibodies. A fixed amount of β_2 subunit (6.75 μg) is incubated with increasing amounts of each monoclonal antibody in 0.1 mL of incubation buffer (buffer A). After 15 min of incubation at room temperature, 0.9 mL of assay medium is added, and the enzymatic activity is measured. The residual activity is expressed as the ratio between the activity found in the assay and the activity obtained without any antibody. The residual activity (in percent) is plotted as a function of the quantity of antibody added: (A) 46-9 (○), 136-3 (□), and 179-2 (△); (B) 68-1 (◇) and 93-6 (▽).

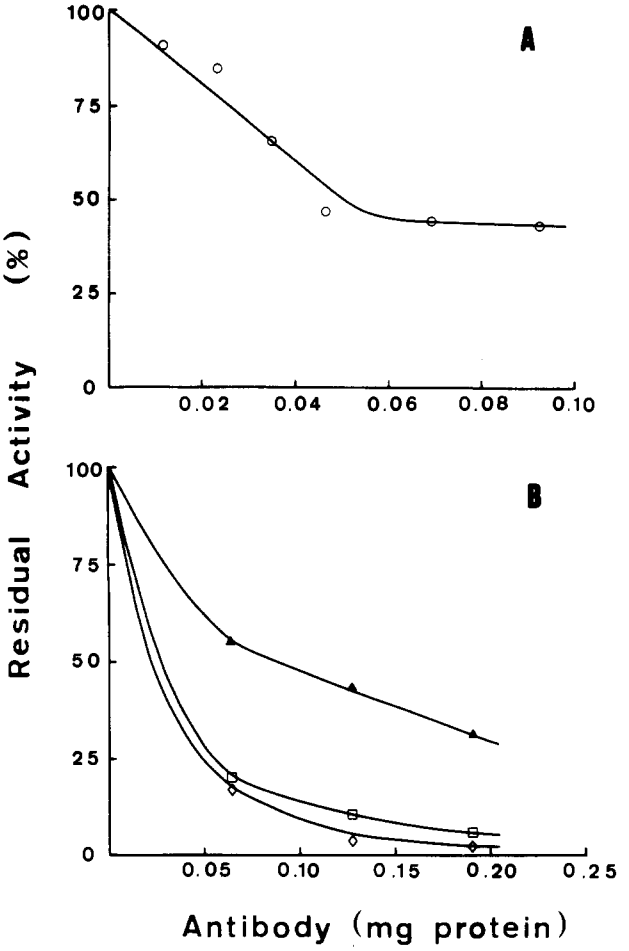

FIGURE 3: Inhibition by a second antibody of the residual activity of the immune complex between β_2 and the first antibody. A fixed amount of β_2 subunit (13.5 μg) is first incubated with about twice the stoichiometric amount of antibody 68-1 (protein content; 0.115 mg) or 93-6 (protein content, 0.075 mg) for 15 min at room temperature. Then, various amounts of a second antibody are added and allowed to bind to the already constituted complex (in 0.1 mL of incubation buffer A). After 15 min of incubation, 0.9 mL of assay medium is added, and the enzymatic activity is measured. The residual activity obtained for each assay is expressed as the ratio between this activity and the activity obtained for the complex in the absence of the second antibody. The residual activity in percent is reported as a function of the quantity of the second antibody added. Panel A represents the inhibition by 68-1 of β_2 complexed with 93-6. Panel B represents the inhibition by 136-3 of β_2 complexed with 68-1 (□) and 93-6 (◇), respectively; the curve of the inhibition of β_2 alone by 136-3 (▲) is also represented.

bodies distinct in epitope specificity are inhibitory. The other antibodies produce no significant inhibition of the serine deaminase activity of β_2 as compared to the control. In addition, the mixture of five antibodies (total protein content 0.75 mg) belonging to each noninhibitory class was not inhibitory under the same experimental conditions.

For each inhibitory antibody, inhibition curves were determined for the serine deaminase activity and are shown in Figure 2. It can be seen (Figure 2A) that the three antibodies of class 3 lead to very similar inhibition curves, with a plateau of inhibition reached only at high amounts of immunoglobulins. On the contrary, antibodies 68-1 and 93-6 (Figure 2B) lead to an inhibition plateau at much lower immunoglobulin concentrations. This can be explained by a higher affinity of these two last antibodies for the β_2 subunit rather than by a different content in specific immunoglobulins, since all the IgG preparations were obtained in the same way. A purification by immunoadsorption of the specific IgG molecules would be needed, however, to confirm this interpretation.

The inhibition efficiency of each inhibitory antibody class thus appeared to be quite different, and in two cases (68-1, 93-6), a nonnegligible residual activity was found at saturation. For each of the three antibody classes, the residual activity at saturation was not changed when the incubation time with β_2 was varied from 5 to 60 min. This reflects their fast binding to β_2.

Because these three classes are directed toward three distinct epitopes on β_2, it was tempting to consider that the inhibition mechanism stems from conformational effects rather than from a direct interaction restricted to the catalytic site. To test such conformational effects, it seemed interesting to study the cumulative action of a second antibody added to a stoichiometric mixture of the β_2 subunit with the first antibody. Three pairs of antibodies have been tested. The results are described in Figure 3. In the three cases, the addition of the second antibody resulted in an increase of the maximum inhibition value. The antibody 68-1 inhibits the residual activity of the 93-6–β_2 complex (Figure 3A) at the same ratio of β_2/antibody concentrations as for the inhibition of β_2 alone (Figure 2B). On the contrary, the antibody 136-3 (belonging to class 3) inhibits the residual activity of the 68-1–β_2 or 93-6–β_2 complexes at lower concentrations (Figure 3B) as compared to the

inhibition by 136-3 of β_2 alone. This phenomenon could be explained by a better binding connected to a better affinity of 136-3 for the immune complexes than for the β_2 subunit. Indeed, such an enhancement of the affinity of an antibody for a protein when the first monoclonal antibody is already bound has been reported for human chorionic gonadotropin (Ehrlich et al., 1982) and for the histocompatibility antigen HLA-A2 (Holmes & Parham, 1983).

The absence of inhibition of β_2 after 15 min of incubation with the seven other monoclonal antibodies could be explained by a lack of interaction between β_2 and these antibodies in solution. To test this possibility, an exclusion chromatography of the β_2–antibody mixture was performed. These antibodies (one from each distinct class and the controls) were incubated with holo-β_2 for 15 min under conditions similar to those described in Table I. The mixture was eluted on an exclusion chromatography column, and the serine deaminase activity of β_2 was tested in the eluted fractions. With all the antibodies, the elution profile for the activity was superimposable with that obtained for β_2 alone. On the contrary, when the same experiment was repeated with the monoclonal antibody 68-1 which inhibits β_2 after 15 min, the residual activity of the 68-1–β_2 complex was found in the dead volume of the column. These results clearly show that, after 15 min, the antibodies that were not inhibitory were not bound to holo-β_2.

Therefore, the antibodies were incubated for 24 h with β_2, and the enzymatic activity was measured. Table I shows that the antibodies can inhibit, at different levels, the serine deaminase activity of β_2. With the four of them (30-2, 69-4, 169-3, and 172-3) for which a noticeable amount of activity remained even after 24 h of incubation, exclusion chromatography was performed and showed that this activity was essentially imputable to free β_2.

(*b*) *Tryptophan Synthase Activity.* It is known (Bartholmes et al., 1976; Wilhelm et al., 1982) that conformational changes of the β_2 subunit occur upon formation of the $\alpha_2\beta_2$ complex. To see whether these changes in conformation affect the antigen–antibody interactions, the effects of monoclonal antibodies on the tryptophan synthase activity of the $\alpha_2\beta_2$ complex were studied. First, it was verified that the monoclonal antibodies which did not affect the serine deaminase activity of β_2 after 15 min of incubation are also noninhibitory for the tryptophan synthase activity of $\alpha_2\beta_2$. This was observed for each antibody taken alone (see Table I) and for a mixture of the five antibodies, as was the case for the serine deaminase activity. The antibodies which were inhibitory after 15 min for the serine deaminase activity also inhibit the $\alpha_2\beta_2$ complex. However, with the three antibodies of class 3 (46-9, 136-3, and 179-2), the $\alpha_2\beta_2$ complex is less inhibited than β_2, even in the presence of a large excess of antibody. This suggested that α and the antibodies of class 3 might compete for binding to β_2. To test this possibility, the residual activity of β_2, in the presence of each antibody and various concentrations of α, was studied. When 6.75 μg of β_2 was incubated with 0.3 mg (protein content) of 46-9 and various amounts of α subunit under the conditions described in Table I, the residual activity increased from 50% (stoichiometric mixture of α and β_2) to 95% (10-fold excess of α over β_2). Likewise, when a similar experiment was performed with the monoclonal antibody 68-1 at a nearly saturating concentration (Ig content; 0.023 mg of protein), 97% of the enzymatic activity was recovered with a 10-fold excess of α subunit. On the contrary, the monoclonal antibody 93-6 interacting with β_2 at a nearly saturating concentration as defined in Figure 2B (Ig content, 0.015 mg of protein) leads to the same inhibition even when the α subunit is added in 10-fold excess. It therefore appears that, while the α subunit is unable to antagonize the inhibitory effect of antibody 93-6 which recognizes the F_2 domain, it is able to restore the enzymatic activity of β_2 in the presence of antibodies 46-9 and 68-1 which recognize the F_1 domain. Two main mechanisms could be envisaged to account for this restoration. One possibility would be that α, β_2, and antibodies form a ternary complex which is active. Another possibility was that the bindings of α and of the antibody to β_2 are mutually exclusive. To distinguish between these two possibilities, the following experiment, aimed at detecting the presence of a ternary complex, was performed. An immunoadsorbent made with rabbit antibody anti-mouse IgG was prepared as described under Materials and Methods. With or without a 3-fold excess of α, 6.75 μg of β_2 was mixed with 0.029 mg (protein content) of 68-1 in 100 μL of incubation buffer B. After 30 min of incubation at room temperature, the residual tryptophan synthase activity was measured. It was found to be 25% and 60%, respectively, in the absence and presence of α. The addition of α thus caused an increase of 35% of the residual tryptophan synthase activity. A similar experiment was then performed where the incubation was made in the presence of an amount of immunoadsorbent sufficient to retain the totality of the monoclonal antibody. After centrifugation to remove the immunoadsorbent, the supernatant was tested: 50% of the initial activity was found in the supernatant when α was present in the incubation medium and only 10% when α was absent. Hence, the addition of α results in the recovery in the supernatant of 40% of the initial activity. Thus, the increase of β_2 activity produced by the addition of α (35%) is very close to the amount of β_2 (40%) released by α from the immunoadsorbent, and hence from 68-1. This allows us to conclude that α and 68-1 cannot bind simultaneously onto β_2.

Discussion

The results reported above can be discussed in relation to the immunogenicity, the structure, and the function of the β_2 protein. With regard to immunogenicity; the ELISA additivity test used to characterize the specificities of the monoclonal antibodies has enabled us to define seven classes of molecules which can, at least in pairs, bind simultaneously to β_2. According to Sachs et al. (1972), this would correspond to a minimum of seven antigenic determinants on β_2. Except in one case (class 7), the antibodies of a given class defined by the ELISA additivity test have a similar behavior in their action on β_2 enzymatic activity, which strengthens the significance of these classes. In addition, the two antibodies D_4-B_6 and 93-6 of class 7, though unable to bind simultaneously to the antigen, produce very different effects when interacting with β_2. While the former binds very slowly to β_2, the latter binds quite rapidly and strongly inhibits both activities of β_2. Thus, these two antibodies appear different and are therefore likely to recognize two distinct epitopes of β_2.

Therefore, a total of eight different epitopes have been defined for the β_2 protein by using monoclonal antibodies. That only 12 randomly selected positive clones could yield antibodies directed against eight epitopes suggests that the total number of antigenic sites on β_2 must be considerably larger than 8. This finding was not in line with the commonly accepted view quoted by Lerner (1982) that "most globular proteins contain fewer than five antigenic sites". Indeed, from the correlation reported by Louvard et al. (1976), one would expect the β_2 protein (M_r 88 000) to carry only about six to seven antigenic determinants.

To account for the large number of determinants found on

β_2, one could assume that this protein, of bacterial origin, is much more immunogenic than most animal proteins usually studied by immunochemists. Another possibility is that during the immune response in vivo, many epitope specificities will ultimately not be expressed in the antiserum because of a negative selection, a process which may be eliminated by the in vitro procedure used for obtaining and isolating clones of specific hybridomas.

Nevertheless, the fact that five of the eight antibodies react only very slowly with the native antigen in solution though they recognize quite rapidly the adsorbed antigen β_2 in the ELISA test prompts a more likely interpretation. Indeed, it can be proposed that these antibodies recognize antigenic determinants normally hidden in the native protein but which become exposed when the protein is coated in the solid-phase procedure. Such antibodies can be produced if, during the immunization, part of the enzyme is proteolyzed and/or denatured and elicits anti-sequence antibodies, comparable to the anti-polypeptide antibodies of Green et al. (1982). If this interpretation is correct, then the ELISA test with a coated protein may lead to the binding of antibodies not specific for the native conformation of the antigen.

Indeed, it has recently been observed (Chaffotte & Goldberg, 1983) that an antiserum raised against the purposely denatured β chain reacts, of course, with the denatured protein but also, though with a lower average affinity, with native β_2. The same study suggested that the conformational flexibility of the enzyme is probably involved in this cross-reactivity between the native and the denatured antigens, since various modifications of the protein (binding of ligands, reduction of the aldimine linkage between the protein and its cofactor) known to stabilize the native conformation reduce the cross-reactivity. Since holo-β_2 is a fairly stable form of the protein, it then seems reasonable to assume that the three monoclonal antibodies which rapidly inactivate it recognize epitopes present at the surface of the native holo-β_2 protein. On the contrary, the slowly binding monoclonal antibodies would recognize antigenic determinants normally buried within the protein; these determinants would then be able to react with the corresponding monoclonal antibodies only after a local, more or less extended, denaturation of the protein. Experiments with the slowly binding monoclonal antibodies have been undertaken to test this interpretation and should yield information on the conformational dynamics of the β_2 protein.

Finally, the functional consequences of the antibody–enzyme interactions yield some valuable information on the mechanisms of the inactivation of β_2 by antibodies. First, monovalent Fab fragments made from polyclonal antibodies show the same inhibitory effects as intact precipitating immunoglobulins. This clearly demonstrates that the basic mechanism of the inhibition is not simply the formation of the network of the immune complex. Second, the inactivation of β_2 by an immune serum had already been reported but had been observed only by use of the enzymatic activity of the $\alpha_2\beta_2$ complex of tryptophan synthase. It therefore could have been assumed that the serum might contain a class of antibodies specific for the site of β_2 which interacts with the α chain; in that case, this antibody would prevent the formation of the $\alpha_2\beta_2$ complex, thus leading to a much reduced activity of β_2 in the assay. This hypothesis is ruled out by our finding that the L-serine deaminase activity of β_2, which is measured in the absence of the α subunit, is inhibited by monoclonal or polyclonal antibodies at least as much as the tryptophan synthase activity of the $\alpha_2\beta_2$ complex.

How then do antibodies inhibit the enzymatic activities of β_2? Two simple mechanisms could readily account for that inhibition. One is that a polyclonal immune serum would contain antibodies directed toward residues of the active site, or very near the active site of β_2, thus forbidding access of the substrates to the catalytic site. The second possible mechanism is that the antibodies, having been obtained by injecting the animals with the native β_2 protein in the absence of its substrates, would bind to β_2 only when that protein has the conformation it adopts in the absence of substrates. If the enzyme has to undergo conformational changes, or structural fluctuations, during a catalytic cycle, then the antibodies might block its activity by "freezing" the structure of the molecule, thus preventing the necessary conformational rearrangements. Its originally seemed easy to us to settle this question by examining the effects of individual monoclonal antibodies on the activity of β_2. Indeed, it would be predicted that if inactivation of β_2 were caused exclusively by antibodies directed at the region of the active site, only a very small minority of the antibody classes would be inhibitory since the catalytic site is likely to cover only a very minor fraction of the protein surface. On the contrary, if the antibodies inactivated β_2 by freezing its conformation, most antibodies should be inhibitory, and very probably should have synergistic effects. In fact, screening the eight classes of monoclonal antibodies has given an apparently clear-cut answer: all the antibodies which were shown to bind to β_2 were also found to reduce its enzymatic activity.

If the slowly binding antibodies indeed recognize and "trap" a denatured form of β_2, their mechanism of action is obvious. The inactivation by the three rapidly binding antibodies is probably more intimately related to the native conformation of β_2. First, it is very unlikely that the inhibition results only from a direct interaction of these antibodies with residues at the active site. Indeed, it seems unreasonable to assume that the active site of native β_2 is large enough to be directly involved in the simultaneous binding of three antibody molecules. Thus, even if one of the monoclonal antibodies might perhaps inactivate β_2 by direct contact (or steric hindrance) with the active site, an alternate model must be imagined for explaining the mechanism of the inactivation by the three rapidly inhibitory antibodies.

A straightforward explanation of the experimental results we obtained may be found by assuming that conformational changes of the β_2 protein are involved in the inactivation process. Indeed, it is known (Bartholmes et al., 1976; Wilhelm et al., 1982) that, upon binding to α, β_2 undergoes a conformational change which is accompanied by a large increase of its enzymatic activity. A nearly as large increase of enzymatic activity (and hence probably the same conformational change) can be elicited in β_2 alone by high concentrations of ammonium ions (Hatanaka et al., 1962). This conformational change may affect a region of β_2 which is recognized by some antibodies; these antibodies, when bound to β_2, will then hinder its conformational rearrangements and thus inhibit the tryptophan synthase activity.

In a similar way, antibodies might affect other structural changes involved in the serine deaminase activity of β_2. Indeed, it is likely that the conformation of β_2 during its catalysis of the serine deamination reaction is different from that of "native" β_2 as suggested by the finding of Miles (1970) that this activity of β_2 is increased by a factor of 2–3 by reacting a specific cysteine of the β chain with *N*-ethylmaleimide.

The experiments conducted with monoclonal antibody 68-1 confirm its conformational influence on β_2 by showing that this antibody and α cannot bind simultaneously to β_2, since this mutual exclusion of α and 68-1 probably does not result from their mere competition for the same site on β_2. This

conclusion is supported by the finding that, when α is added after the formation of the antibody–β_2 complex, the reactivation of β_2, and hence its dissociation from the antibody, is completed in less than 15 min. This is certainly faster than that usually observed for the rate of spontaneous dissociation of an antibody–antigen complex. Thus, the α chain seems to first bind to the immune complex and then modify the conformation of the β_2 subunit through an "induced fit" which would result in the release of the antibody, and in the recovery of the tryptophan synthase activity.

The inactivating effects of antibodies may thus be interpreted in terms of conformational changes which they either prevent by "freezing" or promote by "pulling" β_2 in an active state.

Monoclonal antibodies appear as fairly precise and sensitive probes of the protein conformation and allow one to study in some detail the structure–function relationships within a complex globular protein. A very elegant example of such a study deals with the understanding of the antibody-mediated activation of the inactive β-D-galactosidase produced by some mutants of *E. coli* (Rotman & Celada, 1968). Using an activating monoclonal antibody isolated by Accolla et al. (1981), it has been possible to interpret in detail the mechanisms of the activation. The main conclusion reached by Celada and his co-workers (Celada & Strom, 1983) is that the mutant β-galactosidase molecule has a "quaternary conformation dependent determinant" to which the activating antibody can bind; that this determinant exists in equilibrium between "two shapes", and that the antibody binds preferentially to the nativelike shape of the determinant. Consequently, either by selecting the few nativelike molecules which preexist, thus pulling the equilibrium toward the native conformer, or by inducing a rearrangement of the mutant molecule after an initial weak binding to the inactive conformer, the monoclonal antibody would force the molecule into the active conformation. This mechanism very much resembles that which we postulate for the inactivation of the β_2 subunit of tryptophan synthase and well account for the frequently observed interference of antibodies with the functional properties of the proteins to which they bind.

For this reason, activating and inhibitory monoclonal antibodies might well become a precious tool for investigating the local conformational dynamics involved in the function of proteins.

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