Conformational Effects of Ligand Binding on the β_2 Subunit of *Escherichia coli* Tryptophan Synthase Analyzed with Monoclonal Antibodies†

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ABSTRACT: Five monoclonal antibodies recognizing five different epitopes of the native β_2 subunit of *Escherichia coli* tryptophan synthase (EC 4.1.2.20) were used to analyze the conformational changes occurring upon ligand binding or chemical modifications of the enzyme. For this purpose, the affinities of each antibody for the different forms of the enzyme were determined by using an enzyme-linked immunosorbent assay which allows measurement of the dissociation constant of antigen–antibody equilibrium in solution. The fixation of the coenzyme pyridoxal 5′-phosphate and the substrate L-serine modifies the affinity constants of most of the antibodies for the enzyme, thus showing the existence of extended conformational rearrangements of the protein. The association of the α subunit with the β_2 subunit, which brings about an increase of the tryptophan synthase activity and abolishes the serine deaminase activity of β_2, is accompanied by an important conformational change of the N-terminal domain of β_2 (F_1) since none of the anti-F_1 monoclonal antibodies can bind to $\alpha_2\beta_2$. Similarly, chemical modifications of β_2 which are known to produce significant effects on the enzymatic activities of β_2 result in changes of the affinities of the monoclonal antibodies which can be interpreted as the acquisition of different conformational states of the enzyme.

The refined structural models of proteins obtained by X-ray crystallography give a precise picture of the three-dimensional configuration of these complex macromolecular edifices. However, the rigid models which have been presented usually describe the three coordinates of each atom but fail to take into account a fourth parameter, time. Yet, a good knowledge of the time dependence of the protein conformation, or "protein molecular dynamics", is essential for understanding the functional properties of the protein in solution (Pain, 1983). For instance, "hinge bending" in multidomain enzymes (Mc Cammon et al., 1976; Janin & Wodak, 1983) or local movements of active-site residues upon ligand binding (Hartsuck & Lipscomb, 1971) have been reported by X-ray crystallographers and postulated to pertain to the catalytic mechanism of the protein. Similarly, protein structural dynamics reflecting the conformational flexibility of some regions of a protein have been evidenced by the high-temperature factor in the electron density map (Frauenfelder et al., 1979), and recently, these regions of the protein have been tentatively correlated to their higher antigenicity compared to more rigid areas of the molecule (Westhof et al., 1984; Tainer et al., 1984).

Thus, the dynamic properties of a protein in its native state may be of crucial importance for the expression of its biological function. Therefore, coenzyme binding or zymogen activation, which drastically modifies the functional properties of a protein, may act by changing the molecular dynamics of the polypeptide chain as well as by inducing large structural rearrangements of the molecular edifice.

Most of the detailed results on protein conformational changes have been obtained from studies of X-ray diffraction by protein crystals (Ringe & Petsko, 1985). On the other hand, a variety of physicochemical approaches have been used, including nuclear magnetic resonance (NMR),[1] time-resolved fluorescence, small-angle X-ray diffraction, and theoretical calculations (Citri, 1973; Welch et al., 1982), to investigate the molecular dynamics of proteins in solution (i.e., under the "physiological" conditions where their function is usually studied). But even if detection or prediction of conformational changes has become possible, a clear correlation between an observed signal and the dynamic properties investigated can hardly be established.

Immunochemical approaches have already been used to help in overcoming this difficulty. Indeed, polyclonal antibodies have been used to analyze conformational equilibria (Sachs et al., 1972; Furie et al., 1975; Hurrell et al., 1977; Madar et al., 1980; Nagy et al., 1982) or to characterize intermediates trapped during protein folding (Creighton et al., 1978). It therefore seemed possible to take advantage of the high specificity and sensitivity of these biological "reagents" to investigate the minute, but fundamental, conformational changes that may occur during the catalytic process or in the last steps of the folding of a protein. However, a major difficulty encountered in the use of polyclonal antibodies as conformational probes came from the heterogeneity (both in specificity and in affinity) of antisera even after attempts to fractionate specific antibodies. This difficulty no longer exists if one uses *monoclonal* antibodies as conformational probes. Thus, monoclonal antibodies have been used to investigate structural transitions occurring upon activation of factor V (Foster et al., 1983) or calcium binding by two different proteins (Kilpatrick et al., 1982; Lewis et al., 1983).

Therefore, we undertook an investigation of the conformational dynamics of an enzyme (the β_2 subunit of *Escherichia*

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[1] Abbreviations: α or β_2, α or β_2 subunit of *Escherichia coli* tryptophan synthase (EC 4.2.1.20); apo or holo, form free of pyridoxal 5′-phosphate or saturated with pyridoxal 5′-phosphate, respectively; β_2-NEM, β_2-labeled with *N*-ethylmaleimide on cysteine-170; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; F_1, N-terminal fragment of β chain (M_r 29 000); F_2, C-terminal fragment of β chain (M_r 12 000); Fab, fragment antigen binding of immunoglobulin; FPLC, fast protein liquid chromatography; IgG, immunoglobulin G; K_D, dissociation equilibrium constant; K_A, association equilibrium constant; NEM, *N*-ethylmaleimide; NMR, nuclear magnetic resonance; pyridoxal-P, pyridoxal 5′-phosphate.

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coli tryptophan synthase) with a panel of monoclonal antibodies specific for different regions of the protein and which have been raised and well characterized in our laboratory (Djavadi-Ohaniance et al., 1984; Friguet et al., 1984).

The β_2 protein has been chosen for this study because it is a rather flexible molecule, known to undergo a variety of conformational changes at different steps of its activation and catalytic process. Thus, this dimeric protein binds two pyridoxal 5′-phosphate (pyridoxal-P) molecules in a cooperative manner, and the binding fits the allosteric model, which strongly suggests the existence of spontaneous conformational equilibria in the apoenzyme (Tschopp & Kirschner, 1980). The conformational change stabilized by the binding of pyridoxal-P has been detected by kinetic as well as by steady-state fluorescence measurements (Tschopp & Kirschner, 1980; Lane, 1983). In addition, as a consequence of its association with the α subunit, β_2 undergoes another conformational change (Wiesinger & Hinz, 1984; Lane et al., 1984) responsible for the enhancement of its tryptophan synthase activity and the loss of its serine deaminase activity (Crawford & Yanofsky, 1958; Crawford & Ito, 1964). Furthermore, the binding of the substrate L-serine to β_2, followed by the formation of a Schiff base with pyridoxal-P, is likely to be accompanied by a conformational event since the second substrate, indole, can bind to β_2 only when L-serine is already present (Lane & Kirschner, 1983b). Finally, our previous studies on the functional effects of enzyme–antibody interactions strongly suggested that, in the case of β_2, enzyme inactivation by antibodies results from perturbations of the molecular dynamics rather than from a direct interferences with the active site (Djavadi-Ohaniance et al., 1984).

To investigate the extent and functional importance of these molecular movements, five monoclonal antibodies recognizing distinct epitopes on β_2 have been selected. The present paper describes experiments, performed with a recently developed enzyme-linked immunosorbent assay (ELISA), for measuring binding constants in solution (Friguet et al., 1985), which allowed us to determine the affinity of each monoclonal antibody for various forms of the β_2 protein. The effects of specific ligands (pyridoxal-P, L-serine, α subunit) or of chemical modifications (maleimidation of cysteine-170, nicking of the polypeptide chain) on the binding constants have been studied. The observed effects will be shown to reflect conformational changes involving most of the protein surface but affecting to different extents the five regions corresponding to the antigenic determinants investigated.

Materials and Methods

The apo-β_2 subunit of tryptophan synthase from *Escherichia coli* K 12 was prepared as described by Högberg-Raibaud and Goldberg (1977a).

Reconstitution of the holoenzyme was achieved by incubation of the apoprotein in the presence of 0.05 mM pyridoxal-P.

Nicked holo-β_2 was obtained by proteolysis of holo-β_2 (Högberg-Raibaud & Goldberg, 1977a) and nicked apo-β_2 by incubation of nicked holo-β_2 with hydroxylamine (Zetina & Goldberg, 1980).

Sulfhydryl modification of holo-β_2 with *N*-ethylmaleimide (NEM) was performed as described by Miles (1970). The resulting NEM protein labeled on cysteinyl residue 170 (Miles & Higgins, 1980) was named holo-β_2–NEM.

The α subunit of tryptophan synthase was prepared as described by Hatanaka et al. (1962).

Monoclonal Antibody Preparation. Murine monoclonal antibodies were produced by the technique of Köhler and Milstein (1975) or that of Buttin et al. (1978) as previously described (Friguet et al., 1983a,b).

The immunoglobulins were obtained by precipitating the ascitic fluids with 18% (w/v) sodium sulfate.

Preparation of fragment–antigen binding was performed as described previously (Porter, 1959; Mariuzza et al., 1985).

Protein and Enzymatic Activity Assays. Protein concentrations were determined by the method of Lowry et al. (1951) or by measuring absorbance. The extinction coefficients used were $\epsilon_{280nm}^{mg/mL} = 0.6$, 0.65, 0.46, 1.4, and 1.5 for apo-β_2, holo-β_2, α, immunoglobulin G (IgG), and Fab, respectively (Miles, 1970; Porter, 1959; Onoue et al., 1965).

The activity of the β_2 subunit was measured in the indole to tryptophan reaction in the presence of an excess of α subunit as described by Faeder and Hammes (1970) and in the serine deamination reaction as reported previously by Crawford and Ito (1964).

ELISA Techniques. To determine the epitope specificity of monoclonal antibodies and to define whether an antibody recognizes the native or a denatured form of the antigen in solution, the additivity test and the competition test we have set up (Friguet et al., 1983a, 1984) were used.

The dissociation constants (K_D) of antigen–antibody equilibrium in solution were determined by the enzyme-linked immunosorbent assay (ELISA) described recently (Friguet et al., 1985).

For the holo forms of the enzyme, pyridoxal-P or/and L-serine were added to the incubation buffer at respective concentrations of 0.1 and 100 mM. To avoid fixation of pyridoxal-P to the bovine serum albumin (BSA) present in the incubation buffer, acetylated BSA (0.02% w/v) was used.

Absorption measurement of the product of *o*-nitrophenyl β-D-galactopyranoside (ONPG) hydrolysis in the final step of the ELISA allows measurement of dissociation constants down to only about 10^{-9} M. Since we had to measure dissociation constants as low as 10^{-11} M, it was necessary to increase the sensitivity of the assay by using a fluorogenic substrate of β-galactosidase and measuring fluorescence instead of absorption. The fluorogenic substrate was 4-methylumbelliferyl β-D-galactopyranoside used at 0.2 mM. The fluorescence of the product was measured on a Titertek Fluoroscan.

Each affinity measurement was repeated at least 2 times, and in all experiments, each antibody was analyzed in triplicate.

Fast Protein Liquid Chromatography. Exclusion chromatography analysis of Fab–β_2 or IgG–β_2 complexes was performed with the TSK G3000 SW-LKB column and the Pharmacia FPLC system.

The column was previously equilibrated with a 100 mM potassium phosphate buffer (pH 7.2) containing 0.05 mM pyridoxal-P. Samples of 30 μL were injected, and the absorbance was detected at 280 nm by a Pharmacia UV-1 monitor. The flow rate was 1 mL/min.

Results

In the present work, five monoclonal antibodies were chosen on the basis of their ability to recognize different antigenic sites of the native β_2 subunit. Indeed, it was a prerequisite to our study to use a sufficient number of antibodies of different specificities so as to investigate as many distinct regions of the native protein as possible.

Characterization of Monoclonal Antibodies. Three monoclonal antibodies specific to native β_2, 46-9, 68-1, and 93-6, were already available and have been described elsewhere (Djavadi-Ohaniance et al., 1984): 46-9 and 68-1 are directed against two different epitopes of the N-terminal domain (F_1)

Table I: Additivity Indexes[a] Obtained with the Four Anti-F_1 Antibodies

monoclonal antibodies	31-2	46-9	68-1
15-1	45	40	100
31-2		41	85
46-9			80

[a] For each pair of antibodies, an additivity index (AI) (Friguet et al., 1983a,b) 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.

of the β chain, and 93-6 is directed against the C-terminal domain (F_2). All of them recognize the native protein (Friguet et al., 1984). Among a series of recently obtained monoclonal antibodies, two others, 15-1 and 31-2, have been retained. They are directed against epitopes carried by the N-terminal fragment (F_1). They recognize two epitopes different from those recognized by 46-9 and 68-1 since the additivity indexes obtained by using the additivity test (Friguet et al., 1983a) with these four anti-F_1 antibodies were higher than 40% (Table I). Such values of additivity indexes indicate that the four antibodies recognize four distinct antigenic sites of β_2. The competition test (Friguet et al., 1984) has shown that antibodies 15-1 and 31-2 bind rapidly in solution to the native form of the protein since more than 90% of each antibody is trapped by native β_2 after a 15-min incubation.

As in the case of 46-9, 68-1, and 93-6, these two antibodies inhibit the enzymatic activity of β_2: antibody–antigen complex formation reduces the serine deaminase activity of β_2 by 90% for 15-1 and 100% for 31-2 under the assay conditions previously described (Djavadi-Ohaniance et al., 1984).

Stoichiometry of β_2–Fab Interactions. Before starting any quantitative analysis of the antigen–antibody interaction, it was necessary to determine if the epitopes of the two β chains of a β_2 subunit are able to simultaneously bind antibody molecules. Since the antigen is a divalent molecule, the determination of the stoichiometry can be conveniently conducted only with monovalent antibodies. Therefore, Fab fragments of each monoclonal antibody were prepared, and their binding to β_2 was quantitatively studied. The titration of the antibody binding sites on β_2 was performed by using FPLC. A known amount of purified Fab (10^{-5} M) was incubated with various concentrations of β_2 (from 0.5×10^{-5} to 2×10^{-5} M in terms of the β chain). Then, 30 μL of the mixture was injected in the FPLC column; the formation of the β_2–Fab complex and the disappearance of the free Fab fraction were followed on the elution profile. From this study, it has been found that one β_2 dimer is able to bind two Fab fractions in the case of 31-2 and 68-1 but only one in the case of 15-1, 46-9, and 93-6. The behavior of these last three antibodies could be easily explained by steric hindrance if the corresponding epitopes are close enough to the association area between the two β chains, recalling that each Fab fragment is larger than each β chain.

Conformational Changes of β_2 Induced by Fixation of Specific Ligands. The affinity of an antibody for its antigen depends on the structural complementarity of the two molecules. It will therefore be sensitive to the conformation of the antigen, at least, in the vicinity of the epitope recognized by the antibody when the antigen is a macromolecule. To detect conformational changes of the β_2 subunit induced by the fixation of its specific ligands (pyridoxal-P, L-serine, and the α subunit) and eventually characterize these changes in terms of their amplitude and localization on β_2, we have determined the affinity constants of each of the antibodies described above with β_2 in the absence and presence of these ligands. This was

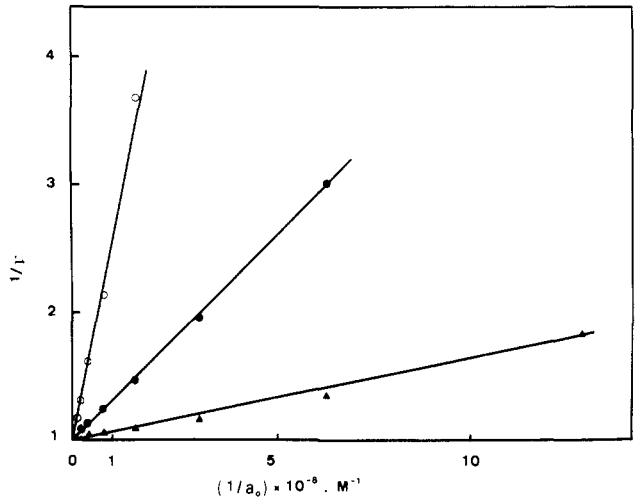

FIGURE 1: Klotz plot of the binding of the monoclonal antibody 46-9 to apo-β_2 and holo-β_2 in the absence and presence of L-serine. The dissociation constant K_D is deduced from the equation $1/\nu = 1 + K_D/a_0$; ν is the fraction of bound antibody and a_0 the total antigen concentration. $1/\nu = i_0/(i_0 - i)$, where i_0 and i are respectively the total and free antibody concentration, is obtained from measurement of the absorbance (or fluorescence) in our ELISA method (Friguet et al., 1985). In this method, $1/\nu = A_0/(A_0 - A)$ where A_0 is the absorbance (or fluorescence) measured in the ELISA for the antibody in the absence of antigen and A the absorbance (or fluorescence) measured for the antibody with a given concentration (a_0) of antigen. The affinity constants (K_A) ($K_A = 1/K_D$) are 15×10^8 M^{-1} for apo-β_2, 3.2×10^8 M^{-1} for holo-β_2, and 0.7×10^8 M^{-1} for holo-β_2 in the presence of L-serine.

Table II: Affinity Constants[a] of the Monoclonal Antibodies for Apo-β_2 and Holo-β_2 in the Absence and in the Presence of Serine

		holo-β_2	
monoclonal antibodies	apo-β_2	−serine	+serine
15-1	40	5.3	1.9
31-2	50	5.5	1.5
46-9	15	3.2	0.7
68-1	100	100	35
93-6	25	5.9	4.2

[a] The affinity constants (K_A) were calculated as described in Figure 1 and are expressed in $M^{-1} \times 10^{-8}$.

done with a method we described previously (Friguet et al., 1985) which uses an ELISA and allows precise measurement of the affinity of the antigen–antibody complex in solution without the need for highly purified antibody preparations. Immunoglobulin fractions obtained by an 18% sodium sulfate precipitation were used. The principle of the method is as follows: for each antibody, various amounts of antigen are incubated with a constant amount of antibody in solution. After the equilibrium is reached (15-h incubation at 20 °C), the proportion of antibody which remains unsaturated is measured by a classical indirect ELISA. The results, analyzed as proposed previously (Friguet et al., 1985), are plotted in the representation of Klotz (see Figure 1) which allows us to calculate the affinity constant from the reciprocal of the slope of the straight line obtained. In the calculations, the antigen concentration was that of β_2 for antibodies 93-6, 15-1, and 46-9 and twice that of β_2 for antibodies 31-2 and 68-1 (see above).

(A) Effect of the Coenzyme Pyridoxal 5'-Phosphate. The first ligand investigated was pyridoxal-P. To analyze its effects on the conformation of β_2, the affinity constants of each monoclonal antibody to the apo- and the holoenzyme were determined and are reported in Table II. The results clearly show the existence of two different conformational states of β_2 since, for the antibodies 46-9, 15-1, 31-2, and 93-6, the

affinity constants are 5–10-fold higher for apo-β_2 than for holo-β_2. Conversely, antibody 68-1 has a similar affinity for the apo- and holoenzymes. This suggests that no conformational rearrangement occurs, upon binding of pyridoxal-P, in the region of β_2 carrying the antigenic determinant recognized by antibody 68-1. Alternatively, in the case of 68-1 the nature of the antibody–β_2 interaction might be such that the binding of the antibody to the antigen would not depend much on the local conformation of the polypeptide chain. The latter interpretation seems unlikely, however, in view of the sensitivity of the binding to other factors (see below).

(*B*) *Effect of the Substrate* L-*Serine.* It was known that apo-β_2 does not bind L-serine. We therefore first verified that the binding of antibodies to apo-β_2 is not affected by serine. Indeed, we found no difference in affinity of the antibodies for apo-β_2 in the absence and in the presence of serine.

The influence of one of its substrates, L-serine, on holo-β_2 was then tested by measuring the affinity constant of each holo-β_2–antibody complex in the absence and in the presence of serine. The results reported in Table II show that all have a 3–5-fold higher affinity for holo-β_2 in the absence of serine than in the presence of serine. On the contrary, the presence of serine does not significantly change the affinity of the anti-F_2 antibody (93-6) for the enzyme.

(*C*) *Effect of the Binding of* α *to* β_2. We had previously reported that α and the antibody 68-1 cannot bind simultaneously to β_2. We therefore first investigated the ability of the other antibodies to bind to the $\alpha_2\beta_2$ complex. This was done by FPLC gel filtration experiments in the following way. The $\alpha_2\beta_2$ complex was first formed by adding to holo-β_2 (2×10^{-5} M) a 2-fold molar excess of α subunit. Then, a 2-fold excess of antibody was added. After 30 min of incubation, the mixture was analyzed by FPLC. With antibody 93-6 which recognizes F_2, the elution diagram showed a large peak corresponding to the immune complex. Furthermore, the height of the peak corresponding to free α indicated that no α had been released from β_2 upon addition of the antibody. This demonstrates that 93-6 can bind to $\alpha_2\beta_2$. On the contrary, the 15-1, 31-2, and 46-9 antibodies recognizing the F_1 domain failed to give rise to a peak of immune complex. Moreover, with the anti-F_1 antibodies, when the antibody–β_2 complex was first formed, the addition of α led to the slow release of the antibody from the complex. This is illustrated by the experiment reported in Figure 2. It shows that the addition of α produces an increase of the peak corresponding to the free antibody and the $\alpha_2\beta_2$ complex and a simultaneous decrease of the peak corresponding to the antibody–β_2 complex.

Since none of the anti-F_1 antibodies can bind to $\alpha_2\beta_2$, the affinity of the antibody for the holo–$\alpha_2\beta_2$ complex was measured only for 93-6. The value obtained, 1.7×10^8 M^{-1}, is only 3-fold lower than for holo-β_2 ($K_A = 5.9 \times 10^8$ M^{-1}), thus showing that the F_2 domain of β_2 probably does not undergo a drastic conformational change when α binds to holo-β_2. Moreover, as reported above for holo-β_2, the presence of serine does not modify the affinity of 93-6 for $\alpha_2\beta_2$.

Conformational Changes of β_2 *Induced by Chemical Modification of the Protein.* The conformation of β_2 as shown above is affected by the binding of ligands. It seemed interesting to study how chemical modifications of the protein known to alter its functional properties could be related to conformational changes.

(*A*) *Nicked Apo- and Holo-*β_2. Mild proteolytic treatment of the β_2 subunit by trypsin results in a nick in the polypeptide chain in the histidine-273–lysine-283 region which corresponds to the hinge peptide between the two domains, F_1 and F_2, of the protein. The resulting protein, though enzymatically inactive, retains a quasi-native structure, remains dimeric, and is able to bind the coenzyme pyridoxal-P and the substrate L-serine and even to give rise to the formation of an intermediate in the catalytic mechanism (Högberg-Raibaud & Goldberg, 1977b). However, the binding constants of the ligands are modified (Högberg-Raibaud & Goldberg, 1977b), and the fixation of the coenzyme is no more cooperative (Tschopp & Kirschner, 1980). It has been postulated that the latter property results from the freezing of β_2 in an inactive conformation, caused by the "nick" (Tschopp & Kirschner, 1980; Chaffotte & Goldberg, 1984). Since the monoclonal antibodies might reveal conformational differences between the intact and the nicked protein, the affinity constants of the five monoclonal antibodies for nicked apo-β_2 and nicked holo-β_2 in the absence and in the presence of serine were determined. The results are reported in Table III.

In the case of the apoprotein, all the antibodies except 68-1 have a 7–17-fold higher affinity for apo-β_2 than for nicked apo-β_2. On the contrary, in the case of the holoprotein, no significant difference of affinity of the antibodies for either

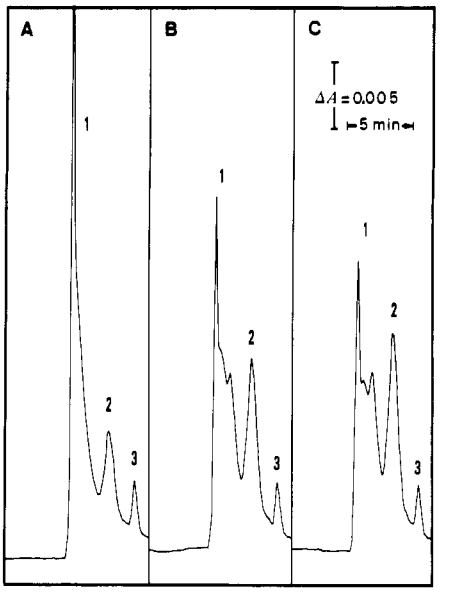

FIGURE 2: FPLC elution profile of the antibody 68-1–β_2 complex at different times of incubation with the α subunit. Holo-β_2 at 0.6×10^{-5} M was incubated at 20 °C with the monoclonal antibody 68-1 at 1.2×10^{-5} M in 100 mM potassium phosphate buffer (pH 7.2) containing 0.05 mM pyridoxal-P and 50 mM L-serine. After 15 min, the α subunit at 2.4×10^{-5} M was added, and 30-μL samples were injected in the FPLC column. (A) Immediately after the addition of α; (B) 15 min after the addition of α; (C) 30 min after the addition of α. In the elution profile, peak 1 corresponds to the 68-1–β_2 complexes, and it exhibits, in the presence of α (B and C), two shoulders representing low molecular weight complexes. Peaks 2 and 3 correspond respectively to antibody 68-1, which is eluted at the same time as the $\alpha_2\beta_2$ complex, and the α subunit.

Table III: Affinity Constants[a] of the Monoclonal Antibodies for Nicked Apo-β_2 and Nicked Holo-β_2 in the Absence and in the Presence of Serine

monoclonal antibodies	nicked apo-β_2	nicked holo-β_2	
		−serine	+serine
15-1	2.9	5.3	2.4
31-2	2.9	3.8	2.9
46-9	1.6	2.9	1.6
68-1	67	140	140
93-6	1.3	4.3	4.3

[a] The affinity constants (K_A) were calculated as described in Figure 1 and are expressed in $M^{-1} \times 10^{-8}$.

Table IV: Affinity Constants[a] of the Monoclonal Antibodies for Holo-β_2 and Holo-β_2–NEM in the Absence and in the Presence of Serine

monoclonal antibodies	holo-β_2		holo-β_2–NEM	
	–serine	+serine	–serine	+serine
15-1	5.3	1.9	2.8	1.4
31-2	5.5	1.5	2.1	1.1
46-9	3.2	0.7	1.5	0.8
68-1	100	35	100	100
93-6	5.9	4.2	5.9	5.5

[a] The affinity constants (K_A) were calculated as described in Figure 1 and are expressed in $M^{-1} \times 10^{-8}$.

holo-β_2 or nicked holo-β_2 is observed.

It should be pointed out that the apo–holo structural transition, which was clearly evidenced with the intact protein, does not seem to be very significant for the nicked enzyme as shown by the small differences between the affinity constants found for nicked apo- and nicked holo-β_2 with all the antibodies. Likewise, only very small effects of serine on the affinity constants could be observed for nicked holo-β_2 protein. Thus, the conformation of nicked-β_2 seems to be much less sensitive to ligand binding than that of uncleaved, native β_2.

(*B*) *Holo-β_2-N-Ethylmaleimide.* Modification of one sulfhydryl residue (Cys-170) of holo-β_2 by NEM leads to the loss of the indole → tryptophan activity and the increase (2–3-fold) of the serine deaminase activity (Miles, 1970). Since the rates of these two reactions catalyzed by the β_2 subunit are so differently affected by NEM modification, it seems likely that the effects of the modification are not limited to interfering with a specific catalytic residue but may involve either a conformational change which affects the active center or a modification of the environment of the active site caused by the presence of NEM. To distinguish between these possibilities, we compared the affinities of the monoclonal antibodies for holo-β_2 and holo-β_2–NEM in the absence and in the presence of serine. The affinity constants of the antibodies for holo-β_2–NEM are reported in Table IV.

For antibody 68-1, the modification of β_2 by NEM does not affect the affinity constant in the absence of serine. However, while the presence of serine modified the interaction of 68-1 with intact β_2, it does not affect the affinity of this antibody for holo-β_2–NEM. Similarly, serine hardly affects the affinities of the three other anti-F_1 monoclonal antibodies with holo-β_2–NEM, while it did reduce their affinities for intact holo-β_2. Therefore, the conformation of holo-β_2–NEM seems hardly sensitive to serine. Moreover, while the conformation of holo-β_2–NEM seems closer to that of holo-β_2 in the presence of serine when probed with antibodies 15-1, 31-2, and 46-9, it looks similar to holo-β_2 in the absence of serine when probed with antibody 68-1.

Discussion

The results described in the present report lead to two important conclusions. First, they show that, in the β_2 subunit of *E. coli* tryptophan synthase, the binding of each of the specific ligands we studied (the coenzyme pyridoxal-P, the substrate L-serine, and the activating α subunit) brings about changes in the conformation of the protein significant enough to modify the affinity of specific monoclonal antibodies for the enzyme. Second, they show that these changes are not limited to a small region of the protein, near the ligand binding site. Rather, they affect an extended area of the protein surface since each ligand modifies the association constant of at least four monoclonal antibodies recognizing distinct epitopes, situated far enough from each other to allow the simultaneous binding of the corresponding antibodies to the antigen.

Though easily detected by undoubtedly significant modifications of the affinity constants, the changes undergone by the protein are likely to be only small alterations of its conformation. Indeed, except in the case of the α chain, the binding of a ligand or the chemical modification of β_2 (NEM labeling or "nicking") changes the affinities by factors ranging from about 2 to 20. This corresponds to a decrease of at most 1.5 kcal/mol of the free energy of interaction between the antibody and β_2. Such a small variation of the interaction energy can be accounted for by the disappearance of as little as one hydrogen bond or by the reduction by about 50 Å^2 of the contact area between the antibody and the antigen (Chothia & Janin, 1975). This is a relatively small modification when compared to the large contact area reported in the single case where the structure of a protein–antibody complex has been solved by X-ray crystallography (Amit et al., 1985). Therefore, unless one admits that each of the five monoclonal antibodies is extremely flexible and able to easily adapt its binding site to large modifications of the corresponding antigenic determinants, the conformational changes of β_2 probably have small amplitudes.

Two interpretations can account for the differences observed between the various conformations of β_2 depending on how one views its interaction with a specific antibody. If the two proteins are considered as fairly rigid molecules, our results would indicate that the atoms of β_2 involved in the five antigenic determinants studied undergo noticeable (though small as discussed above) relative movements under the influence of ligand binding or chemical modification. Alternatively, the antigen can be considered as undergoing a conformational adaptation when binding to the antibody; the affinity changes observed in the presence of ligands would then indicate that the ligands (or chemical modifications) used in this study promote changes in the flexibility of the β_2 protein. Whatever the interpretation may be, the structural or flexibility differences between the various states of β_2 are likely to be quite small since, as discussed above, the affinity changes observed are relatively small.

One could ask whether these conformational changes are functionally important ones and hence can be related to those inferred from previous studies on the kinetics or equilibrium of binding of β_2 to its various ligands: pyridoxal-P (Tschopp & Kirschner, 1980), L-serine (Lane & Kirschner, 1983a), and the α chain (Lane et al., 1984). For instance, it had been concluded that the cooperative binding of pyridoxal-P to β_2 may reflect a structural change of the protein (Tschopp & Kirschner, 1980). The affinity differences observed between apo- and holo-β_2 for the binding of four monoclonal antibodies (Table II) could, at first sight, be thought to reflect the allosteric transition which had been postulated. This view is not compatible, however, with the results obtained with nicked β_2. Indeed, both the binding properties (Tschopp & Kirschner, 1980) and the accessibility to water of the tryptophan residues in nicked β_2 (Chaffotte & Goldberg, 1984) suggested that the nicked protein is "frozen" in a conformational state similar to that of apo-β_2. While nicked β_2 indeed seems frozen when probed with our antibodies (pyridoxal-P does not affect significantly the binding of the antibodies to the nicked protein), its conformation resembles that of holo-β_2 more than that of apo-β_2 as judged by the values of the affinity constants. Therefore, either the relatively large affinity changes observed upon binding pyridoxal-P to intact β_2 reflect a loss of flexibility of the polypeptide chain rather than a shift from one structure

to another or the nicking of β_2 results in a hybrid conformation in which the antigenic sites observed would be "holo-like" while the coenzyme binding site and the tryptophanyl residue environment would be "apo-like".

The binding of L-serine to holo-β_2 is known to be followed by a rearrangement at the active site of the β_2–pyridoxal-P–serine complex (Lane & Kirschner, 1983b) which is involved in the rate-limiting step of the β_2-catalyzed enzymatic reaction (Lane & Kirschner, 1983a). The results we obtain with monoclonal antibodies do confirm that β_2 undergoes a conformational change when binding L-serine. Moreover, they indicate that this change affects primarily the F_1 domain since the affinities of all anti-F_1 antibodies are reduced by L-serine, while that of the anti-F_2 antibody 93-6 remains unchanged (Table IV). In addition, they may provide a clue to understanding why NEM labeling of the single cysteinyl residue accessible per β chain in holo-β_2 results in a 2–3-fold increase of the serine deaminase activity of β_2 (Miles, 1970). Indeed, with three out of the four antibodies recognizing sites carried by F_1, the affinity for holo-β_2–NEM in the absence of serine is close to that of holo-β_2 in the presence of serine. Furthermore, the same antibodies detect only very small changes of holo-β_2–NEM in the presence of serine. This suggests that, even in the absence of serine, the NEM-labeled protein might already be close to the conformation that holo-β_2 adopts only in the presence of L-serine. By maintaining β_2 in this conformation, NEM would then eliminate the rate-limiting isomerization involved in the serine deaminase activity of unmodified β_2 and therefore increase the enzymatic activity of β_2. This interpretation implies that the serine-induced conformational change of β_2 detected with the monoclonal antibodies coincides with the isomerization detected by rapid-kinetics measurements (Lane & Kirschner, 1983a,b).

Our observations that the binding of nicked β_2 to all the monoclonal antibodies is hardly affected by pyridoxal-P or L-serine (Table III) led to the conclusion reached also by Kirschner and his co-workers (Tschopp & Kirschner, 1980) on the basis of kinetic studies that nicked β_2 corresponds to a frozen conformation of β_2. Nicked β_2 thus appears as more rigid than unmodified β_2. This property may explain why nicked β_2 is inactive if, as suggested earlier (Djavadi-Ohaniance et al., 1984), molecular movements are involved in the catalytic mechanism of β_2.

The α chain, when it binds to β_2, brings about a strong increase in the tryptophan synthase activity and abolishes the serine deaminase activity of β_2 (Crawford & Yanofsky, 1958). This has been related to an isomerization of β_2 characterized by fast kinetics methods (Lane et al., 1984). Our studies with monoclonal antibodies clearly show that this isomerization corresponds to a large conformational change of the F_1 domain within β_2. By "large", it is meant that the amplitude of the deformation of each antigenic site is important, as judged from the complete lack of association of $\alpha_2\beta_2$ with the four anti-F_1 monoclonal antibodies. However, it is also meant that the conformational change extends throughout a large area of the F_1 domain, as shown by the fact that none of these four monoclonal antibodies can bind to $\alpha_2\beta_2$. Indeed, a direct competition between each antibody and α for the same site of β_2, or steric hindrance between α and the antibodies, can certainly not account for this multiple antagonist effect of α. Since the four monoclonal antibodies can bind simultaneously to β_2, the corresponding antigenic sites of β_2 must be sufficiently distant from each other to rule out the possibility of significant steric hindrance between α (which is even smaller than an IgG molecule) and each of the four antibodies; therefore, the mutual exclusion of α and of all anti-F_1 antibodies clearly reflects the loss of antigenic sites on β_2 in the $\alpha_2\beta_2$ complex, and hence an extended conformational change of the F_1 domain upon binding the α subunit. On the contrary, since the 93-6 antibody binds to $\alpha_2\beta_2$ nearly as well as to β_2, it seems very likely that the F_2 domain is not affected by α and that α binds to a site carried by F_1.

From the above considerations, it appears that the changes of affinity toward specific monoclonal antibodies which β_2 exhibits upon ligand binding or chemical modification are closely related to the structural changes which had been inferred from the functional properties of the protein. Hence, monoclonal antibodies turn out to be appropriate probes for detecting small modifications of the structure, or of the molecular flexibility, involved in the biological function of a protein. For this purpose, precise quantitative measurements of the association constants between the protein and antibodies must be made because, as seen in the present study, the antigen–antibody interaction may be only weakly modified by the presence of the ligands. However, a hint at the existence of functionally important molecular movements can be given by a quick screening of the catalytic activity of enzyme–antibody complexes. If an enzyme is highly flexible, several monoclonal antibodies will be sensitive to ligand-related conformational changes and will therefore probably interfere with the catalytic activity of the protein by preventing its normal molecular dynamics. This is likely to occur in *E. coli* tryptophan synthase where each monoclonal antibody we studied inhibits the enzymatic activity of β_2 (Djavadi-Ohaniance et al., 1984). If, on the contrary, the protein is rigid, neither substrates nor specific antibodies will modify its structure, and bound antibody molecules will not inhibit the catalytic activity, as is, for instance, the case for *E. coli* β-D-galactosidase (Cohn & Torriani, 1952).

Thus, through an analysis of the functional properties of protein–monoclonal antibody complexes, and through precise measurements of the association constants of these complexes in the presence of protein ligands, it is possible to detect and characterize intramolecular movements of a protein related to its biological function. In particular, because each monoclonal antibody is a probe of a specific region of the protein, the conformational changes induced, or stabilized, by a given ligand can be assigned to the region carrying the antigenic sites recognized by the monoclonal antibodies sensitive to the ligand. Such assignments may be prominently interesting if the localization of the antigenic sites in the three-dimensional structure of the protein is known. Monoclonal antibodies might then usefully supplement the information obtained from X-ray crystallography for understanding the role of fluctuations in the protein structure. When the X-ray structure of tryptophan synthase is available (Ahmed et al., 1985), it is expected that the results reported above will help in the elucidation of the dynamic aspects of protein conformation in the enzymatic activity of its β_2 subunit.

Added in Proof

While this paper was in press, experiments to ascertain the stoichiometry of β_2–Fab complexes were done by using ion-exchange FPLC on Mono-Q (Pharmacia), rather than exclusion FPLC, to achieve better resolution of the peaks. They showed that, with Fab from antibodies 15-1, 31-2, or 46-9, the complex between one β_2 and two Fabs can also be formed, but it requires high concentrations of free Fab. At the protein concentrations used in our affinity measurements, the second site of β_2 remains largely unsaturated and thus does not affect the binding constants reported for 15-1 and 46-9. However,

for Fab 31-2, the affinity constants originally calculated on the basis of two sites per β_2 should all be multiplied by a factor of 2, which does not affect our comparative interpretations.

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Registry No. Pyridoxal-P, 54-47-7; tryptophan synthase, 9014-52-2; L-serine, 56-45-1; L-cysteine, 52-90-4.

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