Immunochemical Evidence for Conformational Flexibility and Its Modulation by Specific Ligands in the β_2 Subunit of *Escherichia coli* Tryptophan Synthase†

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ABSTRACT: The immunochemical reactivity of unfractionated antibodies elicited by denatured β_2 subunits of *Escherichia coli* tryptophan synthase [L-serine hydro-lyase (adding indole) EC 4.2.1.20] with the homologous antigen and with the native enzyme is examined. These antibodies recognize the native apoenzyme nearly as well as the denatured protein. On the contrary, after binding of its cofactor, pyridoxal 5′-phosphate, the protein exhibits a much lower immunoreactivity toward these antibodies. This decrease of affinity becomes even more pronounced when the β_2 protein interacts with the α subunit. Similarly, reduction of the Schiff base formed between the cofactor and the protein leads to a strong decrease of immunoreactivity. To account for these results, it is proposed that apo-β_2 must be a dynamic flexible structure that easily exposes to the solvent regions of its polypeptide chain that normally are buried in its interior. The increase in rigidity of this structure upon binding of the cofactor, reduction of Schiff base, and formation of the $\alpha_2\beta_2$ complex would then account for the decreased immunoreactivity of these various states of the native β_2 protein.

The native state of a globular protein in solution generally cannot be considered a rigid structure. Rotation exists around the single bonds in the backbone and side chains of the molecule, as evidenced by important temperature factors observed in protein crystallography studies (Frauenfelder et al., 1979; Sternberg et al., 1979). This conformational mobility is essential, at least in some cases, for the function of proteins. The large movement of tyrosine-248 in carboxypeptidase A upon the binding of the substrate (Lipscomb, 1971) illustrates the general "induced-fit" model proposed by Koshland (1958). In some cases, the flexibility is not limited to a few residues in direct contact with substrates or ligands, since it can be propagated through the polypeptide chain from one site to the others. Such long-range conformational changes are required for regulation processes involving allosteric interactions or cooperative effects in oligomeric proteins. In the two classical models proposed by Monod et al. (1965) and by Koshland et al. (1966) to account for the cooperative phenomenon, the monomeric unit is assumed to exist in two conformational states. In fact, these two states must be considered as extreme conformations; other conformations are likely to exist. For instance, in the case of glycogen phosphorylase from rabbit skeletal muscle, the native protein in the absence of ligand is in spontaneous equilibrium between at least four different conformations (Buc et al., 1973).

The first evidence for the conformational flexibility in proteins was obtained by hydrogen-exchange experiments (Linderstrøm-Lang, 1955). However, this indirect method often does not lead to clear interpretations because changes in the rigid structure of the protein, as well as changes in the flexibility around one "average" conformation, would affect the kinetics of exchange in the same manner.

Spectrometric methods such as nuclear magnetic resonance or fluorescence have also been widely used to study the dynamics of proteins, particularly the folding process, because they provide quantitative informations. However, the lack of sensitivity frequently limits their utilization for investigating the conformational flexibility because under conditions in which the protein is in the native state, the transient nonnative species are usually present in very small proportions, hence the need for much more sensitive methods.

In the immunological approach suggested by Sachs et al. (1972), the limitation due to sensitivity is not encountered. In their studies, they measured the interactions of native staphylococcal nuclease and of a peptide isolated from this enzyme, with antibodies specific for a limited antigenic region of the native protein. Assuming that the antibodies used can bind exclusively to the native conformation of the specific region, they proposed an experimental method for determining the conformational equilibrium constant, "K_{conf}", characterizing the spontaneous interconversion of the isolated fragment between native and nonnative conformations. This immunological approach has been used frequently during the last 10 years (Hurrel et al., 1977; Vita et al., 1982; Nagy et al., 1982) and allows one to study the flexibility of specific regions of the protein. Similarly, using antibodies elicited with an unfolded fragment of staphylococcal nuclease, Furie et al. (1975) studied the conformational equilibrium of the corresponding region in the native enzyme. On the contrary, Creighton et al. (1978) used unfractionated antibodies to investigate the conformational properties of the entire polypeptide chain of trapped folding intermediates of bovine pancreatic trypsin inhibitor.

A combination of the approaches of Furie et al. (1975) and Creighton et al. (1978) seemed to us particularly adapted for studying the flexibility of a native polypeptide chain. Indeed, the flexibility of a native protein can be defined as the ease with which the polypeptide chain undergoes a conformational "adaptation" to structural constraints exerted by the binding of ligands. Another related aspect of flexibility is the ease of spontaneous fluctuations between several conformational states, some of which may be quite different from the "average native" state depicted by X-ray crystallography. According to these two aspects of flexibility, we thought that antibodies specific for "nonnative" conformations of a protein might be used as probes for investigating its flexibility. The binding of these

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antibodies to the native protein should reflect the ease with which the polypeptide chain can adopt the conformation to which these antibodies can bind. Antibodies raised against the irreversibly denatured protein seemed particularly suitable for this approach. Such antibodies have been shown to be elicited by proteins irreversibly alkylated on their cysteines under denaturing conditions. This method, first used simultaneously by Arnon & Maron (1971) and by Arnheim et al. (1971) has allowed others to detect sequence homologies among proteins (Zakin et al., 1978; Chaffotte et al., 1980; Zakin et al., 1980a; Mouhli et al., 1980; Pekkala-Flagan & Ruoslahti, 1982), thus bringing convincing evidence that the chemical modification of buried cysteines leads to an exposure of the inner part of the native protein, even after eliminating the denaturing agent.

Therefore, we decided to use antibodies directed against a denatured alkylated protein to investigate the conformational flexibility of the corresponding native protein. The system we chose was the β_2 subunit of tryptophan synthase from *Escherichia coli*. This protein could be expected to exhibit some degree of flexibility because it appears to undergo conformational change both upon the binding of its cofactor pyridoxal-P[1] (Bartholmes et al., 1976; Tschopp & Kirschner, 1980) and when associating with the α subunit (Faeder & Hammes, 1970, 1971), the second component of the functional enzymatic complex.

In this paper, we report the use of antibodies raised against the fully alkylated (on its five cysteines) β chain to compare the immunoreactivity of five states of the β_2 protein: nonnative, alkylated β chain; native apo-β_2 subunit; native holo-β_2 subunit; borohydride-reduced holo-β_2 subunit; $\alpha_2\beta_2$ complex. The results obtained confirm the validity of the proposed immunochemical approach for investigating protein flexibility and indicate that apo-β_2 is a very flexible structure whereas the binding of pyridoxal-P or of the α subunit leads to a decrease in flexibility of the native conformation.

Materials and Methods

Proteins. The apo-β_2 subunit of tryptophan synthase from *E. coli* K 12 was purified, crystallized, and reactivated as described by Högberg-Raibaud & Goldberg (1977a). Reconstitution of the holoenzyme was achieved by adding 10^{-4} M pyridoxal-P during the reactivation. Reduced holo-β_2 was prepared by incubating holo-β_2 for 10 min at room temperature with a quantity of sodium borohydride sufficient to inactivate at least 95% of the enzyme. The reduced protein was dialyzed at 4 °C against standard phosphate buffer: 0.1 M potassium phosphate–2×10^{-3} M Na_2EDTA, adjusted to pH 7.8 and supplemented with 5×10^{-3} M 2-mercaptoethanol. The α subunit of tryptophan synthase was prepared as described by Hatanaka et al. (1962).

Alkylation of the β chain of tryptophan synthase was carried out in 6 M guanidine hydrochloride, with *N*-ethylmaleimide or with iodoacetic acid, according to Zakin et al. (1978). After the alkylation step, the protein was dialyzed against distilled water and lyophilized. The protein residue was then dissolved in 0.025 N sodium hydroxide and immediately diluted into the desired buffer, directly at the very low antigen concentration needed for the various assays (i.e., below 50 μg/mL). Under these conditions, the alkylated protein did not precipitate and could be conveniently used in the immunochemical assays. β-Galactosidase from *E. coli* was kindly supplied by A. Ullmann and N. Guiso.

Activity and Protein Assays. The activity of the β_2 subunit in the indole → tryptophan reaction was determined with the spectrometric assay of Faeder & Hammes (1970). The β-galactosidase activity was assayed according to Pardee et al. (1959). When the assay was measured in the microtitration plates, the following modifications were applied: after addition of 0.2 mL of assay mixture to each well, the plate was sealed with an adhesive film to prevent evaporation of *o*-nitrophenol and its redissolution into neighboring wells. The plate was incubated at 37 °C for the appropriate time, and the reaction was stopped by addition of 50 μL of a 1.5 M sodium carbonate solution. The absorbance was recorded at 415 nm with a Multiskan Titertek automatic ELISA plate analyzer (Flow Laboratories, France). Protein concentrations were determined according to Lowry et al. (1951) or by absorbance at 280 nm, using 0.58 and 0.65 as extinction coefficients ($\epsilon_{1cm}^{mg/mL}$) for apo-β_2 and holo-β_2, respectively (Miles, 1970).

Antisera. The antisera (and their respective IgG fraction) raised against native β_2 and alkylated (with *N*-ethymaleimide) β chain were those previously used by Zakin et al. (1980b) and Chaffotte et al. (1980), respectively.

Immunological Assays. Complement fixation experiments were performed as described by Zakin et al. (1978), according to the original procedure of Osler et al. (1952). Binding experiments were performed according to an immunoenzymatic method derived from the classical radioimmunoassay principle and from the ELISA test described by Engwall & Pearlmann (1971). In the first step, the immunoenzymatic conjugate, at a constant concentration, was incubated with various concentrations of soluble antigen (the buffer used is described below). After a 15–18-h incubation at 4 °C, 0.15-mL aliquots were transferred into wells of polystyrene microtitration plates previously coated with the homologous antigen at constant concentration. After a 15–18-h incubation at 4 °C, the liquid phase was removed, and the well was rinsed. The amount of immunoconjugate linked to immobilized antigen was estimated by the β-galactosidase activity measured in each well as indicated above. The binding was expressed as the fraction of saturation, which was calculated by the following formula:

$$y = (A_0 - A_x)/A_0$$

where A_x is the activity corresponding to the test in which the immunoconjugate was incubated with a concentration x of antigen during the first incubation and A_0 is the activity corresponding to the reference test (i.e., the test in which no antigen was added to the immunoconjugate during the first incubation).

A control of the antigen specificity was made by testing the interaction between the immunoconjugate and the α subunit of tryptophan synthase. No reactivity has been observed. Coating of the microtitration plates with the homologous antigen in 0.1 M sodium carbonate at pH 9.6 was carried out by incubation for at least 15 h at 4 °C.

The antigens and the immunoconjugate were incubated in phosphate buffer saline, pH 7.4, containing 0.05% (w/v) Tween 20 and 1% (w/v) bovine serum albumin. In the experiments where holo-β_2 was used as the antigen, the holoprotein was first reconstituted as described above and then diluted into the saline (containing Tween and serum albumin) supplemented with 5×10^{-5} M pyridoxal-P. It has been verified that, under these conditions, at least 90% of the β_2 protein is still saturated with the coenzyme after 24 h at 4 °C, thus ensuring that holo-β_2 is indeed the major antigen present

[1] Abbreviations: Na_2EDTA, ethylenediaminetetraacetic acid disodium salt; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; pyridoxal-P, pyridoxal 5′-phosphate; reduced holo-β_2, sodium borohydride reduced holo-β_2 subunit.

in the reaction mixture. The same buffer without serum albumin was used for the washing of the plates.

The immunoconjugates were prepared according to Avrameas et al. (1978). The coupling of β-galactosidase to the IgG fraction of the antisera was carried out according to the one-step procedure using glutaraldehyde. The IgG fraction was obtained from a 40%-saturated ammonium sulfate precipitation followed by a chromatography on DEAE-52-cellulose; the excluded fraction, after concentration, was directly used without specific fractionation.

The efficiency of antigen coating was estimated as follows: a first plate was coated with 0.15 mL/well alkylated protein at 0.375 μg/mL in 0.1 M Na_2CO_3, pH 9.6, for 15 h at 4 °C. The content of each well was transferred in the wells of a second microtitration plate that was incubated at 4 °C for an additional 15 h. After the first plate was washed, 0.15 mL of 5×10^{-7} M specific immunoconjugate was added to the wells, and the plate was incubated for 15 h at 4 °C. After a washing, the amount of immunoconjugate bound was determined by measuring the β-galactosidase activity present in the wells. The same procedure was applied to the second plate.

If one assumes that the concentrations of antigen to be coated are far from saturation and the efficiency is independent of the antigen concentrations, the coating efficiency p was estimated from the β-galactosidase activity measured in the two plates by the following formula:

$$p = (A_1 - A_2)/A_1$$

where A_1 and A_2 are the activities measured in the wells of the first and the second plates, respectively. When coating was run at 0.375 μg/mL in the two plates, a coating efficiency of 98% was determined according to this calculation.

Thermal Denaturation Experiments. Holo-β_2 and reduced holo-β_2 were adjusted to 0.2 mg/mL in standard phosphate buffer supplemented with 2 mM 2-mercaptoethanol and, when required, 5×10^{-5} M pyridoxal-P. Samples of 0.2 mL were brought to the required temperature in a water bath controlled within ±0.05 °C for the appropriate time and then rapidly cooled in a mixture of ice and water. After a centrifugation at 9600*g* for 15 min in a Microfuge B (Beckman), the activity and the protein concentration in the supernatant were determined.

Results

Complement-Fixation Experiments. Because only two out of the five cysteines present in the β protomer of the native apo-tryptophan synthase are accessible to thiol reagents such as dithiobis(nitrobenzoic acid) (Miles, 1970), we reasoned that the irreversible alkylation of all the cysteines under denaturing conditions would prevent refolding of the protein after elimination of the denaturing agent. Hence, the antisera raised against either the native or the alkylated protein should contain some classes of antibodies recognizing quite distinct antigenic determinants. To examine this hypothesis, we compared the immunochemical reaction of native holo-β_2 and the alkylated protein with antiserum raised against the native protein. As shown in Figure 1, the maximum complement fixation of 100% for the homologous reaction and 90% for the heterologous one is reached at similar antigen concentration (i.e., about 1 nM vs. 4.2 nM). However, it should be noted that for the reaction performed with the homologous antigen, a saturating concentration of antiserum was used. At a greater dilution, the difference between the two profiles would be more accentuated. Thus, in spite of the presence of a strong cross-reactivity between the two forms of the protein, the irreversible alkylation of all the cysteines has led to a modification of the immunogenic behaviors of the β polypeptide chain.

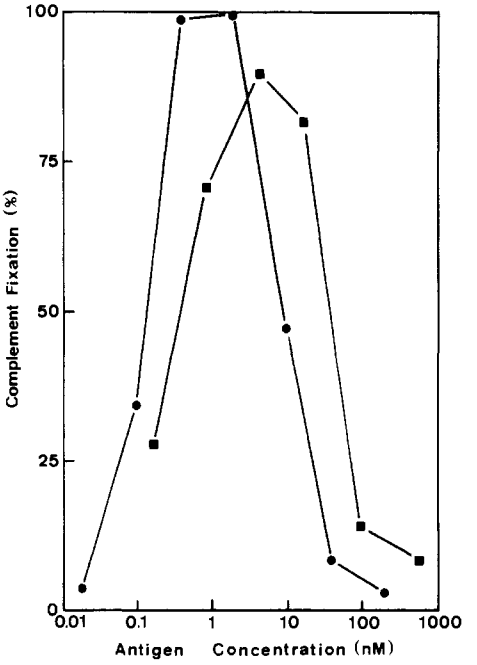

FIGURE 1: Comparison of immunochemical reactivity of alkylated and native holo-β_2 proteins with an antiserum directed against native β_2. Complement fixation assays were performed in duplicate with a 1:1000 dilution of the antiserum. (●) Holo-β_2; (■) alkylated protein.

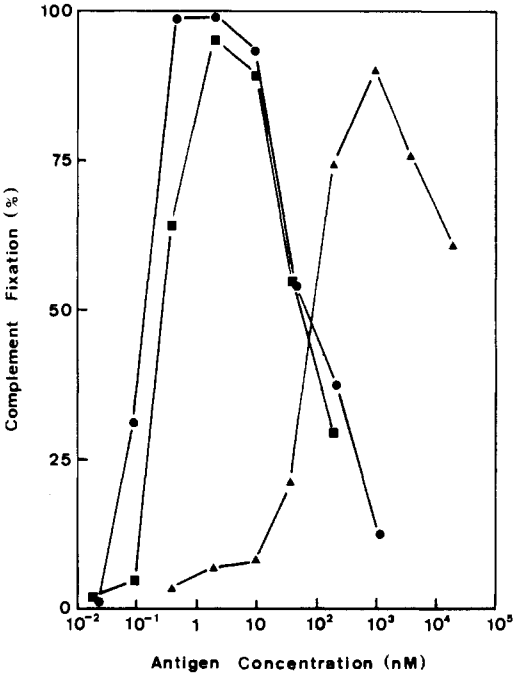

FIGURE 2: Comparison of immunochemical reactivity of alkylated β chain, native apo-β_2, and native holo-β_2 with an antiserum directed against alkylated β chain. Complement fixation assays were performed in duplicate with a 1:500 dilution of the antiserum. (●) Alkylated protein; (■) apo-β_2; (▲) holo-β_2.

The difference between the immunochemical reaction of the apoenzyme and the alkylated protein was tested with an antiserum raised against the alkylated protein. The results in Figure 2 show that the difference between the alkylated protein and the native holoenzyme is much more evident than with antiserum specific for the native protein (see Figure 1) since the optimal concentrations for these antigens are in a ratio close to 1000. Hence, the antiserum raised against the alkylated protein is much more sensitive to differences between

native holo-β_2 and alkylated protein than antiserum raised against the native protein.

Another important result is the very strong "cross-reactivity" between the alkylated and the native apoprotein. Therefore, the antiserum directed against nonnative β chains reveals a great difference between the immunochemical reactivities of apo- and holoprotein, in contrast to results previously obtained with an antiserum directed against the native protein (Zakin et al., 1980b).

Direct Binding Assay of Antigen–Antibody Interaction. The differences observed in complement fixation experiments (Figures 1 and 2) cannot be readily interpreted because this method does not directly monitor the binding of antibodies to the antigens. Thus, the comparison of the interactions of each antigenic form with the antibodies had to be examined with a direct-binding method. We therefore developed a direct-binding assay derived from the ELISA test (Engwall & Pearlmann, 1971). This assay consists of determining, for each antigen concentration, the amount of immune complex present at equilibrium, by measuring the amount of free specific antibody and substracting it from the amount of total specific antibody. The free antibody was determined by trapping it on the homologous antigen adsorbed on a solid support (i.e., polystyrene microtitration plates) as described under Materials and Methods.

(*a*) *Optimalization of Experimental Conditions.* Efficiency of adsorption of the protein on the polystyrene plates used in the ELISA may vary considerably, and it has been established that, below a limiting concentration, the relative quantity of antigen adsorbed is constant for a given protein (Cantarero et al., 1980). Therefore, below this limiting concentration, the coating efficiency can be considered constant. With use of this finding, the coating efficiency of the alkylated β chain was determined as given under Materials and Methods. With an antigen concentration of 0.375 μg/mL (i.e., 8.5 nM), the coating efficiency was found to be 98%. By a simple calculation in which the alkylated β chain is assumed to have a spherical shape, the maximum area occupied by the antigen in the well is found to be 11% of the total available area. This suggests that under these conditions, no steric hindrances should occur during the binding of the immunoconjugate to the immobilized antigen.

In order to find proper conditions for monitoring the amount of free antibody present in the ELISA, calibration curves were constructed. Figure 3 shows the binding of the immunoconjugate to the immobilized, homologous alkylated antigen at four different concentrations of antigen. It can be seen that the different binding curves are significantly different, suggesting that at the two lower antigen concentrations, the trapping of the immunoconjugate is not complete. However, the coincidence of the curves corresponding to the two higher antigen concentrations (i.e., 0.375 and 0.75 μg/mL) shows that at concentrations of immunoconjugate below 2 nM and for a coating antigen concentration of 0.375 μg/mL, the immobilized antigenic sites are in an excess sufficient to render the binding of specific antibodies essentially complete. These conditions were used in all the subsequent experiments.

(*b*) *Comparative Binding of Alkylated β Chain, Apoenzyme, Holoenzyme, Reduced Holoenzyme, and $\alpha_2\beta_2$ Complex to Antibodies Specific for Alkylated β Chain.* To confirm the results obtained previously by complement-fixation experiments (Figure 2), we used the binding assay described above, in which the concentration of immune complex corresponding to a given antigen concentration was deduced from the difference between the concentrations of the total specific

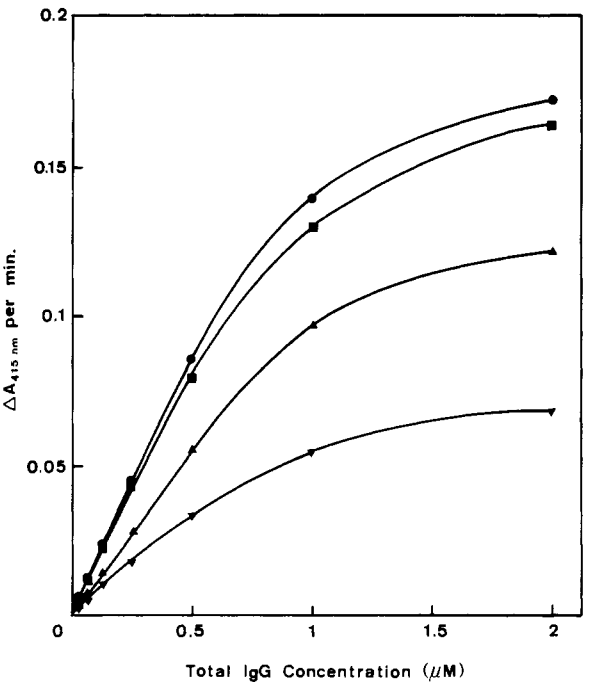

FIGURE 3: Saturation of immobilized alkylated protein with IgG from an antiserum directed against alkylated β chain. The binding was measured by using the β-galactosidase activity of the specifically adsorbed immunoconjugate as described under Materials and Methods. The microtitration plates were coated at four concentrations of the alkylated protein in 0.1 M sodium carbonate, pH 9.6: (▼) 0.094 μg/mL; (▲) 0.188 μg/mL; (■) 0.375 μg/mL; (●) 0.750 μg/mL. The concentration of the immunoconjugate is expressed in terms of total IgG (molecular mass 150 kdalton).

antibody and the free antibody at equilibrium. This assay allowed one to compare the affinity of the alkylated protein to that of the apo- and the holoprotein for antibodies directed against the alkylated protein [which hereafter will be called anti-(alkylated protein) immunoglobulins]. As seen in Figure 4, the homologous binding curve has a hyperbolic shape. However, due to the heterogeneity of the antibodies, the double-reciprocal plot (i.e., reciprocal of the saturation fraction vs. reciprocal of the antigen concentration) does not give a straight line. Hence, no true dissociation constant can be deduced from this binding curve, but the half-saturation concentration can be used to estimate the average affinity of antibodies for the antigen. For the binding of immunoglobulins to the homologous alkylated protein, the plateau value can reasonably be assumed to reach 100%, and thus, the half-saturation concentration is close to 10^{-8} M.

The binding curve corresponding to apo-β_2 is very similar to that of the homologous alkylated protein. The plateau value and the half-saturation concentrations are not significantly different. This result confirms that obtained in complement-fixation experiments (see Figure 2).

The profiles in Figure 4 show a significant progressive decrease of the immunoreactivity of the native β_2 subunit after the following modifications: (i) the binding of the cofactor pyridoxal-P to the active site, (ii) the binding of the α subunit to the holo-β_2 protein, and (iii) the reduction of the Schiff base in the holoenzyme. The binding curves in Figure 4 might suggest a decrease in the number of antigenic determinants. This, however, cannot be concluded from the saturation curves because of the uncertainties concerning the plateau values in the range of concentrations used. Indeed, in experiments in which the total antigen concentration was raised to 8 μM, the values of the saturation fraction obtained for the different heterologous antigens were not significantly different. Hence,

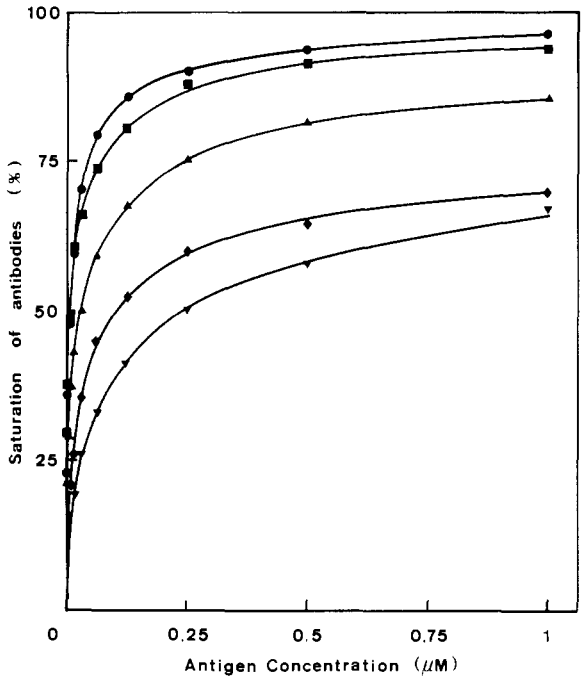

FIGURE 4: Comparative binding of alkylated β chain, apo-β_2, holo-β_2, reduced holo-β_2, and holo-$\alpha_2\beta_2$ complex to unfractionated IgG from an antiserum raised against alkylated protein. Each antigen, at the concentration indicated on the abscissa was incubated at 4 °C for 15–18 h, in a total volume of 500 μL in the presence of 2×10^{-9} M IgG. The antigen concentrations are calculated in terms of β monomer (molecular mass 44 kdalton) for all the species. All the dilutions of holo-β_2 were made in the presence of 5×10^{-5} M pyridoxal-P. Holo-$\alpha_2\beta_2$ was diluted in the presence of 10^{-6} M additional α subunit to avoid spontaneous dissociation of the complex at low concentrations. The samples of reduced holo-β_2 were kept in the dark to prevent photooxidation of the reduced Schiff base. The binding was then assayed and calculated as described under Materials and Methods. (●) Alkylated β chain; (■) apo-β_2; (▲) holo-β_2; (♦) holo-$\alpha_2\beta_2$; (▼) reduced holo-β_2. These experiments were repeated twice as described above and twice at 10^{-8} M IgG with similar results.

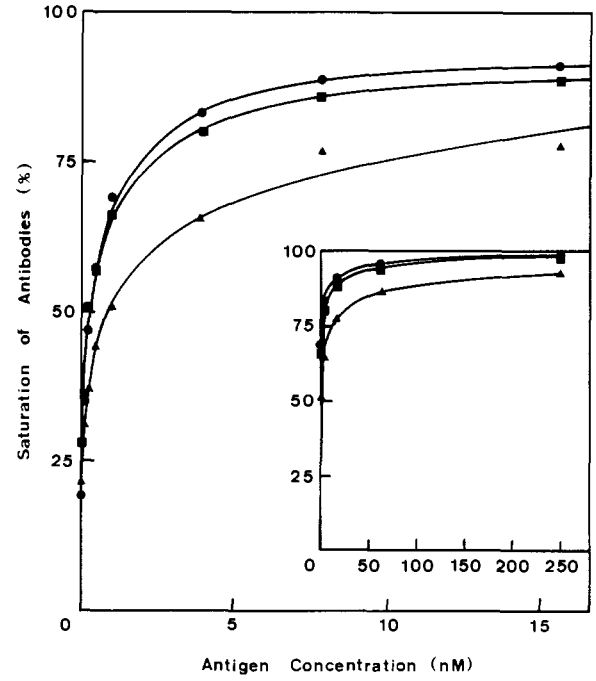

FIGURE 5: Comparative binding of apo-β_2, holo-β_2, and reduced holo-β_2 to unfractionated IgG from an antiserum raised against native β_2 protein. The concentration of immunoconjugate was 3×10^{-9} M as expressed in terms of IgG molarity. The procedure and precautions concerning holo-β_2 and reduced holo-β_2 were the same as in Figure 4. (●) Apo-β_2; (■) holo-β_2; (▲) reduced holo-β_2. These experiments were repeated twice with identical results.

no important difference between the distribution of the antigenic determinants occurs, but the data in Figure 4 reflect a change in the average affinities of the various antigens for the antibodies.

(*c*) *Comparative Binding of Apoenzyme, Holoenzyme, and Reduced Holoenzyme to Antibodies Specific for Native β_2 Protein.* It has been previously reported (Zakin et al., 1980b) that no difference between the immunochemical reactivity of apoenzyme, holoenzyme, and reduced holoenzyme was detected by complement fixation with an antiserum specific for the native state of the protein. Thus, to ascertain the reliability of the technique used here, we applied it to study the binding of apo-β_2, holo-β_2, and reduced holo-β_2 to antibodies against native β_2. As seen in Figure 5, no significant difference is observed between the binding curves of the apo- and the holoenzyme. This is in good agreement with the results previously obtained (Zakin et al., 1980b). On the contrary, the binding curve of the reduced protein shows a decrease of reactivity as compared to the holo-β_2 saturation curve. Though smaller than that observed with anti-(alkylated protein) immunoglobulins (see Figure 4), this decrease is undoubtedly significant and suggests that the reduction of the Schiff base induces some structural changes in the conformation of the holoenzyme. This could have been expected since reduced holo-β_2, upon binding to the α subunit, is a better activator of the α subunit than the native holo-β_2 subunit (Wilson & Crawford, 1965). The decreased reactivity of reduced holo-β_2 toward anti-(native β_2) immunoglobulins was not detected in previous experiments (Zakin et al., 1980b). This was probably due to a lack of sensitivity of the methods used as compared to the ELISA test described here.

Comparative Study of Thermal, Irreversible Denaturation of Holoenzyme and Reduced Holoenzyme. As mentioned above, the binding of pyridoxal-P to apo-β_2 and the reduction of its Schiff base in holo-β_2 produce an important difference of immunological reactivity toward anti-(alkylated β chain) IgG. It is tempting to suggest that this difference reflects a change in the flexibility of the polypeptide chain. This is supported by the correlation in the decrease of immunological recognition with the stabilization of the protein induced by binding of the pyridoxal-P and by reduction of the Schiff base in the enzyme–pyridoxal-P complex. A strong stabilizing effect of pyridoxal-P had already been clearly demonstrated during the study of the thermal inactivation of β_2 (Zetina & Goldberg, 1980). However, no evidence was available to support the assumption that the reduction of the Schiff base increases the stability of the holoprotein. Therefore, we compared the irreversible thermal denaturation of the holoenzyme and of the reduced holo-β_2 protein to see whether the reduction increases the stability.

As shown in Figure 6, no significant difference is observed between the inactivation and the precipitation of holo-β_2, showing that the denaturation can be monitored by measuring the amount of protein remaining soluble after the heat treatment. By this criteria, the reduced holoprotein appears to be considerably more heat stable than holo-β_2, since at 82 °C more than 90% of the reduced holoprotein remained soluble while, at the same temperature, only 20% of holo-β_2 had not precipitated. Furthermore, a semilogarithmic plot of the amount of soluble protein as a function of time of heating at 78 °C showed that both precipitation reactions are described by first-order kinetics. The rate constant corresponding to the holoenzyme precipitation is 0.088 min^{-1}; the rate constant of the precipitation of the reduced protein is 0.004 min^{-1}, about

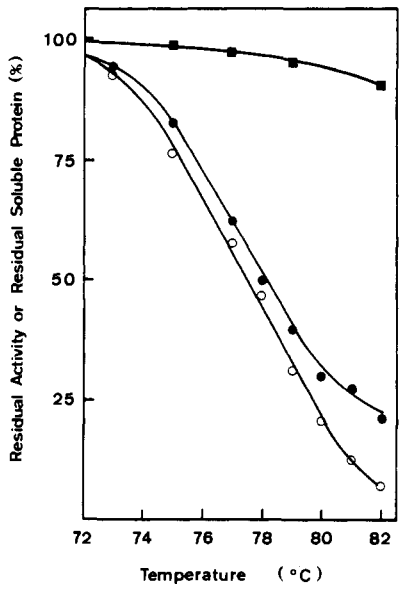

FIGURE 6: Comparison of irreversible thermal denaturation of holo-β_2 and reduced holo-β_2. 0.2-mL samples of holo-β_2 or reduced holo-β_2 were heated for 6 min at various temperatures and then cooled rapidly. Residual activity and soluble protein were measured in the supernatants after centrifugation of the samples. (○) Holo-β_2 activity; (●) holo-β_2 residual soluble protein; (■) reduced holo-β_2 residual soluble protein.

20-fold smaller than that for holo-β_2. This confirms that the reduction of the Schiff base introduces a strong additional stabilization of the protein molecule.

Discussion

The results reported in the present work demonstrate that in the case of the β_2 subunit of tryptophan synthase from *E. coli* antibodies elicited by a nonnative state of a protein may be used to reveal static and/or dynamic structural changes occurring upon protein–ligand or protein–protein interaction. In the present case, the binding of the cofactor pyridoxal-P, as well as the interaction with the α subunit, leads to a decreased binding of the antibodies to the β_2 subunit. On the contrary, antibodies specific for the native protein do not reveal the conformational changes induced by the cofactor pyridoxal-P (Zakin et al., 1980b). This indicates that complete alkylation of the cysteine residues leads to a structural state of the protein that contains antigenic determinants specifically distinct from those existing in the holoenzyme. This could be expected because in the native protomer, three out of five cysteines are buried (Miles, 1970). Moreover, after elimination of the denaturant, the alkylated protein becomes highly insoluble and totally devoid of catalytic activity. This demonstrates that the chemical modification has prevented a refolding of the polypeptide chain to its native structure. However, it is known that the native protomer is composed of two independently folding structural domains, "F_1" and "F_2" (Högberg-Raibaud & Goldberg, 1977a; Zetina & Goldberg, 1982). Furthermore, the single cysteine in F_2 in readily accessible to thiol reagents, even when the isolated F_2 fragment is folded (Goldberg & Högberg-Raibaud, 1979). Thus, it can be expected that after alkylation and elimination of the denaturant, the refolding of the F_2 moiety will occur, leading to a reconstitution of the same antigenic structure as that of the pseudonative isolated F_2 fragment, which is known to cross-react with native holo-β_2 (Zakin et al., 1980b). This probably explains the recognition of the alkylated protein by antiserum raised against the native protein and vice versa.

A nearly total cross-reactivity between native apo-β_2 and the alkylated protein has been observed by two different immunochemical methods (ELISA test and complement fixation) with an antiserum specific for the alkylated protein. Since this antiserum must contain antibody subclasses specific for the nonnative conformations, one can conclude that these nonnative structures must spontaneously exist in the native apoprotein. For reasons already mentioned above, it is very unlikely that apo-β_2 has a static structure identical with or similar to that of the alkylated protein. Thus the almost perfect recognition of apo-β_2 by the anti-(alkylated protein) immunoglobulin must reflect the high frequency of appearance of transient, local nonnative states. Hence, our results reflect the conformational dynamics of the native structure, which appears to behave as a flexible entity.

The binding of pyridoxal-P to the apoenzyme has been shown to bring about a decrease of immunological recognition by antibodies specific for the alkylated protein. Thus, in the native protein, binding of pyridoxal-P induces an important change in the structural properties of the protein. It is unlikely that this decrease in immunoreactivity depends only on the direct masking by pyridoxal-P of residues in contact with the cofactor, even if these residues are involved in an eventual antigenic determinant. The modification of the antigenic structure in holo-β_2 as seen with the anti-(alkylated protein) immunoglobulins is more likely due to a decrease of the frequency of appearance of the nonnative antigenic sites involved in the interactions with the specific antibodies. That pyridoxal-P influences the conformation of β_2 could be predicted from the cooperative aspect of its binding (Barthomes et al., 1976; Tschopp & Kirschner, 1980). This structural effect can also be analyzed in terms of a decrease of the conformational flexibility. Indeed, during a comparative study of the thermal inactivation of apo-β_2 and holo-β_2 (Zetina & Goldberg, 1980), it was concluded that pyridoxal-P would stabilize the F_1 domain but also would increase interdomain and intersubunit interactions, and a "tightening" of each protomer was supposed to be responsible for this stabilization. Similarly, it has been shown (Högberg-Raibaud & Goldberg, 1977b) that pyridoxal-P protects β_2 against complete degradation (as observed with the apoenzyme) by tryptic proteolysis. This observation can be interpreted in terms of a decreased "adaptability" of the polypeptide chain to the catalytic site of the protease. This decrease of the conformational adaptability to the protease may be related to the decrease of conformational adaptability to the antibodies as reported in the present study. Finally, a tightening of the native protein upon binding of pyridoxal-P has also been demonstrated by Wiesinger et al. (1979) in the course of a calorimetric study. Hence, it can be concluded that the changes caused by pyridoxal-P in the immunoreactivity toward anti-(alkylated β chain) immunoglobulins are primarily due to an increase of rigidity of the polypeptide chain by the cofactor.

This structural effect resulting from the binding of the cofactor seems to be enhanced by the reduction of the Schiff base, as shown by the strong decrease of immunoreactivity with anti-(alkylated protein) immunoglobulins. This decrease of immunoreactivity again parallels a difference of thermolability between holo-β_2 and reduced holo-β_2, as shown in the present work (see Figure 6). No direct evidence exists for a conformational change occurring upon the reduction of the Schiff base. However, it has been shown that reduced holo-β_2 is a better activator of the α subunit than nonreduced holo-β_2 (Wilson & Crawford, 1965). Hence, a structural difference is likely to be produced by the reduction. Furthermore, it must be noted that the reduction renders the linkage of pyridoxal-P to the protein strictly irreversible, which is not the case in the

normal Schiff base. This irreversibility may prevent structural fluctuations, thus leading to an even more rigid conformation of the protein.

The effect of the α subunit on the immunochemical properties of holo-β_2 clearly reflects a structural difference in the β_2 subunit when unassociated or integrated in the $\alpha_2\beta_2$ complex. This difference in immunoreactivity might be due to a reduced accessibility, within the $\alpha_2\beta_2$ complex, of an antigenic determinant of the β protomer engaged in the interactions with the α subunit. However, no decrease of the total number of antigenic determinants seemed to occur as compared to holo-β_2. Thus, the difference between the immunological behaviors of holo-β_2 and $\alpha_2\beta_2$ appears related to a structural change occurring in holo-β_2 upon its association with the α subunit. Indeed, the existence of such a conformational change has already been proposed by Faeder & Hammes (1970, 1971) in their kinetic studies and by Bartholmes et al. (1976) on the basis of binding equilibrium studies. Though attractive, the possibility that this change would correspond to a modification of the conformational dynamics of the holo-β_2 subunit upon its interactions with the α subunit remains a precarious hypothesis because no other supporting evidence is available.

In conclusion, the results discussed above confirm the existence of important static or dynamic conformational changes in the β_2 subunit of tryptophan synthase. More generally, they show that the use of antibodies directed against the denatured protein can give valuable qualitative information on the ease of adaptation of the native protein to structural constraints, as well as on the restriction of this conformational adaptability resulting from binding of specific ligands. Quantitative analysis of the modulation of the local flexibility could be made if monoclonal antibodies specific for the denatured protein were used. Alternatively, the conclusions presented here could be refined by the use of a variety of physicochemical methods. Both approaches are currently being explored in our laboratory.

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