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Biochemistry

Partly native epitopes are already present on early intermediates in the folding of tryptophan synthase

(folding intermediates/domain interactions/monoclonal antibodies)

SYLVIE BLOND AND MICHEL GOLDBERG

Unité de Biochimie des Régulations Cellulaires, Département de Biochimie et Génétique Moléculaire, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France

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ABSTRACT Two monoclonal antibodies directed against the native β_2 subunit of *Escherichia coli* tryptophan synthase [L-serine hydro-lyase (adding indoleglycerol-phosphate), EC 4.2.1.20], one recognizing the C-terminal F_1 domain and the other the N-terminal F_2 domain, were used to detect immunoreactive intermediates in the folding of the protein. For that purpose, the association of the monoclonal antibodies with either the β_2 subunit or its isolated domains was studied by using fluorescence energy transfer between tryptophan residues of the antibodies and a dansyl group covalently linked to the antigen. It is shown that the association of both monoclonal antibodies with the antigen occurs within a few seconds after initiation of the renaturation, whereas complete refolding of the β_2 subunit requires several minutes under the same experimental conditions. Thus, immunoreactive intermediates appear to be formed at an early stage of the folding process. While the isolated F_1 domain alone is able to rapidly refold into a conformational intermediate already well recognized by the anti-native-β_2 antibody, it cannot, in the absence of the F_2 domain, reach its native conformation. However, its association with the anti-native-β_2 antibody induces a structural change of F_1 that brings it closer to the conformation it normally adopts when interacting with F_2 inside the native β_2 subunit.

The folding and assembly of large polypeptide chains into the biologically functional, native three-dimensional structure of a protein proceeds through a series of structural intermediates, which can be summarized as follows (1–3). Short stretches of local, barely stable, secondary structures (α-helices, β-sheets) are formed first (4). Some of them interact through more-or-less specific interactions to form a "molten globule" (5, 6), which then condenses further into a stable compact "domain" (7). Two or more domains of a polypeptide chain then assemble through interactions between specific regions on their surfaces to give the final tertiary structure of the polypeptide chain. When the protein is an oligomer, the folded chains finally associate to form the native quaternary structure. It has been pointed out that, at each step on this pathway, the existing structural intermediates appear to undergo conformational rearrangements because of the constraints exerted by newly formed interactions (3). The question asked in this study was to know how important these conformational rearrangements are; in other words, how close is the structure of folding intermediates to that of the same region of the protein in its native state? We thought that this question might be answered by finding out how far on the folding pathway an intermediate must be for it to be recognized by antibodies specific to the native protein.

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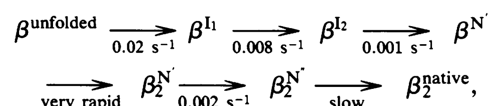
Therefore, we investigated the immunoreactivity of intermediates in the folding of the β_2 subunit of *Escherichia coli* tryptophan synthase [L-serine hydro-lyase (adding indoleglycerol-phosphate), EC 4.2.1.20]. This protein is a dimer (8). Each polypeptide chain (M_r = 42,000) is made of two domains, F_1 (N-terminal, M_r = 29,000) and F_2 (C-terminal, M_r = 12,000), which have been shown to fold independently of one another into native-like structures (9). The kinetics of folding (10, 11), domain association (10), and dimerization of monomers (12) have been extensively studied and have allowed identification of six successive steps in the renaturation of the β_2 subunit. The following scheme summarizes the results of these studies:



$$\beta^{\text{unfolded}} \xrightarrow[0.02\ s^{-1}]{} \beta^{I_1} \xrightarrow[0.008\ s^{-1}]{} \beta^{I_2} \xrightarrow[0.001\ s^{-1}]{} \beta^{N'} \xrightarrow[\text{very rapid}]{} \beta_2^{N'} \xrightarrow[0.002\ s^{-1}]{} \beta_2^{N''} \xrightarrow[\text{slow}]{} \beta_2^{\text{native}},$$

in which $\beta^{I_{1,2}}$ and $\beta^{N'}$ are monomeric and $\beta^{N',N''}$ are dimeric intermediates.

It is important to point out that this scheme represents only a temporal sequence of events. Thus, while the F_1 domain alone (i.e., the M_r 29,000 N-terminal fragment obtained by proteolytic cleavage of the β_2 subunit) is not able to undergo any of the steps from β^{I_1} to $\beta_2^{N''}$(11), it does spontaneously undergo the isomerization responsible for the last, rate-limiting step leading from $\beta_2^{N''}$ to β_2^{native} during the folding of the entire β chain.

Because several of the rate constants indicated above (all determined at 12°C) are fairly small, it seemed possible to establish whether or not some of the intermediates on the folding pathway can be recognized by monoclonal antibodies specific to the native β_2 subunit.

This communication describes the kinetics of association of two such monoclonal antibodies, recognizing two distinct epitopes on the native β_2 subunit with their respective antigens during the refolding of β chains or of the isolated F_1 and F_2 domains. The results obtained will be discussed with respect to protein folding but also in terms of the molecular mechanisms involved in antigen–antibody association.

MATERIALS AND METHODS

Chemicals and Buffer. *N*-Iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS) and pyridoxal 5'-phosphate (pyridoxal-*P*) were obtained from Sigma, and guanidine

Abbreviations: IAEDANS, *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)-ethylenediamine; β_2, the β_2 subunit of *Escherichia coli* tryptophan synthase [L-serine hydro-lyase (adding indoleglycerol-phosphate), EC 4.2.1.20]; F_1, the N-terminal proteolytic fragment of β_2; F_2, the C-terminal proteolytic fragment of β_2; β_2-IAEDANS and F_1-IAEDANS, β_2 subunit and F_1 fragment labeled with IAEDANS on cysteine-170; F_2-IAEDANS, F_2 fragment labeled with IAEDANS on cysteine-340; pyridoxal-*P*, pyridoxal 5'-phosphate.

hydrochloride (Reagente Puro Erba) from Carlo Erba. Unless otherwise stated, experiments were all done in 100 mM potassium phosphate (pH 7.8) containing 2 mM EDTA and 2 mM 2-mercaptoethanol (buffer A).

Preparation of the β_2 Subunit and Its Proteolytic Fragments. The apo (i.e., free of pyridoxal-*P*) and holo (i.e., saturated with pyridoxal-*P*) forms of β_2 and its proteolytic F_1 and F_2 fragments were prepared as described by Zetina and Goldberg (10).

Preparation of Fluorescent Derivatives of β_2, F_1, and F_2. IAEDANS derivatives of the β_2 subunit (β_2-IAEDANS) and the F_1 fragment (F_1-IAEDANS) were prepared as described (12).

Fluorescent labeling of the F_2 fragment at its single cysteinyl residue was achieved as described by Friguet *et al.* (13). The degree of labeling was determined from the absorption spectrum [$\varepsilon_M = 6{,}100\ M^{-1}{\cdot}cm^{-1}$ for IAEDANS at 336 nm (14)] and was found to be 1 ± 0.1 mol of IAEDANS per mol of protein.

Monoclonal Antibodies. Murine monoclonal antibodies were produced, prepared, and characterized as described (15–17), and their antigen-binding proteolytic Fab fragments were obtained as described by Mariuzza *et al.* (18).

Activity and Protein Assay. The activity of the β_2 subunit, used to monitor the cleavage by trypsin, was measured in the presence of the α subunit as described by Faeder and Hammes (19). Protein concentrations were determined by the method of Bradford (20) or spectrophotometrically by using the specific absorbances reported by Högberg-Raibaud and Goldberg (9) for antigens and Onoue *et al.* (21) for antibodies.

Association and Renaturation of the β_2 Subunit and Its Proteolytic Fragments with Antibodies. The denaturation of antigens was achieved with guanidine hydrochloride as described (10). The renaturation was initiated by diluting the denatured protein 1:50–100 into 2.5 ml of buffer A containing an excess of antibodies. The same procedure was followed for association with native antigens.

Fluorescence Measurements. All kinetic measurements were done in a JY-3 (Jobin-Yvon, Longjumeau, France) spectrofluorometer equipped with a thermostat and a built-in magnetic stirrer. In all kinetic experiments, the excitation wavelength was 295 nm and the emission wavelength was 490 nm.

RESULTS

Interactions Between the β_2 Subunit and Monoclonal Antibody 19 Specific to the F_1 Domain. Comparing the uncorrected emission spectrum of apo-β_2-IAEDANS, Ig-19, and the antigen–antibody complex (excitation at 295 nm) shows that the fluorescence of the dansyl group linked to cysteine-170 in the β chain strongly increases when the antigen is associated with the antibody. This fluorescence transfer between tryptophan residues of the antibody and the dansyl group linked to β_2 was used to study the kinetics of association of Ig-19 with apo-β_2. The semilogarithmic plot of the fluorescence change observed after mixing Ig-19 with β_2-IAEDANS could be well fitted by a straight line. This indicates that the reaction obeys pseudo-first-order kinetics under the conditions of the experiments, where the Ig concentration was at least 4-fold higher than the antigen concentration. It was verified that the apparent rate constant, obtained from the slope of such semilogarithmic plots, was proportional to the Ig concentration. The proportionality constant is the true second-order rate constant and was found to be $k = 4.2 \pm 0.4 \times 10^5\ M^{-1}{\cdot}s^{-1}$ at 12°C. To avoid possible artifacts like circular complexes (22) resulting from the existence of two sites both on β_2 and on the IgG molecule, the same kinetic experiments were performed with Fab fragments prepared from Ig-19. The semilogarithmic representations of the fluorescence changes were again linear (Fig. 1*A*), and the second-order rate constant found, $k = 3.0 \pm 0.5 \times 10^5\ M^{-1}{\cdot}s^{-1}$, was not significantly different from that determined above with Ig-19. Thus, all of the following experiments were carried out with purified monovalent Fab fragment.

β_2-IAEDANS, denatured in 6 M guanidine hydrochloride, was diluted in buffer A (to initiate its renaturation) in the presence of an excess of Fab-19 at 12°C. The tryptophan–IAEDANS energy transfer was recorded as a function of time. Semilogarithmic representations of the fluorescence change showed biphasic kinetics (Fig. 1*B*). Graphic decomposition of the kinetics in two elementary phases allowed determination of the apparent rate constants, calculated from the slopes of the straight line obtained for each phase. Fig. 2 shows the concentration dependence of the apparent rate constants determined for the fast and slow phases. The fast phase corresponds to a second-order process, and its rate constant was found to be $k = 2.1 \pm 0.4 \times 10^5\ M^{-1}{\cdot}s^{-1}$, a value not significantly different from the association constant of Fab-19 with native β_2. On the contrary, the Fab-19 concentration didn't affect the value of the slower rate constant, which was found to be $k = 0.007 \pm 0.001\ s^{-1}$. Thus, the slow phase appears to correspond to an isomerization of β. This isomerization could correspond to a conformational change taking place within the already formed antigen–antibody complex. Alternatively, it could correspond to the slow folding of some β chains, which would be rate limiting for the specific binding of Fab-19 onto these slowly refolding molecules. To distinguish between these two possibilities, native apo-β_2 in a 5- to 10-fold molar excess over Fab-19 was added at the end of the fast phase—i.e., 1 min after the dilution of denatured β-IAEDANS in buffer A containing Fab-19. These experiments showed that neither the relative amplitude nor the rate constant of the slow phase was affected by this competition between refolding apo-β_2-IAEDANS and native unlabeled apo-β_2. On the contrary, both the fast and the slow phases disappeared when unlabeled β_2 was added together with β-IAEDANS. This indicates that the slow phase corresponds to an isomerization that takes place in the already formed antigen–antibody complex.

Interactions Between the F_1 Fragment and Fab-19. The refolding of guanidine hydrochloride-denatured F_1-IAEDANS domain in the presence of Fab-19 was then studied by the same method as described above for β-IAEDANS. Biphasic kinetics were again obtained (Fig. 3). As observed for the renaturation of β_2, the rate of the fast phase varied with the Fab concentration, and the second-order rate constant was found at 12°C to be $k = 3.5 \pm 0.5 \times 10^5\ M^{-1}{\cdot}s^{-1}$. The slower phase obeyed first-order kinetics, since it was not affected by the Fab-19 concentration. Furthermore, it was not affected when a large excess of undansylated native F_1 was added at the end of the fast phase. Thus, as found above for β_2-IAEDANS, this slow phase corresponds to an isomerization step taking place in the already formed F_1–Fab-19 complex. Up to this point, β-IAEDANS and F_1-IAEDANS appeared to behave in a similar way. However, when the association of Fab-19 with the "native" (i.e., already refolded) antigens were compared, a major difference was observed. Whereas for native β_2-IAEDANS the fluorescence change obeyed simple second-order kinetics (see above), in the case of F_1-IAEDANS it followed the same biphasic kinetics as during the refolding (Fig. 3). Indeed, the amplitudes and rates of both phases were the same with "folding" and "already folded" F_1-IAEDANS. Similarly, the effects of an excess of unlabeled F_1 were the same. Thus, the binding of Fab-19 onto folded F_1 appears to induce an isomerization that spontaneously occurs during the refolding of β chains, but which F_1 alone is unable to undergo.

Interactions Between F_2 and an Anti-Native F_2 Antibody, Fab-93. Experiments similar to those described above were repeated but with another monoclonal antibody, 93, specific

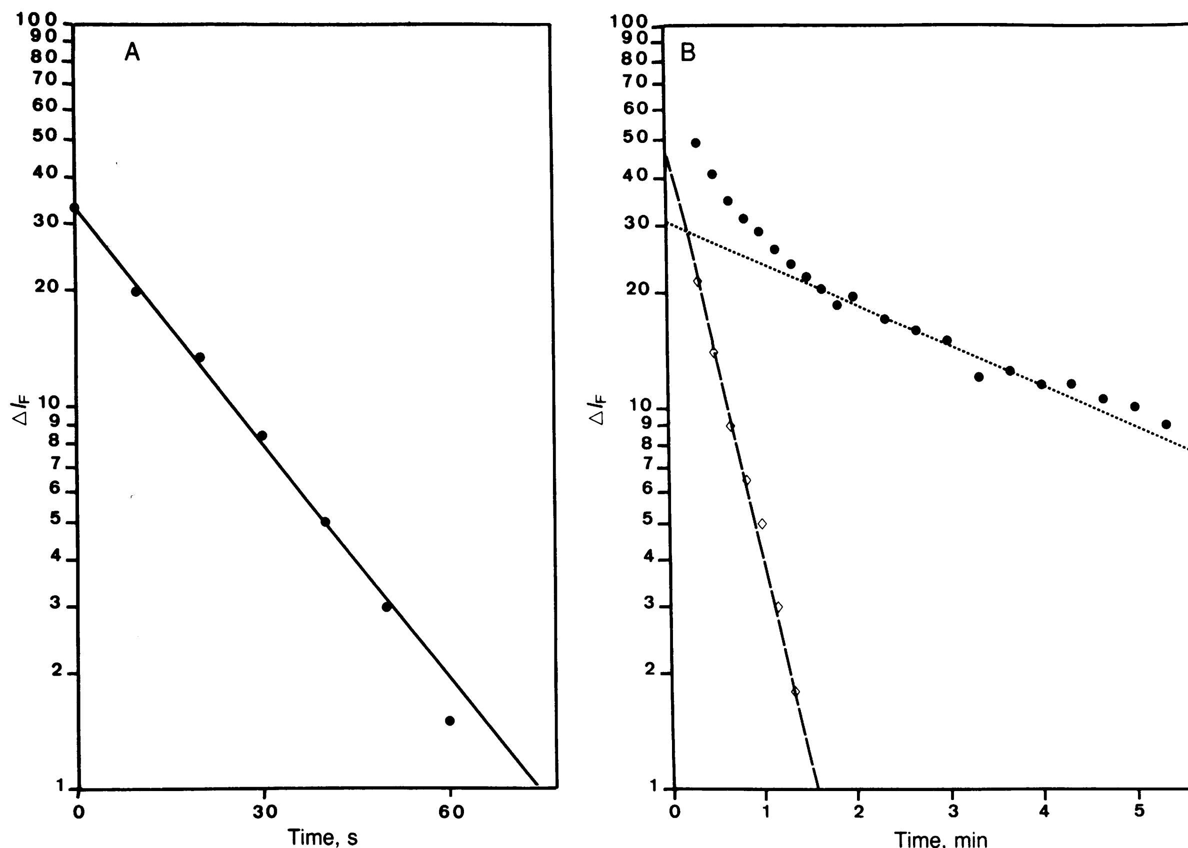


FIG. 1. Kinetics of appearance of the tryptophan-to-dansyl group fluorescence energy transfer during association of Fab-19 and β_2-IAEDANS. Native apo-β_2-IAEDANS (*A*) or denatured β-IAEDANS (*B*) was diluted at 12°C into 2.5 ml of buffer A containing an excess of Fab-19. The fluorescence (excitation wavelength = 295 nm; emission wavelength = 470 nm) was recorded as a function of time. The results are plotted on a logarithmic scale as the change in fluorescence intensity ΔI_F as a function of time. $\Delta I_F = I_\infty - I_t$, where I_t is the fluorescence intensity at time t after dilution, and I_∞ is the fluorescence intensity reached at the end of the reaction. (*A*) Association with native apo-β_2-IAEDANS. The final concentrations were 13.5 nM (in β chains) for the antigen and 150 nM for Fab-19. (*B*) Association with refolding β-IAEDANS. The final concentrations were 9 nM for the antigen and 105 nM for Fab-19. ●, experimental points; ◇, rapid phase obtained by decomposition into two exponentials.

to an epitope carried by the C-terminal F_2 domain of the β_2 subunit.

The tryptophan–IAEDANS fluorescence transfer within the β_2-IAEDANS–Fab-93 complex was too small to be used for studying the interactions of Fab-93 with the β chain. But when isolated F_2 labeled with IAEDANS on its single cysteine interacted with Fab-93, the efficiency of energy transfer was large enough to be used for studying the association of these two molecules. Because the folding of F_2 was expected to be rapid, association and renaturation experiments were performed at +4°C. When the refolded isolated F_2-IAEDANS fragment was mixed with an excess of Fab-93, the increase in fluorescence caused by the energy transfer appeared to be a monophasic pseudo-first-order process because in a semilogarithmic representation, it varied linearly as a function of time. The apparent rate constants, calculated from the slopes of the straight lines in such semilogarithmic representations, were found to be proportional to the Fab-93 concentration. This demonstrates that the process obeys simple second-order kinetics. The corresponding association rate constant at 4°C, obtained from the proportionality constant, is $k = 0.9 \times 10^5\ M^{-1} \cdot s^{-1}$.

When F_2-IAEDANS was first denatured with guanidine hydrochloride and then diluted in buffer A containing Fab-93 to initiate its renaturation, the fluorescence energy transfer appeared with the same kinetics as when folded F_2-IAEDANS was mixed with Fab-93. This shows that an immunoreactive conformation of F_2 is formed rapidly during the renaturation of F_2 because its appearance is not rate-limiting throughout the Fab concentration range investigated, even though the association of F_2 with Fab-93 is fast. Indeed, at the highest Fab-93 concentration investigated (1 μM), the pseudo-first-order rate constant observed for the association was $0.09 \pm 0.01\ s^{-1}$ at 4°C. This rate constant is by far higher than that of any of the folding steps previously identified for the β_2 subunit (11).

DISCUSSION

The results reported above give new information on two apparently distinct yet intimately related problems: protein folding and antigen–antibody interactions. Indeed, using monoclonal antibodies specific to the native β_2 subunit of tryptophan synthase as probes of its conformation, we have been able to pinpoint folding steps directly related to the antigenic properties of this protein. Thus, by observing the fluorescence energy transfer between tryptophan residues and a dansyl group linked to a unique cysteine residue of the antigen, two phases have been observed when IAEDANS-labeled β chains refold in the presence of Fab-19. The fast phase represents the step of association of Fab-19 with a rapidly formed folding intermediate. This is clearly demonstrated by the following observations: this phase is not observed when β-IAEDANS refolds in the absence of Fab-19, and it obeys second-order kinetics (i.e., its apparent rate constant is proportional to the Fab concentration as seen in Fig. 2). The slow phase corresponds to an isomerization occurring within the already formed β–Fab complex, as

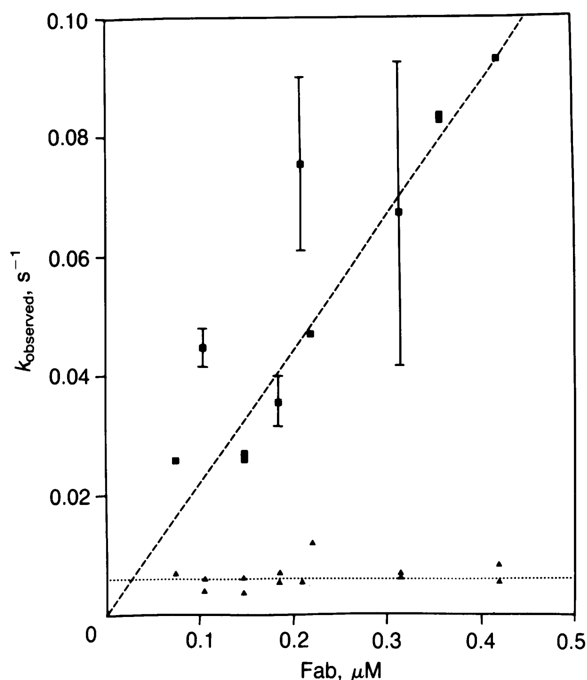


FIG. 2. Kinetics of refolding of β_2 in the presence of Fab-19. Guanidine hydrochloride-denatured apo-β_2-IAEDANS was diluted into 2.5 ml of buffer A containing an excess of Fab-19 at 12°C. The final concentration of β-IAEDANS ranged between 9 nM and 45 nM. The Fab-19 concentration, shown along the abscissa, was always at least 3-fold higher than that of the antigen. The fluorescence intensity was recorded as a function of time. When plotted in a semilogarithmic representation, the fluorescence change can be decomposed in two phases. From the slope of each straight line, the rate constants ($k_{observed}$) were computed and their respective values are shown as a function of Fab-19 concentration during the renaturation: ■, Apparent rate constant of the fast phase; ▲, rate constant of the slow phase.

demonstrated by the finding that this phase is not "quenched" by an excess of native, unlabeled antigen.

The true second-order rate constant found for the fast phase ($k = 2.1 \pm 0.4 \times 10^5\ M^{-1} \cdot s^{-1}$) is very close to that found for the association of Fab-19 with native β_2-IAEDANS ($k = 3.0 \pm 0.5 \times 10^5\ M^{-1} \cdot s^{-1}$). Furthermore, at the highest Fab-19 concentration used (see Fig. 2), the pseudo-first-order rate constant observed at 12°C is about 0.09 s^{-1}, a value 4-fold higher than the rate constant of the folding step leading to the first intermediate, β^{I_1}, thus far identified. It therefore can be concluded that, even before β^{I_1} is formed, β either preexists or folds up rapidly into a structure carrying an epitope recognized by Fab-19, as rapidly as the corresponding epitope on the native β_2 subunit.

Similarly, the isolated F_2 domain is recognized very rapidly by Fab-93, even when it refolds at 4°C, where the $\beta^{unfolded}$ to β^{I_1} step occurs with a rate constant of only 0.007 s^{-1}. Thus, for β chains as well as for its isolated F_1 and F_2 domains, structural intermediates formed very early on the folding pathway of the whole protein carry epitopes recognized by monoclonal antibodies "specific" to the native β_2 subunit.

Nall (23), on the basis of spectroscopic studies, showed that native-like transient intermediates are rapidly formed during the folding of alkaline iso-2-cytochrome *c*. Are such native-like intermediates comparable to our rapidly formed immunoreactive intermediates? At present, we have no indication on the nature of these partly native epitopes and, particularly, on how well they approximate the native epitopes recognized by the two monoclonal antibodies we used. They may involve most of the residues of the native epitope, already reasonably well positioned. Alternatively, they may consist in a few residues contained in a stretch of secondary

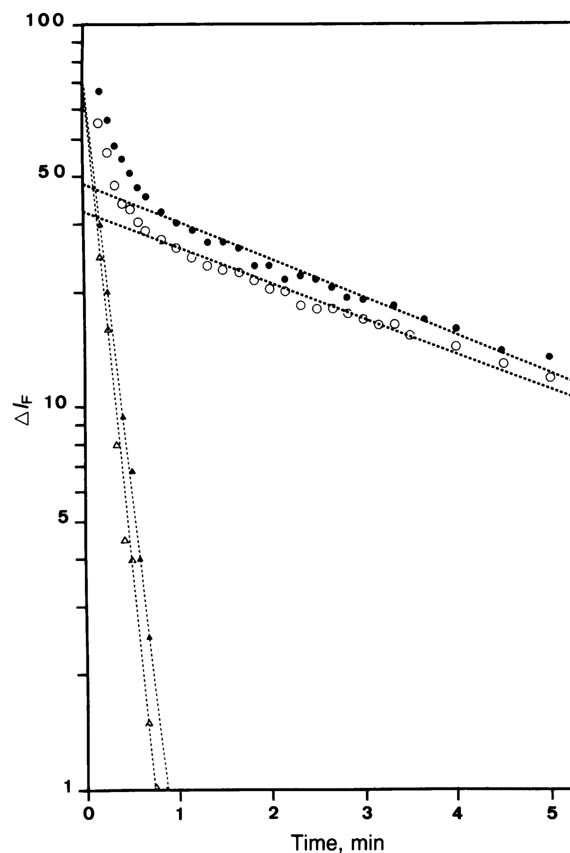


FIG. 3. Kinetic of refolding and association of isolated F_1 fragment with Fab-19. Guanidine hydrochloride-denatured or native F_1-IAEDANS was diluted into 2.5 ml of buffer A containing an excess of Fab-19 at 12°C. Final concentrations were 57 nM and 200 nM, respectively, for the antigen and the antibody. The change in fluorescence intensity ΔI_F (defined as in Fig. 1) is plotted as a function of time in a semilogarithmic representation: ●, Refolding of F_1-IAEDANS in the presence of Fab-19; ○, association of native F_1-IAEDANS with Fab-19.

structure, representing only part of the native epitope. Studies on the rate of appearance of the early epitopes might give an answer to this question. Indeed, at a high-enough Fab concentration, the rate of association should become independent of the Fab concentration, and the plateau should reflect the rate of appearance of the earliest immunoreactive intermediates. But because this rate is clearly too high to be measured by conventional methods (see Fig. 2), its determination will require the use of a stopped-flow. Experiments along this line have just been started.

It has been pointed out above that, for Fab-19, the second-order rate constants of association with the native β_2 subunit and with the early folding intermediates are the same. This might have suggested that, at least in the vicinity of the epitope recognized by Fab-19, the structure of the intermediates is already identical to that of native β_2 and that the various isomerization steps previously described would occur in the protein far enough from that epitope. However, affinity measurements have shown that monoclonal antibodies specific to native β_2 bind more strongly to β_2 than to its isolated native-like F_1 or F_2 domains. Thus, the affinity of Fab-19 is 5-fold higher for apo-β_2 than for isolated F_1, and that of Fab-93 is 50-fold higher for apo-β_2 than for isolated F_2 (24). This clearly indicates that conformational changes affecting the two epitopes recognized by Fab-19 and Fab-93 do occur at later stages of the folding.

How are these conformational changes related to the kinetic and thermodynamic parameters of the antigen–antibody interaction? A clue to answering this question may

be found in the properties of the slow change in fluorescence energy transfer observed when β-IAEDANS refolds in the presence of Fab-19. As discussed above, this slow phase corresponds to an isomerization of β-IAEDANS inside the antigen–antibody complex. In fact, a similar energy-transfer change is observed when β-IAEDANS refolds in the absence of Fab. The fluorochromes involved in this signal were shown to be tryptophan-177 and IAEDANS linked to cysteine-170 within the same β chain (11). This change is associated with the β^{I_1}-to-β^{I_2} folding step. Its amplitude and its rate ($k = 0.008$ s^{-1} at 12°C) are similar to the amplitude and the rate ($k = 0.007 \pm 0.001$ s^{-1} at 12°C) of the slow phase observed when β-IAEDANS refolds in the presence of Fab-19. Hence, the same intrachain isomerization step is likely to be involved in the refolding of β-IAEDANS, either alone or already associated with Fab-19. Therefore, it would be tempting to conclude that the interactions responsible for the association of Fab-19 with β chains do not affect the β^{I_1}-to-β^{I_2} transition.

This conclusion is obviously erroneous as seen when the isolated F_1 fragment interacts with Fab-19. Indeed, F_1 alone is unable to undergo the β^{I_1} to β^{I_2} isomerization; yet this isomerization (as judged by the slow phase of the energy-transfer change seen in Fig. 3) takes place when F_1 interacts with Fab-19. Thus, through an "induced fit" mechanism, the binding of Fab-19 appears to force F_1 closer to the conformation it has in the native β_2 subunit. The free energy consumed in this induced fit must then be subtracted from the antigen–antibody binding energy. This explains, at least in part, why the affinity of Fab-19 is lower for isolated F_1 than for β_2. This mechanism, at least from a kinetic point of view, differs much from that proposed by Sachs *et al.* (25) to account for the immunoreactivity of fragments from staphylococcal nuclease. They postulated that only the perfectly native conformation of the polypeptide chain can be recognized by antibodies raised against the native protein. Therefore, they concluded that the ratio of the affinities of an antibody for a fragment and for the native protein directly reflects the equilibrium constant $K_{conf.}$ of a preexisting conformational equilibrium between the native and denatured states of the fragment. Our results cannot be quantitatively interpreted according to this simple model because binding to F_1 occurs at a rate much higher than that of the steps leading from the early immunoreactive intermediates to the native β_2 subunit.

In conclusion, the results obtained in the present study show that, early on the folding pathway of β_2, intermediates are formed whose structure resembles that of the corresponding region of the native protein enough to be recognized by monoclonal antibodies specific to the native state. However, the epitopes carried by these intermediates are not perfectly native. They can approach their native structure either at later stages of the folding of β_2 or through an induced fit that follows their association with the specific antibody. In this respect, the association of a Fab fragment with a protein fragment is much like a step in protein folding: stereospecific assembly followed by conformational adaptation of the polypeptide chains, as illustrated by the similarities of Fab-19 and F_2 in modulating the structure of F_1.

Such a mechanism for the association of an antigenic protein with a specific antibody requires that the region of the protein that carries the epitope be somewhat flexible. It supports the first of the two suggestions of Wilson *et al.* (26), who proposed that "a conformational change is induced by antibody binding or that the antibody binds to a minor conformational state of the protein." Indeed it explains why antibodies raised or selected with a flexible, nonnative peptide isolated from a protein will be able to react with the entire protein only if the corresponding epitope lies in a flexible region of the native protein (27) or if the protein unfolds (17, 28). It does not rule out that antigen–antibody association can take place without conformational changes if the native antigen is rigid and if the antibody has been elicited and selected with the native antigen, as reported for the lysozyme–Fab D1.3 complex (29). However, it predicts that intermediates in the folding of lysozyme or lysozyme fragments would be recognized by this monoclonal antibody even if their conformation somewhat differs from that of the native enzyme. In this respect, carefully chosen monoclonal antibodies may turn out to be exquisitely precise reagents for investigating the possible connections between antigen–antibody recognition and nucleation centers in protein folding (30).

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