Kinetics of Appearance of an Early Immunoreactive Species during the Refolding of Acid-Denatured *Escherichia coli* Tryptophan Synthase β_2 Subunit†

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ABSTRACT: A reversible acid-denaturation process of the β_2 subunit of *Escherichia coli* tryptophan synthase has been set up. The acid-denatured state has been physically characterized: though not in a random-coiled conformation, it is extensively denatured. The renaturation of this denatured state of β_2 has been observed in a stopped-flow system, in the presence of a monoclonal antibody directed against native β_2. It is shown that the association occurs very early in the folding of β_2. The association rate constants of the antibody with the immunoreactive folding intermediate and with native β_2 are the same (3×10^5 $M^{-1} \cdot s^{-1}$). But at high antibody concentrations the formation of the antigen/antibody complex is rate limited by a rapid (5.4×10^{-2} s^{-1}) isomerization of refolding β chains. This isomerization appears to reflect the formation of at least part of the epitope recognized by the antibody during the folding of β_2. Further conformational adjustments occurring later in the folding pathway would then allow the ultimate structuring of the epitope.

Since the first monoclonal antibodies were obtained, in 1975 (Köhler & Milstein, 1975), they have proved to be an all-powerful tool in the study of a variety of biochemical phenomena. In particular, they have given a new impulse to investigations concerning the structure–function relationships that determine the biological properties of proteins. In this general area of investigations, however, monoclonal antibodies have not yet been exploited for studying protein folding. Yet, they may constitute a convenient tool for studying with precision the regain of antigenicity during the acquisition of the native conformation of a polypeptide chain. Indeed, an immunological approach to protein folding had already begun with the use of polyclonal antibodies to follow the return to the native state (Creighton et al., 1978; Ghelis & Yon, 1982; Teale & Benjamin, 1976a; Wichman et al., 1977). In such kinetic studies however, the rate obtained for renaturation was likely to represent an average for several phases, since the antisera used to detect the return of antigenic activity contained antibodies directed against several antigenic determinants on the protein. The use of antibody populations fractionated to monospecificity (i.e., directed against a distinct antigenic determinant of the surface of a protein) allowed this drawback to be partly overcome: folding intermediates have been detected and characterized that correspond to the return to native structure of different parts of the protein (Chavez & Scheraga, 1977, 1980; Sachs et al., 1974; Teale & Benjamin, 1976b, 1977). But these methods remain unsatisfactory because, in spite of this fractionation, the antibodies thus obtained are still heterogeneous and often difficult to characterize in terms of conformational specificity and thus allow an interpretation of the experimental results only in terms of antigenic regions and not in terms of epitopes.

Because of their homogeneity and their very selective reactivity, monoclonal antibodies may give access to quantifiable and much more precise information about molecular events of protein folding. In this way, Blond and Goldberg (1987) have started to investigate the appearance of "native-like" antigenic determinants on intermediate states during the folding of the β_2 subunit of *Escherichia coli* tryptophan synthase. Indeed, this protein (a dimer made by the assembly of two identical polypeptide chains of M_r 44000 each) seemed a suitable model because many studies concerning its folding had led to the characterization of the denatured states of the β_2 subunit and its isolated domains in Gdn·HCl[1] (Zetina & Goldberg, 1980), to a description of the kinetics of domain association (Zetina & Goldberg, 1982) and the dimerization of monomers (Blond & Goldberg, 1985), and to the establishment of a tentative folding pathway composed of at least six steps (Blond & Goldberg, 1986):

$$\beta(\text{unfolded in Gdn·HCl}) \xrightarrow{0.02\ s^{-1}} \beta^{I_1} \xrightarrow{0.008\ s^{-1}} \beta^{I_2} \xrightarrow{0.001\ s^{-1}}$$
$$\beta^{N'} \xrightarrow{\text{very rapid}} \beta_2^{N'} \xrightarrow{0.002\ s^{-1}} \beta_2^{N''} \xrightarrow{\text{rate limiting}} \beta_2^{\text{native}}$$

(rate constants determined at 12 °C). β^{I_1}, β^{I_2}, and $\beta^{N'}$ are monomeric intermediates, whereas $\beta_2^{N'}$ and $\beta_2^{N''}$ are dimeric species, as native β_2.

The first step, leading to β^{I_1}, reflects the folding of the N-terminal F_1 domain of the β chain (F_1, M_r 29000). It is characterized by the burying of the single tryptophanyl residue of the polypeptide chain (Trp-177) into a very hydrophobic environment, the bringing close together of Trp-177 and Lys-87, and an increase in the rigidity of the monomer (Blond & Goldberg, 1986). The second step, leading from β^{I_1} to β^{I_2}, is observed by a fluorescence transfer from Trp-177 to a dansyl group linked to Cys-170 (Blond & Goldberg, 1985). $\beta_2^{N'}$ is the first dimeric species observed on the folding pathway. It has the same overall rotational diffusion coefficient as the native protein (Blond & Goldberg, 1985). Its isomerization

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[1] Abbreviations: IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; α and β_2, α and β_2 subunits, respectively, of *Escherichia coli* tryptophan synthase [L-serine hydro-lyase (adding indoleglycerol-phosphate), EC 4.2.1.20]; β_2-AEDANS, β_2 subunit labeled with IAEDANS on cysteine-170; β^D-acid, β_2 denatured in buffer C, pH 1.5; β^D-Gdn·HCl, β_2 denaturated in 4 M Gdn·HCl; pyridoxal-P, pyridoxal 5'-phosphate; Gdn·HCl, guanidine hydrochloride; EDTA, ethylenediaminetetraacetic acid; β-SH, β-mercaptoethanol; BSA, bovine serum albumin.

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to $\beta_2^{N''}$ was observed during studies on the reassembly of the isolated N-terminal (F_1) and C-terminal (F_2, M_r 12000) folded domains (Zetina & Goldberg, 1982). The final isomerization of $\beta_2^{N''}$ to native β_2 is responsible for the lag phase observed in the renaturation of β_2 and gives to the protein its functional properties (binding of the coenzyme pyridoxal-P and activity). An additional step, occurring early on the folding pathway, was revealed by use of monoclonal antibodies directed against native β_2. It was shown (Blond & Goldberg, 1987) that partly native epitopes can be detected during the refolding of β_2 even before the first step (leading to β^{I_1}) was achieved. Unfortunately, the kinetics of appearance of these "native-like" patterns in refolding β_2 could not be determined, because they seemed too rapid ($k \geq 0.07$ s^{-1} at 12 °C) to be observed without the use of the stopped-flow technology. In the experiments mentioned above, refolding of β_2 was achieved after denaturation of the protein in 6 M Gdn·HCl: this required a 50–100-fold dilution to allow the renaturation to proceed with no (or little) interference from the residual guanidine. Only a manual mixing seemed to allow such a dilution.

That is why we decided to set up a denaturation process of β_2 that would allow renaturation by a simple pH jump, easily feasible in a stopped-flow apparatus. Alkaline conditions had to be avoided since they are known to extensively modify the covalent structure of proteins (Tanford, 1968). The present paper will therefore describe an acid denaturation of β_2, show that this denaturation is reversible, and characterize this acid-denaturated state of the protein. Then, it will report the association of refolding β chains with a monoclonal antibody directed against native β_2 and demonstrate that the kinetics of this association are limited by a rapid folding step of the β chains, which seems to correspond to the local appearance of the epitope recognized by the antibody.

Experimental Procedures

Chemicals and Buffers. *N*-(Iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS) and pyridoxal 5'-phosphate (pyridoxal-P) were obtained from Sigma. All other chemicals were of reagent grade. Buffer A was 100 mM potassium phosphate and 2 mM EDTA, pH 7.8; buffer B was 200 mM potassium phosphate and 4 mM EDTA, pH 7.8; buffer C was 1 mM potassium phosphate and 20 μM EDTA, pH 7.8; buffer D was 100 mM potassium phosphate, 0.5 mM indole, 2 mM EDTA, 5 mM β-SH, 50 mM L-serine, 20 μg/mL α, 0.05 mM pyridoxal-P, and 100 μg/mL bovine serum albumin, pH 8.

Preparation of the β_2 Subunit. The apo-β_2 protein was purified, crystallized, and reactivated as described earlier (Högberg-Raibaud & Goldberg, 1977a) by use of as starting material *E. coli* transformed with an expression plasmid directing the synthesis of the tryptophan synthase $\alpha_2\beta_2$ complex (C. R. Zetina, and A. F. Chaffotte, unpublished data).

Preparation of the Fluorescent Derivative of β_2. β_2-AEDANS was prepared as described previously (Zetina & Goldberg, 1982).

Monoclonal Antibodies. Murine monoclonal antibody IgG19, previously characterized (Djavadi-Ohaniance et al., 1984, 1986; Friguet et al., 1983, 1984), was supplied by Drs. Friguet and Djavadi-Ohaniance. The antigen-binding proteolytic Fab fragments were obtained as described by Mariuzza et al. (1985).

Titration of Antibody Sites. The Fab-19 was titrated with β_2-AEDANS before each experiment, according to the fluorescence transfer assay described (Friguet et al., 1985).

Activity and Protein Assay. The activity of β_2 was measured at 25 °C in the presence of α subunits as described by Faeder and Hammes (1970), in buffer D. Protein concentration was determined by the method of Lowry et al. (1951) or spectrophotometrically by use of the specific absorbances reported by Miles (1970) for β_2 and by Onoué et al. (1965) for antibodies.

Denaturation and Renaturation of β_2. β_2, initially in buffer C, was denatured by bringing the pH to 1.5 through the addition of 25 μL of 2 N HCl to 1 mL of protein solution, followed by a 30-min incubation at room temperature. The renaturation was initiated by mixing the acid–protein solution volume to volume with buffer B. This restores buffer A (optimal conditions for β_2).

Stopped-Flow Measurements. The mixing was performed by using either a rapid manual mixing apparatus (SFA-11 Hi-Tech Scientific, Salisbury, England), connected to a Haake cryothermostat, or a stopped-flow module SFM3 (Bio-Logic, Echirolles, France) equipped with a thermostat, and connected to a PC/XT/AT Tandon Microcomputer. All experiments were done at 12 °C. For both apparatus, the two solutions to be mixed arrive at the bottom of the observation cell. Since they have different densities (1 for acid-protein solution and 1.019 for buffer B), the density of the acid–protein solution was adjusted to that of buffer B by addition of 0.164 M (56.1 g/L) saccharose: diffusion of reagents from top to bottom of the cell after the mixing due to differences in their densities was therefore avoided.

Unless stated otherwise, all kinetic measurements were carried out with a double-monochromator spectrofluorometer, Perkin-Elmer MPF4, equipped with a Servotrace-Sefram PE recorder. The fluorescence cell of the SFA-11 Hi-Tech was placed in the thermostated cell holder of the fluorometer. The SFA-11 was connected to a Servotrace recorder, so that the stopping syringe triggers the data capture system. In this way, the dead time was about 0.5 s. In all kinetic experiments, the excitation wavelength was 280 nm, and the emission wavelength was 460 nm. The absorbance of the samples was maintained below 0.1 throughout the excitation and emission wavelength range, to minimize internal filter effects.

For performing the multimixing experiments, a Bio-Logic double mixer stopped-flow SFM3 was used. A delay line of 150 μL was introduced between the two mixers. The dead time was below 50 ms. The excitation wavelength was selected with a monochromator (280 nm), and the emitted light was observed through an Orion high-pass filter ($\lambda_{50\%}$ transmission = 450 nm) and recorded with an Apple IIe microcomputer interfaced to the SFM-3 photomultiplier by means of the AI/3 (Interactive Structures Inc.) analog–digital converter and the Fast A/D converter option for ADALAB by Interactive Microwave Inc.

Analytical Centrifugations. The centrifugations were carried out at 50000 rpm, 12 °C, in a Centriscan 75 (MSE, England) analytical centrifuge equipped with a monochromator, using the ultraviolet absorption scanning system at 280 nm. Cells with 20-mm light path were used. This enabled us to keep the protein concentration low (260 μg/mL) so as to minimize the nonideality of the solution and especially the charge effects.

Circular Dichroism Measurements. The CD spectra were recorded with a Jobin-Yvon Mark III dichrograph connected to a MICRAL 31 microcomputer. The samples were analyzed at 12 °C in 1 mm wide cells. Ellipticities in deg·cm^2·dmol^{-1} were expressed as $\Delta\theta$ (mean residual molar ellipticity). For $\Delta\theta$ calculation, the molecular weight of the β_2 subunit (397 amino acid residues) was assumed to be 45000.

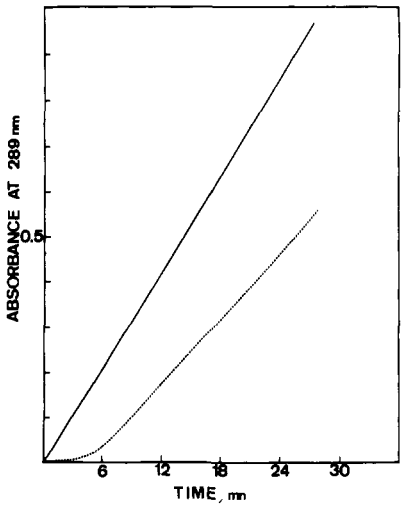

FIGURE 1: Activity assay of β_2. A total of 10 μL of a protein solution (0.43 mg/mL) either native in buffer A (—) or acid denatured at pH 1.5 for 30 min (···) is diluted in 1 mL of buffer D thermostated at 25 °C. Then, the increase of absorbance at 289 nm, corresponding to synthesis of L-tryptophan, is recorded as a function of time.

RESULTS

Acid Denaturation and Renaturation of β_2. Three conditions had to be satisfied to denature β_2 in an acidic environment: First, the pH should be far below the isoelectric point of the protein (5.4) to minimize aggregation by taking advantage of the electrostatic repulsion between the fully protonated polypeptide chains. Second, the solution should be of low buffering and ionic strength, in order not to reduce the electrostatic repulsions mentioned above and to permit the pH to be easily raised for initiating the renaturation. Finally, a polyacid should not be used to lower the pH, in order to avoid ions with two or three negative charges that could act like bridges between the positive groups of the polypeptide chains. Thus, β_2 has been incubated in 1 mM potassium phosphate containing 20 μM EDTA, pH 7.8 (buffer C), supplemented with concentrated HCl to lower the pH to 1.5. The question was then to know if β_2 was actually denatured under these conditions. To answer this question, four parameters of acid-treated β_2 have been analyzed that are representative of the physical state of the protein: its enzymatic activity, sedimentation coefficient, fluorescence spectrum, and circular dichroism.

Enzymatic Activity of β^D-acid. It was first verified that, after the acid treatment, the enzyme was inactivated, as far as its ability to catalyze L-tryptophan synthesis was concerned. When β^D-acid is diluted in the assay buffer (buffer D), which immediately brings the protein back to a neutral pH, no enzymatic activity could be detected at first. This demonstrates that β_2 is indeed inactivated at pH 1.5. However, the activity increased with time after the dilution, to reach its highest level after about 10 min at 25 °C (Figure 1). The comparison of this final level of activity with the activity of native β_2 at the same concentration, in the same assay conditions, gives the yield of renaturation (74% in the experiment of Figure 1). This shows that the acid inactivation is reversible, since β_2 regains activity when returned to neutrality. This experiment has been repeated under different conditions of denaturation (concentration of β_2 and incubation time at acidic pH) (data not shown). Since the yield of renaturation decreases with increasing concentrations of β_2, it seems that aggregation is responsible for the practically irreversible denaturation observed at high concentrations of protein. Moreover, aggregation apparently increases with denaturation time. Incubation for 30 min at 20 °C and pH 1.5 was satisfactory for both ensuring complete inactivation of the protein and minimizing the subsequent aggregation. Therefore, in all the experiments described later, the protein has been denatured at pH 1.5 for exactly 30 min, at concentrations below 0.3 mg/mL and at 20 °C. But whereas the renaturation temperature was 25 °C when enzymatic activity was used (as above) to monitor refolding of β^D-acid, all the other renaturation experiments were conducted at 12 °C because the whole folding pathway of β_2 has been thus far analyzed at 12 °C. In these conditions reactivation yields above 90% were always obtained for β^D-acid.

Quaternary Structure of β^D-acid. To determine whether the protein was or was not dissociated into monomers, its sedimentation coefficient was measured by analytical centrifugation. The value obtained $s_{20,pH1.5}$ = 1.9 S, can be considered as close to the $s_{20,w}$ value, since the density and viscosity of the solution were very close to those of pure water. Though electrostatic repulsion between the protonated side chains in the low ionic strength solution may interfere with the sedimentation of the protein, this effect is likely to be small in view of the low protein concentration used in this experiment. Thus, the experimental value $s_{20,pH1.5}$ = 1.9 S found here compares well with the $s_{20,w}$ values reported for β chains in 0.1 M glycine/HCl, pH 2.3, plus 4.5 M Gdn·HCl and in 4 M Gdn·HCl, respectively 1.7 S (Groha et al., 1978) and 2 S (C. R. Zetina and M. E. Goldberg, unpublished results), while it drastically differs from that of native β_2 in buffer A, 5.1 S (Högberg-Raibaud & Goldberg, 1977b). β^D-acid therefore appears to be completely dissociated into monomers, and the quaternary interactions within the protein are destroyed under the denaturation conditions.

Tertiary Structure of β^D-acid. The fluorescence emission spectrum of the single tryptophanyl residue of the β chain (Trp-177) has been used extensively to analyze the denaturation/renaturation transition of β_2 (Blond & Goldberg, 1986; Zetina & Golberg, 1980). Its maximum is shifted from 328 to 355 nm when β_2 is denatured at pH 1.5 in buffer C (data not shown). Moreover, at pH 1.5, the intrinsic fluorescence of Trp-177 drastically decreases compared to that observed in native β_2: the fluorescence intensity of β^D-acid at 355 nm is only about 30% of that of native β_2 at 328 nm. Since the fluorescence emission spectrum of *N*-acetyl-L-tryptophanamide in buffer C brought to pH 1.5 also has a maximum at 355 nm, it appears that Trp-177, which is buried in a very hydrophobic environment in native β_2, becomes completely accessible to the solvent in β^D-acid. β^D-acid has therefore lost most of its tertiary structure, at least in the environment of the tryptophan residue.

Secondary Structure of β^D-acid. Finally, the secondary structure of β^D-acid was investigated by means of the circular dichroism in the far UV. The circular dichroism spectra of native β_2 in buffer A and β^D-acid in buffer C, pH 1.5, are shown in Figure 2. They clearly indicate that β^D-acid has lost a large part of its secondary structure. However, unlike β^D-Gdn·HCl, which is in a random-coiled conformation (C. R. Zetina, personal communication), β^D-acid still exhibits some organized secondary structure: its circular dichroism spectrum has a distinct maximum at 193 nm and two minima at 210 and 222 nm, typical of α-helices. Two hypotheses can account for the presence of these secondary structures: either they may be some of the α-helices that exist in native β_2, and which failed to be completely destroyed at acidic pH, or they may be new helices, irrelevant to the native state, due to the acidic environment and to the positive charges thus created all along the

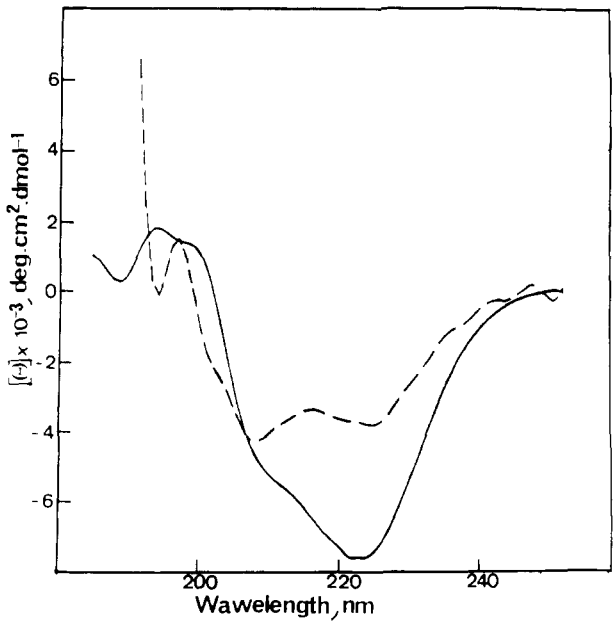

FIGURE 2: Circular dichroism spectra of native β_2 and β^D-acid at 20 °C. The data are reported as the difference between the circular dichroisms of the protein solutions and of the solvent of each solution alone. (—) Native β_2 (0.5 mg/mL in buffer A); (--) β^D-acid (0.5 mg/mL in buffer C, pH 1.5, after 30 min of incubation).

polypeptide chain. This will be discussed later.

It thus appears that β^D-acid is largely denatured since it is inactive, monomeric, and devoid of most of the optical signals characteristic of the secondary and tertiary structures present in native β_2. Although it is not completely in a random-coiled conformation, β^D-acid was thus also likely to have lost the antigenic determinants specific for the native state. It therefore appeared to be a suitable model for investigating the reappearance of the antigenicity during the refolding of β_2.

Interaction between an Anti-Native β_2 Monoclonal Antibody and Acid-Denatured β Chains during Their Refolding. The following experiments were aimed at studying the appearance, during the refolding of acid-denatured β chains, of early intermediates already carrying an epitope recognized by a monoclonal antibody (IgG19), previously characterized as "specific" for the native conformation of β_2 (Djavadi-Ohaniance et al., 1986). In fact, Fab fragments rather than the antibody itself were used to avoid complications arising from the bivalence of the IgG. AEDANS-labeled β_2 was first acid denatured in buffer C at pH 1.5, and its renaturation was initiated by mixing, in a stopped-flow apparatus, the denatured protein with either buffer B or buffer B containing the monovalent Fab-19 fragments. As a control experiment, acid-denatured β-AEDANS was first allowed to renature in the absence of Fab. A fluorescence transfer signal (λ_{ex} = 280 nm; λ_{em} = 460 nm) was then found to appear according to monophasic first-order kinetics (data not shown), with a rate constant of 0.009 s^{-1} at 12 °C, the temperature of all our previous studies on the folding pathway of β_2 (Blond & Goldberg, 1985, 1986, 1987; Zetina & Goldberg, 1980, 1982). This isomerization is very likely to correspond to tryptophan-177 and cysteine-170 coming closer together, which has already been observed with a very similar rate constant (0.008 s^{-1}) during the refolding of guanidine-denatured β_2, either in the absence of Fab-19 (Blond & Goldberg, 1986) or in its presence (Blond & Goldberg, 1987).

The renaturation of acid-denatured β-AEDANS in the presence of Fab-19 was then analyzed. As reported earlier (Blond & Goldberg, 1987), the association of Fab-19 with

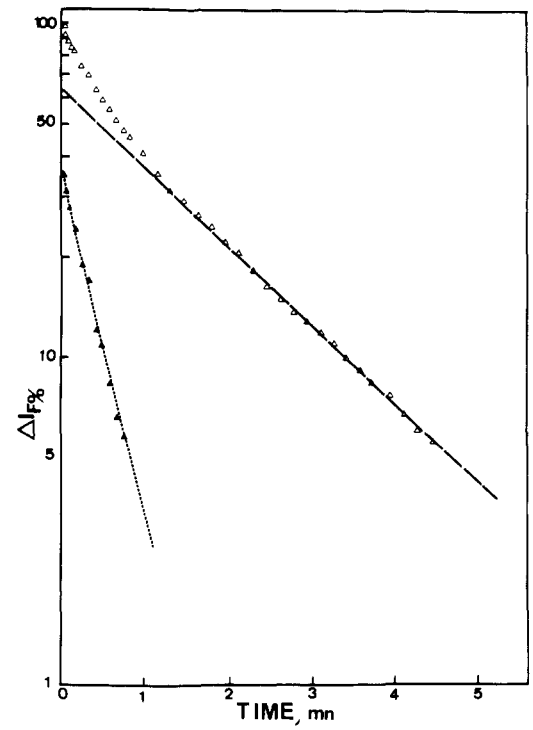

FIGURE 3: Kinetics of appearance of the tryptophan to AEDANS energy transfer during association of Fab and refolding-AEDANS-β^D-acid. AEDANS-β^D-acid (44 nM) was rapidly mixed in the SFA-11 manual stopped-flow with an equal volume of buffer B containing Fab-19 (420 nM). The reagents before mixing and the observation chamber were maintained at 12 °C. The fluorescence (λ_{ex} = 295 nm; λ_{em} = 470 nm) was recorded as a function of time at 12 °C. The results are plotted on a logarithmic scale as the change in fluorescence intensity $\Delta I_{F\%}$ as a function of time: $\Delta I_{F\%} = (I_\infty - I_t)/(I_\infty - I_0) \times 100$, where I_t is the fluorescence intensity at time t after dilution, I_∞ the fluorescence intensity reached at the end of the reaction, and I_0 the fluorescence intensity just after the dilution, obtained by extrapolation. The final concentrations were 22 nM for the antigen and 210 nM for Fab-19. (△) Experimental points; (▲) rapid phase obtained by decomposition into two exponentials.

β_2-AEDANS is accompanied by the appearance of a fluorescence transfer signal involving tryptophan residues of the Fab as energy donors and the AEDANS group of the labeled antigen as energy acceptor. This signal was used to monitor the antigen/antibody association occurring in the stopped-flow system. These mixing experiments were all performed at 12 °C. The initial concentration of Fab-19, determined by titration with native β_2-AEDANS, as described under Experimental Procedures, was always at least 10-fold higher than that of β chains and could therefore be considered as practically constant during each experiment. Figure 3 shows a semilogarithmic representation of the kinetics of appearance of the fluorescence transfer signal obtained in one such experiment. They clearly appear biphasic and can be easily decomposed graphically into two first- (or pseudo-first-) order phases, the apparent rate constants of which can be obtained from the slopes of the corresponding straight lines (Figure 3). By repeating such measurements at various initial Fab concentrations, the concentration dependence of the apparent rate constants was examined, both for the slow and for the rapid phases of the kinetics (Figure 4A). It was found that the slow phase has a rate constant independent of the Fab-19 concentration, k = 0.009 s^{-1}. Thus, this phase, which behaves as a first-order (in β chains) reaction, must correspond to an isomerization of the β chains independent of the presence of the Fab-19.

As opposed to the slow phase, the fast phase observed during the refolding of acid-denatured β_2 does depend on the Fab-19

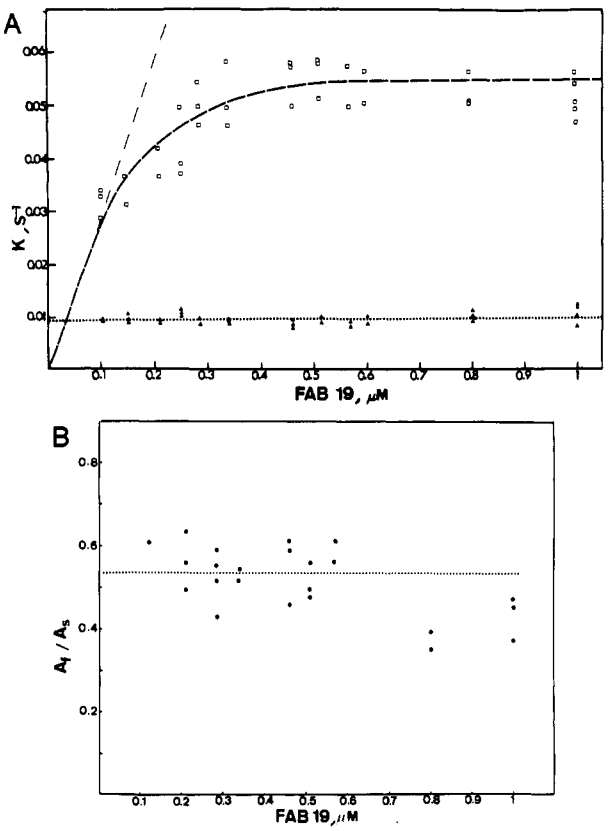

FIGURE 4: Concentration dependence of the rapid and slow phases observed upon refolding of AEDANS-β^D-acid with Fab-19. Apo-AEDANS-β^D-acid was diluted twice in buffer B containing an excess of Fab-19, at 12 °C as in Figure 3. The final concentration of AEDANS-β^D-acid ranged between 11 and 22 nM. The Fab-19 concentration, shown along the abscissa, was always at least 10-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 (see Figure 3). (A) Concentration dependence of the rate constants of slow and rapid phases. From the slope of each straight line, the rate constants (k) 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. (B) Concentration dependence of the rapid-phase amplitude. The amplitudes of the fluorescence signals could not be compared for experiments conducted at different Fab and β^D-acid concentrations because of differences in internal filter effects and in the setting of the fluorometer (emission slit width, amplification factors, etc.). Therefore, the observed amplitude A_f of the fast phase is compared, in each experiment, to the amplitude A_s of the corresponding slow phase. Since A_s is independent of the absence or presence of the Fab (see Table I), it will, for each setting of the apparatus, depend only on the AEDANS-protein concentration and thus can be used as an internal standard. Therefore, the amplitude of the fast phase is expressed on the ordinate as the ratio A_f/A_s.

concentration. At low Fab concentrations (i.e., below 0.1 μM), it behaves as a second-order reaction with a rate constant of about 3×10^5 $M^{-1} \cdot s^{-1}$. This is the same value as that found for the second-order rate constant of association of Fab-19 with native β_2. At higher concentrations of Fab-19 (above 0.4 μM), the apparent rate constant of the first phase becomes invariable, with a maximum value of 5.4×10^{-2} s^{-1}. This seemingly complex Fab concentration dependence of the apparent rate constant can easily be accounted for by a sequence of two coupled reactions: an isomerization of β chains (with a first-order rate constant of 5.4×10^{-2} s^{-1}) followed or preceded by an association reaction (with a second-order rate constant of 3×10^5 $M^{-1} \cdot s^{-1}$). Indeed, at low Fab concentrations, the association would be rate limiting and thus be the observed reaction. On the contrary, at high Fab concentrations, the association would be rapid compared to the isomerization, and therefore, the fast phase of the fluorescence transfer appearance would reflect the kinetics of this isomerization. As suggested above, the isomerization could either follow or precede the association of Fab-19 with the refolding β chains. Indeed, one possibility is that the Fab could bind to β chains before the isomerization occurs, but without giving rise to the fluorescence transfer signal; an "intracomplex" isomerization of β chains would then bring the AEDANS group close enough and properly oriented relative to the tryptophan of the Fab so as to give rise to the fluorescence transfer. Alternatively, the Fab might be unable to bind the refolding β chains unless the latter have already undergone the isomerization. If such were the case, this isomerization would indeed correspond to the appearance of the first folding intermediate structured well enough (compared to native β_2) to be recognized by Fab-19.

In order to sort out these two possibilities, double mixing experiments could be performed according to the following rationale. If one mixes AEDANS-labeled acid-denatured β chains with Fab-19 at neutral pH to initiate refolding and association, at Fab concentrations high enough to render the association very rapid compared to the isomerization, and if one then adds an excess of unlabeled native β_2 at a time when the association of refolding β-AEDANS with Fab-19 should be nearly completed (as calculated from the association rate constant determined above), then the fast phase of the fluorescence transfer kinetics should still be observed only if the isomerization follows the association. On the contrary, if the isomerization has to occur before the antigen can be recognized by the Fab-19, very few antigen/antibody complexes will have had time to be formed before the unlabeled native β_2 protein is added, and all the Fab will be saturated with unlabeled native antigen, thus preventing the later association of Fab-19 with refolding β-AEDANS chains. Such an experiment was performed as follows: β_2-AEDANS denatured in buffer C, pH 1.5, at a concentration of 4.4×10^{-7} M in β chains, was placed in the first syringe, and Fab-19 (4.6×10^{-6} M) in buffer B was placed in the second syringe. Unlabeled native β_2 (10^{-5} M in buffer A) was placed in the third syringe. A total of 150 μL of AEDANS-β^D-acid and 150 μL of Fab-19 were first mixed together and injected in the delay line. Six seconds later, 125 μL of this mixture was mixed with 125 μL of unlabeled β_2. The fluorescence (λ_{ex} = 280 nm; $\lambda_{em} > 450$ nm) was recorded as a function of time. The kinetics thus obtained reflected a monophasic, slow (k = 0.009 s^{-1}) reaction, the same as that observed when AEDANS-β^D-acid refolds in the absence of Fab (Figure 5). No fast phase reflecting the association could be detected. A control experiment was conducted in the double-mixing stopped flow, in which AEDANS-β^D-acid was first mixed with Fab-19 as above and, after the same delay, buffer A (instead of unlabeled β_2) was added. To reproduce exactly the same internal filter effect as that when native unlabeled β_2 is added, bovine serum albumin (0.78 mg/mL) was added to buffer A so that the final absorbance in the stopped-flow observation cell is the same (0.52) in both multimixing experiments. There, the fast phase of association did appear clearly in the kinetics of fluorescence transfer change (Figure 5). That the single phase observed in the presence of the native β_2 competitor indeed corresponds to the isomerization that takes place in free refolding β chains is confirmed by a detailed comparison of the amplitudes and rates of variation of the transfer signals. Table I shows that the single phases observed for β^D-acid refolding alone, or in the presence of Fab-19 with the delayed addition of native β_2, have the same rate constants and the

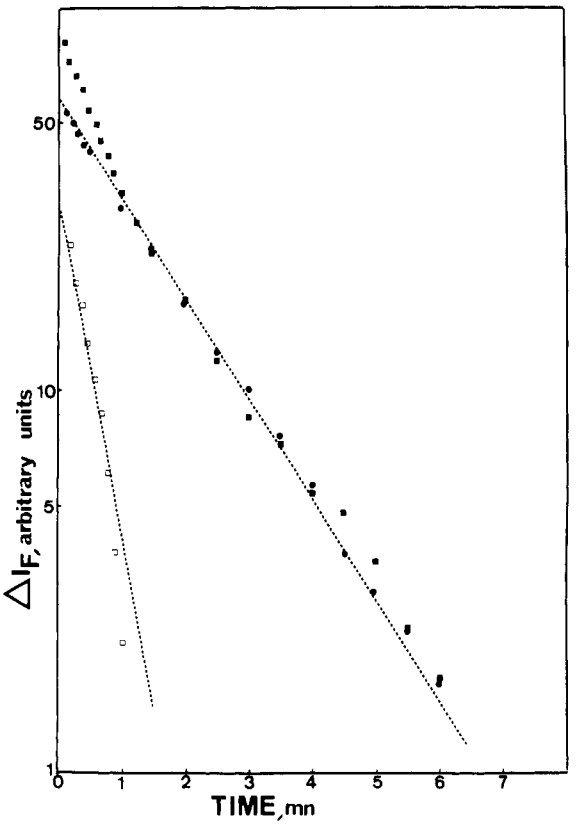

FIGURE 5: Kinetics of appearance of the tryptophan to AEDANS energy transfer upon refolding of AEDANS-β^D-acid in the presence of Fab-19, during multimixing experiments. A total of 150 μL of AEDANS-β^D-acid (4.4×10^{-7} M in β chains) was first rapidly mixed with 150 μL of Fab-19 (4.6×10^{-6} M) and injected into the delay line. Six seconds later, 125 μL of this mixture was mixed with 125 μL of unlabeled native β_2 (10^{-5} M) (●) or with 125 μL of buffer A containing bovine serum albumin (0.78 mg/mL) (■). The reagents before mixing and the observation chamber were maintained at 12 °C. The fluorescence (λ_{ex} = 295 nm; λ_{em} = 470 nm) was recorded as a function of time at 12 °C. The results are plotted on a logarithmic scale as the change of fluorescence intensity ΔI_F versus time ($\Delta I_F = I_\infty - I_t$, where I_∞ and I_t are defined as in the legend to Figure 3). (●) Refolding of AEDANS-β^D-acid in the presence of Fab-19 (first mixing) and then in the presence of unlabeled native β_2. The ratios of the various proteins in the final mixture are 1 AEDANS-β^D/10 IgG binding sites/100 native β^2 antigenic sites. (■) Refolding of AEDANS-β^D-acid in the presence of Fab-19 (first mixing) and then mixed with buffer A containing bovine serum albumin (experimental points). (□) Rapid phase obtained by decomposition in two exponentials.

same amplitudes. Furthermore, in the multimixing experiment performed with Fab-19 and serum albumin, the slow phase has also the same amplitude and rate constant as above, indicating that the corresponding isomerization occurs with the same kinetics for free and bound β chains.

Together with the disappearance of the fast phase caused by the delayed addition of the native β_2 competitor, these observations clearly rule out that the association could occur before the isomerization responsible for the fast phase ($k = 5.4 \times 10^{-2}$ s^{-1}). Indeed, it can be calculated that if this isomerization was not a prerequisite for the association, the half-life of the association ($k = 3 \times 10^5$ M^{-1}·s^{-1}) would have been only 1 s at the Fab concentration (2.3×10^{-6} M) after the first mixing. Thus, refolding β-AEDANS/Fab-19 complexes would have been formed during the 6 s before the addition of native unlabeled β_2, and the fluorescence transfer then reflecting an intracomplex "fast" isomerization ($k = 5.4 \times 10^{-2}$ s^{-1}) would have been observed. That this signal was not observed therefore leads to the unambiguous conclusion

Table I: Amplitude and Rate Constants of the Kinetic Phases in Multimixing Experiments[a]

syringe 1	AEDANS-β^D	AEDANS-β^D	AEDANS-β^D
syringe 2	buffer B	Fab-19	Fab-19
syringe 3	native β_2	native β_2	BSA
rapid-phase amplitude[b]	0	0	34
slow-phase amplitude[b]	55	57	55
slow-phase rate constant[b]	0.009	0.009	0.009

[a] The contents of syringe 1 (AEDANS-β^D-acid, 4.4×10^{-7} M in β chains) and syringe 2 (Fab-19, 4.6×10^{-6} M, or buffer B alone as indicated) were mixed (150 μL of each) in the delay line. Six seconds later, 125 μL of this mixture was mixed with 125 μL of the contents of syringe 3 (either unlabeled native β_2, 10^{-5} M, or 0.78 mg/mL serum albumin in buffer A to produce the same inner filter effect as β_2). The reagents before mixing, the delay line, and the observation chamber were at 12 °C. The fluorescence was recorded and plotted as in Figure 5. [b] The amplitude in arbitrary units of each phase was estimated by extrapolation to time zero of the straight lines obtained either directly (no Fab-19, or Fab and native β_2) or after decomposition of the kinetics (no native β_2).

that the isomerization, shown above to be required for the appearance of the fluorescence transfer ($k = 5.4 \times 10^{-2}$ s^{-1}), actually represents the rate-limiting step in the recognition of refolding β chains by Fab-19.

Finally, the amplitude of the fast phase does not increase with the Fab concentration (Figure 4B) between 10^{-7} and 10^{-6} M. This indicates that the immunoreactive intermediate is saturated by the Fab in this concentration range. The equilibrium dissociation constant for the intermediate/Fab complex must therefore be below 10^{-7} M. Hence, the rate constant of the dissociation of the complex ($k_{diss} = Kk_{assoc}$) must be below 3×10^{-2} s^{-1}. The early intermediate thus appears to already be tightly bound to the antibody.

DISCUSSION

The main finding reported in this paper is the determination of the rate of appearance of the first intermediate (on the folding pathway of the tryptophan synthase β_2 subunit) that possesses an antigenic determinant efficiently recognized by a monoclonal antibody specific for native β_2. This was made possible because conditions were found under which β_2 could be denatured at low pH and renatured by rapidly raising the pH to neutrality. Since our study can be considered as meaningful only if β_2 is actually denatured (rather than slightly altered in its conformation) during this acid treatment, we shall first discuss the extent of unfolding which β_2 may undergo during its stay at low pH. Several criteria show that the structure of β_2 has been strongly altered at pH 1.5. At first, its secondary structure is drastically altered at pH 1.5, as shown by the circular dichroism spectrum in the 180–240-nm range. It cannot, however, be considered as a random-coil state, because of the presence of a significant amount of α-helices revealed by the circular dichroism spectrum of β_2 at pH 1.5. As already suggested under Results, these α-helices may have two distinct origins: either they may be part of the original secondary structure present in native β_2, which the acid treatment would fail to disrupt, or they may be entirely irrelevant to the native state, and formed de novo at pH 1.5, perhaps because of new interactions created between protonated side chains. Several observations reported in the literature are in line with the second hypothesis:

(i) First, α-helices seem to be present at acidic pH in other proteins: for instance, Zettlmeissl et al. (1979) have shown that lactic dehydrogenase in 1 M glycine–H_3PO_4, pH 2, was also denatured and had a circular dichroism spectrum indicative of a high content of α-helices. Even more striking is

the observation that denaturation of bovine erythrocyte carbonic anhydrase B at pH 1 increases the α-helix content of the protein, as compared to that of the native state (McCoy & Wong, 1981).

(ii) Second, several poly-α-amino acids spontaneously fold into α-helix at acidic pH, like, for instance, poly(L-glutamic acid) in 0.1 M NaCl, pH 4.28, and copoly(L-Glu^{42}-Lys^{28}-Ala^{30}) in 0.1 M NaF, pH 3.1 (Fasman, 1967). Moreover, poly(L-glutamic acid) was shown to undergo an helix $\rightarrow$ coil transition with increasing pH (Fasman, 1967). Similarly, the ribonuclease S–peptide–α-helix stability has been examined as a function of pH: an acidic pH (below 4) was shown to cause an important stabilization of this α-helix (Nelson & Kallenbach, 1986).

(iii) Third, the acid-denatured state obtained directly from native carbonic anhydrase B was compared to the acid-denatured state obtained via its random-coiled state (McCoy & Wong, 1981): they exhibit identical far-UV circular dichroism spectra. Thus, the two acid-denatured states possess the same secondary structure. This strongly suggests that the formation of secondary structures in carbonic anhydrase at acidic pH (essentially α-helices, as reported above) is independent of the presence of secondary structures in native conformation, since the latter have been lost in the intermediate conditions giving the random-coiled state.

Although not demonstrated, a similar situation may exist for acid-denatured β_2, which suggests the possibility that the α-helices exhibited by this protein at pH 1.5 might not be part of structural patterns of native β_2 that would have been preserved in spite of the acidic pH. Anyway, whatever the origin of these α-helices may be, several other criteria clearly show that the conformation of β_2 is drastically disorganized at acidic pH. Indeed, tryptophan-177 is readily exposed to the solvent, while it is buried in the hydrophobic core of the protein in the native state of β_2, as shown by its fluorescence spectra. Furthermore, the sedimentation coefficient observed at pH 1.5 (s = 1.9 S) clearly indicates that the protein has dissociated into monomers and strongly suggests that these monomers are largely unfolded: obviously, such a low sedimentation coefficient is not compatible with that expected for a folded globular protein having the molecular weight of the β chain (M_r 44 000), as demonstrated by the following examples: α-glycoprotein from pleural fluid (M_r 45 230, $s_{20,w}$ = 3.15 S), α-trypsin inhibitor from human serum (M_r 44 980, $s_{20,w}$ = 3.41 S), peroxidase II from horseradish (M_r 44 050, $s_{20,w}$ = 3.85 S), α-amylase from pig pancreas (M_r 45 200, $s_{20,w}$ = 4.5 S), and ovalbumin (M_r 43 000, $s_{20,w}$ = 3.6 S) [data from *Handbook of Biochemistry* (1968)]. And finally, the acid conformation of β chains is catalytically inactive and not recognized by the anti-native β_2 monoclonal antibody IgG19. This is demonstrated by the fact that activity and antigenicity both require folding steps to occur before they are restored after the pH jump to neutrality. Taken together, these observations leave little doubt that β^D-acid indeed represents an extensively denatured state of β_2.

The studies reported in this paper on the refolding of β^D-acid upon raising the pH yield three pieces of information:

(i) β_2 can be renatured, since active enzyme is recovered with high yield after neutralization.

(ii) The step leading from β^{I_1} to β^{I_2} (see the introduction), which is detected by an intrachain fluorescence transfer from tryptophan-177 to AEDANS on cysteine-170 during the refolding of Gdn·HCl-denatured β chains (Blond & Goldberg, 1986), also occurs, and with the same rate constant, during the refolding of acid-denatured β chains.

(iii) An isomerization step, identified as being the rate-limiting step in the recognition of refolding β chains by the monoclonal antibody 19, has been characterized kinetically.

The first two points will be studied in more detail and discussed elsewhere (A. Murry-Brelier and M. E. Goldberg, unpublished results). The third will now be examined. The results we have reported here confirm that an early intermediate in the refolding of β_2 is efficiently recognized by Fab-19 ("specific" of native β_2). This had already been reported for the refolding of Gdn·HCl-denatured β chains (Blond & Goldberg, 1987). However, in our previous studies we were unable, for technical reasons (i.e., the time needed to dilute out the denaturing agent by hand mixing), to follow the appearance of this immunoreactive intermediate. We therefore had no information about the structure of this early epitope. Was it just a hapten-like, very local, antigenic determinant already present in significant amounts in the denatured state (say, for instance, a small sequential determinant)? Or conversely, was it formed by bringing close together residues which are distant in the amino acid sequence but adjacent on the surface of native β_2 (a conformational determinant)? The finding reported here that β^D-acid is not recognized by Fab-19 immediately after neutralization rules out the former hypothesis. And the latter is further supported by the rate constant found for the kinetics of formation of the epitope, $k = 5.4 \times 10^{-2}$ s^{-1} at 12 °C. This value corresponds to a half-time of reaction of about 13 s, a value compatible with the complete folding of a small globular protein and much too long to involve only the formation of a local secondary structure.

Thus, this isomerization does correspond to a real folding step, which controls the appearance of the first antigenic (with respect to Fab-19), intermediate during the renaturation of β_2. This immunoreactive species has two striking features. First, it is formed, during the renaturation, a long time before β_2 has recovered its really native structure. In fact, it even precedes by far *all* of the five intermediates identified in the folding pathway before the use of monoclonal antibodies, including that corresponding to the burying of the tryptophan residue. Second, even though it is clearly not entirely native, this early antigenic intermediate binds to Fab-19 with a second-order rate constant of association ($k = 3 \times 10^5$ M^{-1}·s^{-1} as determined at low Fab concentrations) identical with that of native β_2 (Blond & Goldberg, 1987). And yet, IgG19 has been shown to recognize preferentially native β_2 (Djavadi-Ohaniance et al., 1986); therefore, Fab-19 is only likely to bind to structural patterns close to those of the epitope it recognizes on native β_2. How can one explain that an anti-native β_2 antibody binds a nonnative state of the antigen so efficiently? Probably, the immunoreactive folding intermediate already possesses at least a part of the native epitope. This "subepitope" must be large enough to allow for the efficient binding of Fab-19, through some of the specific interactions established in the native β_2/Fab-19 complex. Then, the translational and rotational constraints on the relative positions of the Fab and the antigen required for their association would be more or less the same for native β_2 and the early immunoreactive intermediate. This would explain why the association rate constants are the same for both native and refolding β_2. On the contrary, the dissociation constants should then be different: if the epitope is only partly structured in refolding β^D-acid, a larger area will be involved in the association of Fab-19 with native β_2 than with the intermediate, and more interactions will strengthen the binding, thus decreasing the dissociation constant. The affinity of Fab-19 for

the folding intermediates of β^D-acid is therefore likely to be lower than for native β_2. This reasoning assumes that conformational changes that occur later in the folding pathway of β_2 are responsible for the ultimate fashioning of the epitope, which results in the optimum interactions between the antigen and the antibody. This point of view is supported by the fact that Fab-19, which is directed against the N-terminal F_1 domain of β_2, has a 5-fold higher affinity for apo-β_2 than for isolated F_1 (Djavadi-Ohaniance et al., 1986). Yet, the rate constants of association of Fab-19 to native β_2 and to isolated F_1 are the same (Blond & Goldberg, 1987). Moreover, it was shown that isolated F_1 is unable to undergo the isomerization leading from β^{I_1} to β^{I_2} (Blond & Goldberg, 1986) but that this isomerization occurs in the presence of Fab-19 (Blond & Goldberg, 1987). This antibody then induces a conformational change in F_1 that brings it closer to the conformation it has in native β_2. This clearly demonstrates that in the case of isolated F_1 an imperfect epitope is sufficient to ensure the rapid binding of the antibody and that the later structuring of the epitope, either spontaneous as in β chains or induced by the antibody as in isolated F_1, results in an increased affinity of the antibody for the protein. This is exactly the behavior predicted above for the immunoreactive folding intermediate. It strongly suggests that the dissociation rate constant is actually the factor that determines the affinity constant of an antibody for the various states of its antigen, as well as, probably, the variations of this affinity observed through conformational changes of the antigen (Djavadi-Ohaniance et al., 1986).

In summary, it can be concluded that the use of a monoclonal antibody to investigate the folding of β_2 gave some insights into the basic kinetic properties of the antibody/antigen interaction and enabled us to determine the rate of appearance of the first immunoreactive species, which turns out to be the earliest intermediate thus far detected, in the renaturation pathway of β_2.

However, because the secondary structures present in the acid-denatured form of the protein may perhaps influence its refolding pathway one may ask the two following questions: where is the immunoreactive intermediate located on the folding pathway of acid-denatured protein? And how do the kinetics of appearance of this intermediate compare during the renaturation of the acid- or guanidine-denatured β chains? Work in progress in this laboratory will answer these questions by comparing in more detail all the observable steps during the refolding of β_2 after acid or guanidine denaturation.

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