Kinetic Characterization of Early Immunoreactive Intermediates during the Refolding of Guanidine-Unfolded *Escherichia coli* Tryptophan Synthase β_2 Subunits†

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ABSTRACT: This paper deals with stopped-flow studies on the kinetics of the regain of immunoreactivity toward five distinct monoclonal antibodies during the folding of the guanidine-unfolded β_2 subunit of *Escherichia coli* tryptophan synthase and of two complementary proteolytic fragments of β, F_1 (N-terminal; M_w = 29 000) and F_2 (C-terminal; M_w = 12 000). It is shown that, while selected as being "specific" for the native protein, these antibodies are all able to recognize early folding intermediates. The two antigenic determinants carried by the F_2 domain and the antigenic site carried by the hinge peptide linking F_1 and F_2 are present so early during the folding process that their kinetics of appearance could not be followed. On the contrary, the rate constants of appearance of two "native-like" epitopes, carried by F_1, could be determined during the folding of β chains. The rate constant of appearance of the epitope to antibody 19 was found to be k = 0.065 s^{-1} at 12 °C. This value is very similar to that we reported previously for the appearance of an early epitope to the same antibody during the folding of acid-denatured β chains. Thus, in spite of the important structural differences between guanidine-unfolded and acid-denatured β chains, the same early folding events seem to be involved in the appearance of this epitope. The rate constant was found to be significantly smaller (k = 0.02 s^{-1} at 12 °C) for the appearance of the epitope to antibody 9. This shows that the regain of immunoreactivity is not concerted within the F_1 domain. Finally, it was observed that the epitopes to antibodies 9 and 19 appear significantly more rapidly during the folding of the isolated F_1 fragment than during the folding of complete β chains. Thus, in the complete protein, the rest of the polypeptide chain interferes with the folding of the F_1 domain even in the early stages leading to the appearance of the immunoreactivity.

The β_2 subunit of *Escherichia coli* tryptophan synthase has been the subject of extensive studies aimed at understanding the molecular mechanisms that lead the polypeptide chain to spontaneously acquire the complex three-dimensional structure characteristic of its native state. Thus, it has been shown that, after being completely unfolded with concentrated guanidine hydrochloride, β chains refold through a temporal sequence of several intermediates that have been kinetically characterized by use of a variety of physical–chemical criteria (Zetina & Goldberg, 1982; Blond & Goldberg, 1986). With the aim of gaining some insight into the degree of structural organization of these intermediates, we undertook, some years ago, a series of investigations on their immunoreactivity toward monoclonal antibodies that had been prepared with native β_2 subunits and selected as being specific for the native conformation of β_2.[1] This study led us to the unexpected observation that even the first intermediate previously observed by classical optical methods was already immunoreactive to two different monoclonal antibodies (Blond & Goldberg, 1987). The appearance of the immunoreactivity was too fast to be observed through the "hand-mixing" method then used to initiate the refolding of β chains by a 50–100-fold dilution of the guanidine. However, by use of a conventional stopped-flow machine, we could study the refolding of acid-denatured β chains and show, with one of the monoclonal antibodies (mAb 19), that while the denatured β chains were not immunoreactive, an immunoreactive intermediate rapidly appeared as a result of a folding step. This step was also faster (k = 0.05–0.06 s^{-1} at 12 °C) than the appearance of any other folding intermediate thus far detected with β chains (Murry-Brelier & Goldberg, 1988).

However, the significance of the conformational change involved in the appearance of the immunoreactivity during the refolding of acid-denatured β chains was not entirely clear, because acid-denatured β chains are not completely unfolded: they exhibit a far-UV circular dichroism spectrum characteristic of the presence of a significant amount of α-helix (Murry-Brelier & Goldberg, 1988). Therefore, the rate-limiting step in the appearance of the immunoreactivity could correspond to the disruption of a "wrong" structural pattern that must precede the formation or demasking of the epitope; alternatively, it could indeed represent the folding step leading

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[1] Abbreviations: β_2, β_2 subunit of *E. coli* tryptophan synthase [L-serine hydro-lyase (adding indoleglycerol-phosphate), EC 4.2.1.20]; IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; β_2-AEDANS and AEDANS-β, native β_2 dimer and monomeric unfolded β chains, respectively, labeled with IAEDANS on cysteine-170; F_1-AEDANS and F_2-AEDANS, F_1 and F_2 proteolytic fragments of β_2 labeled with IAEDANS on cysteine-170 and cysteine-340, respectively; EDTA, ethylenediaminetetraacetic acid; pyridoxal-P, pyridoxal 5'-phosphate; GuHCl, guanidine hydrochloride; β-SH, β-mercaptoethanol; mAb, monoclonal antibody.

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to the native-like epitope. The first aim of the work described here was to solve this alternative by studying the regain of immunoreactivity to mAb 19 during the refolding of β chains that were completely unfolded with guanidine; we shall report here that the rate constant found for guanidine-unfolded β chains is the same as that previously reported for acid-denatured β chains. This proved that we observed a real folding step (that which is rate limiting for the regain of immunoreactivity) and not the disruption of a wrong structural pattern present in acid-denatured β chains.

This pointed to monoclonal antibodies as a tool of choice for investigating the appearance of native-like local structural patterns during the folding of polypeptide chains. We therefore thought it of interest to investigate the kinetics of appearance of other antigenic determinants located in distinct regions of the protein. The β_2 subunit of tryptophan synthase seemed a particularly convenient system for carrying out such a study. Indeed, each β chain is made of two regions, F_1 (N-terminal; M_w = 29000) and F_2 (C-terminal; M_w = 12000), which can be easily separated after proteolytic cleavage by trypsin of the native protein (Högberg-Raibaud & Goldberg, 1977a) and which behave as autonomously folding domains (Högberg-Raibaud & Goldberg, 1977b). Several, well-characterized, monoclonal antibodies are available and were shown to recognize distinct epitopes of native β_2, located either on F_1 or on F_2 (Friguet et al., 1983, 1984; Djavadi-Ohaniance et al., 1984). In this paper, we shall describe investigations on the association of five such antibodies (two against F_1, two against F_2, and one against the hinge between these two domains) during the refolding of the guanidine-unfolded β chain or its proteolytic F_1 and F_2 fragments, with the aim of answering the two following important questions: Is the appearance of the overall antigenicity of a domain concerted (i.e., do all its conformation-dependent epitopes appear simultaneously)? Do the early folding steps that lead to the native-like immunoreactivity occur exclusively within a domain, or are they influenced by the rest of the polypeptide chain?

Materials and Methods

Chemicals and Buffers. IAEDANS and pyridoxal-P were obtained from Sigma. Ultrapure GuHCl was from Schwarz/Mann. All other chemicals were reagent grade. Buffer A was 100 mM potassium phosphate, 2 mM EDTA, and 5 mM β-SH, pH 7.8. Deuterated buffer A was buffer A but prepared with pure deuterated water (D_2O) so that the final D_2O content was 90%.

Preparation of the β_2 Subunit and Its Proteolytic Fragments. The apo-β_2 protein was purified, crystallized, and reactivated before use as described earlier (Högberg-Raibaud & Goldberg, 1977b) with as a starting material a culture of *E. coli* transformed with an expression plasmid directing the synthesis of the tryptophan synthase $\alpha_2\beta_2$ complex (gift from Dr. C. R. Zetina). The F_1 and F_2 proteolytic fragments of β were prepared as previously described from holo-β_2 reconstituted with pyridoxal-P (Zetina & Goldberg, 1982).

Preparation of the Fluorescent Derivatives of β_2 and Its Proteolytic Fragments. β_2-AEDANS and F_1-AEDANS were prepared as described previously (Zetina & Goldberg, 1982) while F_2-AEDANS was prepared according to Friguet et al. (1985).

Monoclonal Antibodies. Murine monoclonal antibodies were provided by Drs. Djavadi-Ohaniance and Friguet. All of them recognize native β_2 in solution; antibodies 9 and 19 recognize epitopes carried by F_1 and were shown, by the additivity test previously described (Friguet et al., 1983), to bind simultaneously to β_2, and hence to recognize distinct epitopes. Antibodies 93 and D4B6 recognize epitopes carried by F_2 (Friguet et al., 1983). Antibody 164 recognizes the hinge peptide that links F_1 and F_2 (Friguet et al., 1989). The antibodies were titrated with the fluorescence transfer assay described by Friguet et al. (1985).

Denaturation of β_2. Apo-β_2 was incubated for at least 4 h in buffer A supplemented with 100 mM β-SH and 6 M GuHCl. The denatured AEDANS-β chains were then diluted into 4 M GuHCl just before the refolding experiments were started. This was shown earlier (Zetina & Goldberg, 1980) to ensure complete unfolding of the β chains, as judged by far-UV circular dichroism measurements.

Activity and Protein Assay. The activity of β_2 was measured as described by Faeder and Hammes (1970), and protein concentrations were measured by the method of Bradford (1976) or spectrophotometrically by use of the specific absorbances reported by Miles (1970) for β_2, by Högberg-Raibaud and Goldberg (1977b) for F_1 and F_2, and by Onoué et al. (1965) for antibodies.

Fluorescence Measurements. Fluorescence spectra and titration experiments were performed at 12 and 20 °C, respectively, in a Perkin-Elmer LS-5B double monochromator fluorometer equipped with a thermostated cell holder. All measurements were done in buffer A. The optical density of the samples was kept below 0.1 throughout the wavelength range investigated, so as to minimize inner-filter effects.

Stopped-Flow Measurements. They were performed with a three-syringe, double-mixer SFM-3 stopped-flow module (Bio-Logic, Echirolles, France) in which the step motors driving the syringes are controlled by a microprocessor that can be programmed at will by a PC/XT/AT Tandon microcomputer. A delay line was introduced, when needed, between the two mixers. One "small" (5-mL total volume) and two "large" (18-mL total volume) syringes were used. The stopped-flow module was equipped with a 200-μL fluorescence chamber that was carefully thermostated at 12 °C, together with the reservoirs, syringes, lines, and mixers, by means of a Haake cryothermostat connected to a TE-4-MD-MC high-flux circulation pump (Little Giant Pump Co., Oklahoma City, OK). The excitation wavelength was selected by the monochromator adjusted at 280 nm. The emitted light was observed at a right angle through an Orion 450 high-pass filter ($\lambda_{50\%}$ = 450 nm). The fluorescence intensity was measured with the photomultiplier, high-voltage supply, and amplifier suppled by Bio-Logic, and the signal was collected and analyzed on the Tandon microcomputer by means of the Bio-Logic (Echirolles, France) Bio-Kine interface and software package.

Results

In order to follow the appearance of antigenic determinants during the folding of β chains, we used the same approach as that described earlier (Murry-Brelier & Goldberg, 1988): by observing the fluorescence energy transfer from the tryptophan residues of the antibody to a fluorescent probe introduced on the antigen, one observes directly the formation of the immune complex. By using high concentrations of antibody, one can render the association reaction (a second-order reaction) very rapid. If a folding step is required to render the β chains reactive and if the association step is faster than this folding step, then the rate-limiting reaction for the formation of the immune complex will be the folding step. This approach requires that a fluorescent label be introduced on the antigen. One can use IAEDANS to covalently and specifically modify the SH group of cysteine-170, and several studies showed that the fluorescent label on β_2-AEDANS does not interfere significantly with the folding, the conformation, and the immu-

noreactivity of the polypeptide chain (Blond & Goldberg, 1986; Friguet et al., 1989).

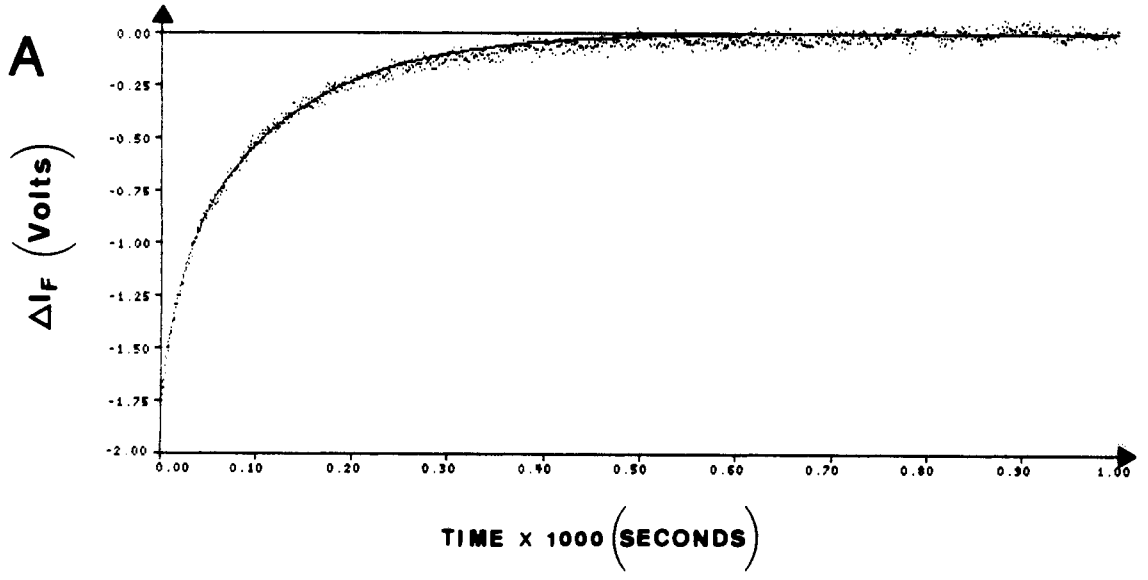
We therefore first investigated our panel of monoclonal antibodies against native β_2 to find out which ones give rise to a usable signal of fluorescence transfer (excitation 280 nm; emission 490 nm) when interacting with native β_2-AEDANS. This was done by recording and comparing, for each antibody, the fluorescence emission spectra of β_2-AEDANS, of the antibody, and of the complex between the antibody and the labeled antigen. Antibodies 9 and 19, which recognize two distinct antigenic sites of the N-terminal F_1 domain of the β chain (see Materials and Methods), gave rise to better fluorescence transfer signals than the other mAbs tested. These two antibodies were therefore chosen to study the regain of immunoreactivity during the refolding of guanidine-unfolded β chains.

Kinetics of Interaction of mAb 19 with Refolding β Chains. The stopped-flow experiments we intended to perform required a double-mixing protocol to achieve large dilution factors and the use of deuterated buffer to avoid the mixing artifacts due to large density differences between concentrated GuHCl solutions and buffers prepared with "normal" (H_2O) water (Blond-Elguindi et al., 1988). It was first checked that this protocol and the use of deuterated water do not bias the rate constant observed for the association of the antigen with the antibody. By use of a double-mixing protocol similar to that designed for the renaturation experiments, native β_2-AEDANS (20 μL at 0.21 mg/mL) was first mixed with deuterated buffer A (230 μL); then, 30 μL of the content of the delay line (total volume 50 μL) was immediately mixed with 180 μL of mAb 19 (binding site concentration varying from 0.5 to 5 μM in deuterated buffer A), and the fluorescence transfer signal was observed. In all these experiments, the appearance of the fluorescence obeyed apparent first-order kinetics (since the mAb was always in at least a 5-fold excess over the antigen). The apparent rate constant was strictly proportional to the antibody site concentration all over the concentration range investigated; the second-order rate constant, which could be obtained from a plot (not shown) of the apparent first-order rate constant versus mAb concentration, was $k_{assoc,19} = 2.8 \times 10^5 \pm 0.3 \times 10^5$ M^{-1} s^{-1} (Blond-Elguindi, 1989). This value is very similar to that obtained previously in normal buffer A and with a conventional mixing protocol (Blond & Goldberg, 1987).

The double-mixing protocol with deuterated buffer could thus be used to initiate the refolding of guanidine-unfolded AEDANS-β chains. When this was done, the fluorescence transfer signal was found to vary according to complex kinetics that clearly exhibited more than a single phase (Figure 1A). When the experimental points of these kinetics were analyzed with the multiexponential data fitting program provided with the Bio-Kine software of Bio-Logic (see Material and Methods), they fitted very well a biphasic curve with two well-separated phases. The rate constants of these two phases were provided directly by the program; their values were plotted as a function of the antibody concentration (Figure 1B). The rate constant of the slow phase did not vary with the mAb concentration; its value was 0.008 ± 0.001 s^{-1}. The rate constant of the rapid phase varies with the mAb concentration in a complex way (Figure 1B). At low (i.e., below about 1 μM) mAb 19 site concentrations, the rate constant increases with the mAb concentration, in a way that we did not try to analyze in detail, but which is compatible with a second-order reaction with a second-order rate constant of at least 1×10^5 M^{-1} s^{-1}. Above 1 μM, the rate constant no longer varies. Its value is 0.065 ± 0.01 s^{-1} (at 12 °C).

Kinetics of Interaction of mAb 9 with Refolding β Chains. Experiments similar to those reported above have been repeated with mAb 9. It was first verified that, upon mixing the native antigen with mAb 9, the appearance of the fluorescence transfer signal obeyed second-order kinetics. The second-order rate constant of the association, at 12 °C, was found to be $k_{assoc,9} = 2.8 \times 10^4$ M^{-1} s^{-1}. It is noteworthy that this value is rather low, compared to that found for mAb 19 at 12 °C (Blond & Goldberg, 1987) or to those found for other mAbs against β_2, which varied from 1.5×10^5 to 15×10^5 M^{-1} s^{-1} at 20 °C (Friguet et al., 1989). Then, guanidine-unfolded AEDANS-labeled β chains were diluted in the presence of mAb 9, with the double-mixing procedure in deuterated buffer A, to initiate their refolding and observe their association with the antibody. Again, the kinetics of appearance of the fluorescence transfer looked biphasic. They indeed could be decomposed in two exponentials, and the variations of the rate constants of the two phases with the mAb concentration are shown in Figure 2. The rate constant of the slow phase does not vary with the mAb concentration. Its value ($k = 0.004 \pm 0.002$ s^{-1}) is significantly smaller than that previously observed for β chains alone or in the presence of mAb 19.

The experimental points for the rate constant of the fast phase exhibit some important scatter. This was caused by the reduced amount of UV light available for exciting the fluorescence at 280 nm in the presence of the rather high concentrations of mAb needed for being in excess over the antigen (the transfer signal was weaker than with mAb 19; thus, the β-AEDANS concentration was higher). Nevertheless, it clearly appears that the rate constant is not proportional to the mAb concentration in the range investigated and that, at least as a first approximation, one can conclude that they do not vary significantly throughout this concentration range. The value of the fast-phase rate constant then would be $k = 0.02 \pm 0.01$ s^{-1}. Thus, in this concentration range, an isomerization seems to be rate limiting in the appearance of the fluorescence transfer signal. For the reason just mentioned, it was not possible to observe the kinetics at mAb concentrations below about 1 μM while remaining in excess over the antigen. We therefore could not perform experiments in which the association reaction would be slower than the isomerization; but the results in Figure 2 set the lower limit of the rate of association to about $k_{assoc,9} = 2 \times 10^4$ M^{-1} s^{-1} (since the pseudo-first-order rate constant of the association, $k_{pseudo} = k_{assoc,9}$ [mAb], is higher than 0.02 s^{-1} at 1 μM mAb 9). This value is of the same order as that reported above for the association of mAb 9 with native β_2. Furthermore, the *relative* amplitude of the fast phase did not vary when the mAb concentration increased from 1.5 to 3.7 μM (Figure 2B), and the slight increase in the amplitude observed between 0.8 and 1.5 μM mAb is hardly significant. Because the slow phase corresponds to the intrachain isomerization that gives rise to an intrinsic fluorescence energy transfer, the *absolute* amplitude of the slow phase was not expected to vary with the mAb concentration. It therefore can be concluded that the *absolute* amplitude of the fast phase also does not vary significantly with mAb 9 concentrations between about 1 and 3.7 μM. This indicates that the antigenic determinant was saturated by the mAb in this concentration range, thus showing that the equilibrium association constant of the transient immunoreactive intermediate with mAb 9 is well above 10^6 M^{-1}. Consequently, the dissociation rate constant must be below 2×10^{-2} s^{-1}, indicating that the dissociation is too slow to play



Fit Parameters

N	Rate Constants (1/sec) Real	Amplitudes (mVolts) Real
2	-0.008	-1212.148
	-0.054	-597.875

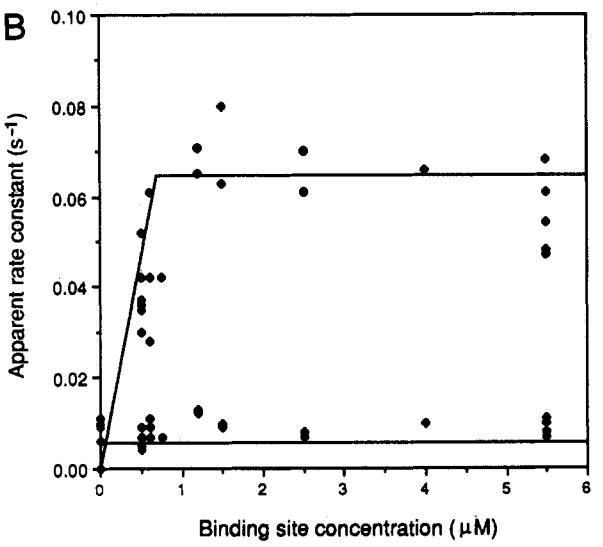


FIGURE 1: Folding/association kinetics of AEDANS-β with mAb19. (A) The syringes of the stopped flow (thermostated at 12 °C) were initially filled as follows: syringe 1 (large 18-mL syringe), deuterated buffer A; syringe 2 (small 5-mL syringe), AEDANS-β (27.6 μM) in buffer A containing 4 M GuHCl; syringe 3 (large 18-mL syringe), mAb 19 (6.5 μM binding sites) in deuterated buffer A. The sequence of injections was as follows: (i) 250 μL from syringe 1 in 50 ms to rinse the lines, mixers, and observation chamber; (ii) same as (i); (iii) 50-ms pause with no injection; (iv) 29 μL from syringe 2 and 232 μL from syringe 1 mixed in 50 ms into and through the 50-μL delay line; (v) 30 μL from syringe 2 (to push, out of the delay line and through the second mixer, the same volume of the previous mixture) and 180 μL from syringe 3 mixed in 50 ms. The reagent concentrations after this sequence of injections were 0.9 μM for AEDANS-β and 5.5 μM for mAb 19. Data recording was triggered at the end of the last injection. The fluorescence energy transfer from tryptophans to AEDANS was monitored by exciting at 280 nm and observing the light emitted at wavelengths higher than 450 nm through a high-pass filter. The figure shows the 1000 experimental points obtained, as a function of time, for the difference ΔI_F (in volts) between the fluorescence at the end of the reaction and the fluorescence at time t. The solid line represents the fit obtained with the multiexponential data fitting program (two exponentials). The print out of the parameters (amplitude and rate constant of each phase) resulting from this fitting is shown under the graph. (B) Experiments similar to that described in (A) were repeated at different antibody concentrations. The rate constants of the fast (open symbols) and slow (closed symbols) phases, obtained by fitting as above, are plotted as a function of the antibody binding site concentration in the observation chamber.

any significant role in the experiments described here. A similar conclusion had been reached previously for mAb 19 during the folding of acid-denatured β chains (Murry-Brelier & Goldberg, 1988).

Having thus characterized kinetically the isomerization involved in the rapid phase of fluorescence transfer (that occurring between the tryptophans of the mAb and the AEDANS of the antigen), it was important to decide whether or not this isomerization precedes (i.e., since it is rate limiting for the transfer signal, is required for) the association of mAb 9 to the antigen. To do that, we followed the same approach as that previously used (Murry-Brelier & Goldberg, 1988)

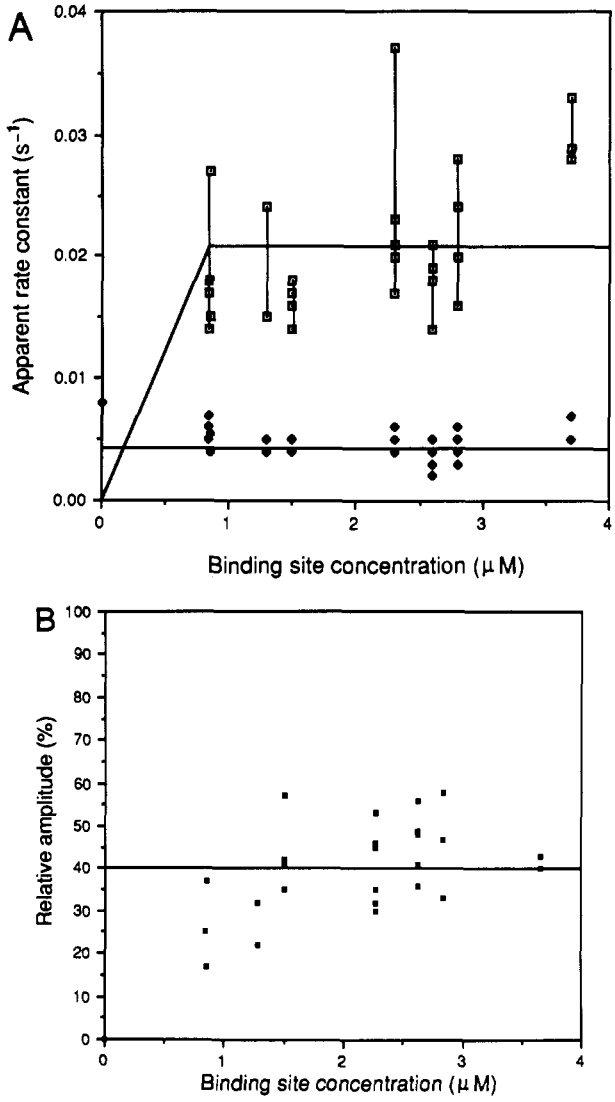


FIGURE 2: Folding/association kinetics of AEDANS-β with mAb 9. Experiments similar to those described in Figure 1 were performed with mAb 9. The final concentration of AEDANS-β chains in the observation chamber was between 0.2 and 0.5 μM; that of mAb 9 varied as indicated in the abscissa and was always in an at least 5-fold excess over that of the antigen. (A) The rate constants obtained by fitting the data to biphasic kinetics (two exponentials) are shown as ordinates. The horizontal solid lines represent the best fit of the data, assuming that each rate constant is independent of the mAb concentration. The third solid line, which passes through the origin, is the best fit of the rate constants observed in parallel experiments for the association of mAb 9 with native β_2-AEDANS (see text). (□) Rate constant of the fast phase; (◆) rate constant of the slow phase. (B) The absolute amplitudes of the fast and slow phases given by the fitting program varied with the concentration of AEDANS-β (direct effect) and with the mAb concentration (inner-filter effect due to the absorbance of the antibody molecules). Rather than trying to correct for these effects, the relative amplitudes of the two phases were calculated as the percent of the total signal change, and the relative amplitude of the fast phase is shown as ordinates as a function of the mAb 9 concentration in the observation chamber.

during studies on the refolding of acid-denatured β chains. This approach relies on the following rationale: guanidine-unfolded AEDANS-β chains are first diluted into renaturation buffer containing mAb 9 to initiate their refolding; they are incubated for a short time in the delay line of the stopped-flow module; the contents of the delay line are now mixed through the second mixer with an excess of unlabeled native β_2. The time of incubation in the delay line is adjusted so that it is short (compared to the isomerization of rate constant 0.02 s^{-1}) but so that the association of the folding chains with the mAb would proceed to nearly completion during that time if the association could occur before the isomerization. If the latter assumption were correct, then the mAb/AEDANS-β chain complex would be formed during the incubation in the delay line and, after the second mixing, the isomerization would take place in the complex, thus giving rise to the fast phase of appearance of the antibody/antigen fluorescence transfer signal. If, on the contrary, the association could occur only after the isomerization, little immunoreactive intermediate, and hence little mAb/AEDANS-β chain complexes, would be formed in the delay line; then, after the second mixing, most mAb molecules would be saturated with the unlabeled antigen in excess, and no antibody/antigen fluorescence transfer would be observed.

Performing this experiment in the two-mixer stopped flow required however that the guanidine be sufficiently diluted even after the first mixing. The double-mixing dilution protocol thus far employed could therefore no longer be used. Hence, we set up a new mixing protocol, using only the first mixer and syringes 1 and 2, which permitted us to obtain a 35-fold dilution with no mixing artifacts (as checked with 3 mM IAEDANS in 4 M GuHCl diluted in deuterated buffer A): this protocol involved a 10-ms phase of acceleration of the two syringes during which 1 μL of the IAEDANS solution from the small syringe and 49 μL of deuterated buffer A from the large syringe were first injected, followed by a second phase, where 5 μL of IAEDANS and 245 μL of deuterated buffer A were mixed in 50 ms. By oberving the fluorescence before and after the mixing, it was found that the signal was perfectly stable and reproducible after the mixing and that the dilution factor thus obtained was 35-fold (Blond-Elguindi, 1989).

Using this dilution protocol, we mixed (through the first mixer and into the 200-μL delay line) 5 μL of guanidine-unfolded β-AEDANS (1.2×10^{-5} M in 4 M GuHCl) with 245 μL of mAb 9 (4.3×10^{-6} M in deuterated buffer A), interrupted the flow for 20 s, and then mixed 125 μL of the contents of the delay line with an equal volume of unlabled native β_2 (8×10^{-6} M in deuterated buffer A). The appearance of the fluorescence transfer signal was then observed. Two control experiments were run in parallel; in one of them (no association at all of AEDANS-β to the antibody), the mAb was omitted from the buffer used for the first dilution. In the second control (no competition with unlabeled β_2 after the second mixing) a solution of bovine serum albumin was used instead of β_2. The concentration of serum albumin was such that its absorbance at 280 nm was the same as that of β_2 in the real experiment (so as to have the same inner-filter effects). When the kinetics were analyzed with the multiexponential fitting program, the results in Table I were obtained. The two controls gave the expected results: a classical single slow phase of intrachain fluorescence transfer in the absence of antibody, and two phases as above (see Figure 2), with the expected rate constants, in the presence of mAb but in the absence of competing native β_2. In the presence of both the mAb and the competing antigen, two phases can still be observed. However, the amplitude of the fast phase is reduced by more than 50%. This indicates that more than half of the refolding β chains had not yet been complexed with the antibody during the 20 s of incubation in the delay line. Moreover, while the rate constant of the slow phase in the absence of competitor is, as expected from the results in Figure 2, indeed reduced by the presence of mAb 9 (to 0.0045 s^{-1}), it is back to its original value in the presence of the competing antigen (k = 0.007 s^{-1}). These two results clearly indicate that the refolding β chains and mAb 9 have not undergone much association during their

Table I: Amplitudes and Rate Constants of the Kinetic Phases in Competition Experiments[a]

first mixture	protein added on second mixing	fast phase		slow phase	
		amplitude (mV)	rate constant (s^{-1})	amplitude (mV)	rate constant (s^{-1})
AEDANS-β + buffer	serum albumin	0		1200 ± 100	0.007
AEDANS-β + mAb 9	serum albumin	1550 ± 250	0.017	1100 ± 100	0.0045
AEDANS-β + mAb 9	native β_2	670 ± 100	0.032	1270 ± 100	0.007

[a] The proteins indicated in the first column were injected through the first mixer into the delay line of the stopped flow, and the flow was interrupted for 20 s; the contents of the delay line was then mixed (through the second mixer) with the protein indicated in the second column (for experimental details, see text), and the change in the fluorescence transfer (excitation at 280 nm; emission above 450 nm) was recorded as a function of time. The amplitudes and rates of the kinetic phases were obtained by fitting the data to double exponentials. The values indicated in the table are the average and error limit of at least three experiments.

20-s incubation after the initial mixing. Thus, isomerization with a rate constant of 0.02 s^{-1} is required for the association to take place. It corresponds to the rate-limiting folding step leading to the appearance of the native-like epitope to mAb 9.

Kinetics of Interaction of mAbs with Refolding Isolated F_1 and F_2 Domains. Experiments similar to those reported above have been performed to follow the kinetics of regain of the epitopes to mAbs 9 and 19 during the refolding of guanidine-unfolded F_1-AEDANS fragments. In both cases, biphasic kinetics were obtained. The rate constants for F_1 and mAbs 9 and 19 are shown, as a function of the antibody concentration, in Figure 3. Results essentially similar to those obtained with intact β chains were found: at high enough antibody concentrations, an isomerization appears to be rate limiting for the fast phase of fluorescence transfer signal, which reflects the antigen/antibody association. The corresponding rate constants are 0.085 ± 0.02 and 0.04 ± 0.01 s^{-1}, respectively, for mAb 19 and mAb 9. The rate constant of the slow phase corresponding to the intrachain fluorescence transfer does not depend on the mAb concentration; surprisingly, it is found to be the same with mAb 9 as with mAb 19 or without antibody, in contrast to what was observed (see above) for entire β chains in the presence of mAb 9.

To study the association of mAbs 93, 164, and D4B6 with refolding F_2 fragments, F_2 was labeled with IAEDANS on cytsteine-340. This chemical modification does not interfere significantly with the interaction between the antigen and the antibodies, and the modified fragment gives rise to a fluorescence transfer signal from the tryptophans of the antibody to the AEDANS moiety of the antigen upon formation of the immune complex (Friguet et al., 1985, 1989). Guanidine-unfolded F_2-AEDANS was then diluted in the presence of these mAbs, and the appearance of the fluorescence transfer signal was recorded as above. With the three mAbs, monophasic, pseudo-first-order kinetics were obtained. The apparent first-order rate constants observed for these kinetics were proportional to the mAb concentration, throughout the concentration range investigated for each mAb (Figure 4), and the proportionality constants gave the following values of the association rate constants: $8.9 \times 10^4 \pm 0.3 \times 10^4$ M^{-1} s^{-1} for mAb D4B6; $17.1 \times 10^4 \pm 0.6 \times 10^4$ M^{-1} s^{-1} for mAb 93; $6.9 \times 10^4 \pm 0.5 \times 10^4$ M^{-1} s^{-1} for mAb 164. We could not, for technical reasons pertaining to the amounts of mAbs available and to the strong inner-filter effect caused by their high absorbance at 280 nm, increase further the mAb concentrations in the stopped-flow experiments (Figure 4).

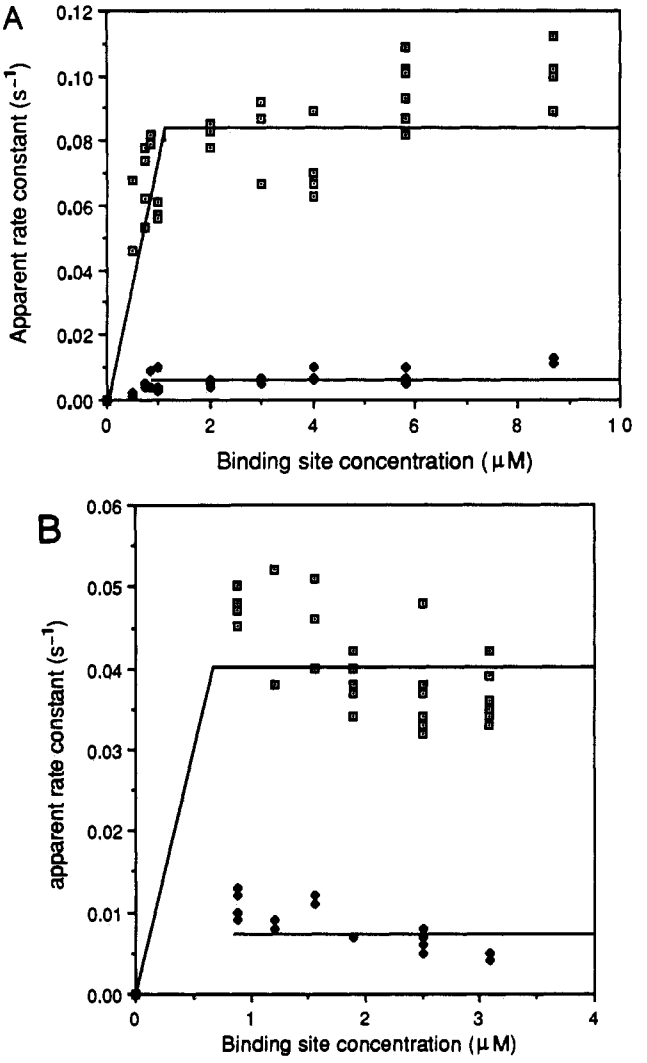


FIGURE 3: Folding/association kinetics of isolated F_1-AEDANS with mAbs 9 and 19. The experiments and the analysis of the data were performed exactly as in Figures 1 and 2. The solid lines were drawn as in Figure 2.

Discussion

In the experiments described above, the kinetics of folding of guanidine-unfolded β_2 subunits from *E. coli* tryptophan synthase have been investigated, using as local probes of native-like conformation the regain of immunoreactivity toward anti-native-β_2 monoclonal antibodies. Stopped-flow studies were conducted with two monoclonal antibodies (mAbs 9 and 19), both recognizing epitopes carried by the F_1 N-terminal domain of the β chain and both giving rise to usable fluorescence signals upon association to β_2-AEDANS. With both antibodies, biphasic kinetics were observed, and the rate constant of each phase for each antibody was determined (Table II). Hand-mixing experiments reported previously (Blond & Goldberg, 1987) had shown that with mAb 19 the slow phase corresponds to an isomerization which the β chain undergoes spontaneously, either alone or already engaged in a complex with the antibody. And it was observed that the corresponding change in the fluorescence signal involves an intrachain fluorescence transfer from the single tryptophan residue of β (tryptophan-177) toward the AEDANS on cys-

Table II: Summary of the Observed Rate Constants during the Folding of β_2 and F_1, Alone or with mAbs 9 and 19

	proteins observed					
	β chains	isolated F_1	β + mAb 19	F_1 + mAb 19	β + mAb 9	F_1 + mAb 9
rate constant of fast phase (s^{-1})	nfp[a]	nfp[a]	0.065	0.085	0.02	0.04
relative amplitude of fast phase (% of total)	nfp[a]	nfp[a]	40 ± 10	70 ± 10	40 ± 10	50 ± 10
rate constant of slow phase (s^{-1})	0.008	nsp[b]	0.0086	0.0074	0.0046	0.0074

[a] nfp, no fast phase is observed in the absence of mAbs. [b] nsp, no slow phase is observed when the isolated F_1 fragment refolds in the absence of a mAb (Blond & Goldberg, 1986).

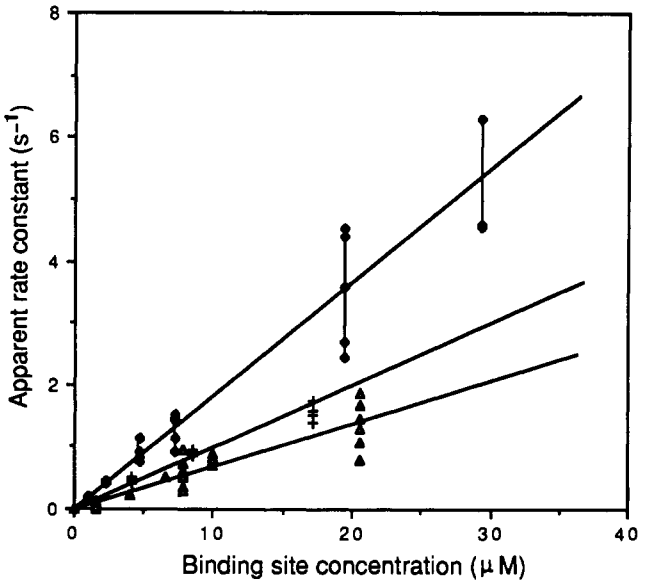


FIGURE 4: Association kinetics of refolding F_2-AEDANS with mAbs 93, 164, and D4B6. Double-mixing stopped-flow experiments similar to those described in Figure 1 were performed with F_2-AEDANS (unfolded in buffer A + 4 M GuHCl) in syringe 2 and varying concentrations of the anti-F_2 monoclonal antibody (in deuterated buffer A) in syringe 3. For each experiment, the data were analyzed with the multiexponential fitting program; the best fit was always obtained with a single exponential, the rate constant of which is shown as a function of the antibody site concentration after the mixings. The antibody site concentration after the mixing was always in an at least 5-fold excess over the antigen concentration. (◆) mAb 93 (the F_2-AEDANS concentration after the mixing ranged between 0.5 and 2.7 μM); (△) mAb 164 (the F_2-AEDANS concentration after the mixing ranged between 0.5 and 1 μM); (+) mAb D4B6 (the F_2-AEDANS concentration after the mixing ranged between 0.75 and 1.8 μM).

teine-170 (Blond & Goldberg, 1986). The fast phase was shown, on the contrary, to require the association with the mAb (Blond & Goldberg, 1987) and thus to result from a fluorescence transfer from the tryptophan residues of the antibody toward the AEDANS linked to the antigen. Also, during the refolding of acid-denatured β chains, the fast phase of fluorescence transfer change (i.e., the association with mAb 19) reflects a folding step resulting in the appearance of the corresponding epitope (Murry-Brelier & Goldberg, 1988). Here, we also obtained biphasic kinetics for the refolding of guanidine-unfolded β chains. And the rate constant we determined here with mAb 19 for the fast phase (0.065 ± 0.01 s^{-1}) is, within experimental error, the same as that observed during the refolding of acid-denatured β chains (0.054 ± 0.01 s^{-1}; Murry-Brelier & Goldberg, 1988). This strongly suggests that, for guanidine-unfolded β chains, the appearance of the fluorescence signal characteristic of the antigen/antibody complex is controlled by a folding step that is probably the same (indistinguishable rate constants) as that required for the regain of immunoreactivity during the renaturation of acid-denatured β chains. Moreover since, in the presence of guanidine at the concentrations used here for denaturation, the β chains are completely unfolded (Zetina & Goldberg, 1980), this step does not reflect the disruption of a "wrong" conformation that might have been present in the residual structure that exists (Murry-Brelier & Goldberg, 1988) in the acid-denatured state of β chains. Rather, it appears to correspond to a common folding step (k = 0.06 s^{-1} at 12 °C) that, in spite of the differences between the structures and folding pathways of acid-denatured and guanidine-unfolded β chains (Murry-Brelier & Goldberg, 1989), would lead on the two folding pathways to a common immunoreactive, native-like conformation of the polypeptide chain in the vicinity of the epitope to mAb 19.

With mAb 9, it has been shown directly here, by a double-mixing delayed-competition experiment, that the fast phase of appearance of the fluorescence transfer signal does correspond to a folding step that is required for the regain of immunoreactivity. Furthermore, the rate constant of this folding step has been determined. Its value (k = 0.02 ± 0.01 s^{-1} at 12 °C) is about 3-fold smaller than that found for mAb 19 under rigorously identical conditions. This demonstrates that the epitopes to mAbs 9 and 19, though both belonging to the same N-terminal F_1 domain, do not reappear simultaneously during the folding of β chains: the mAb 19 reactive epitope refolds faster than the one specific for mAb9. Moreover, if the epitope to mAb 9 had to appear, through an obligatory sequence, only after the epitope to mAb 19, then the kinetics would exhibit a lag, because the rate constants of the two steps are of the same order of magnitude. Thus, since the appearance of the epitope to mAb 9 obeys first-order kinetics with no lag, it is clear that the two epitopes fold independently.

The relatively high values determined for the rate constant of appearance of the two epitopes to mAbs 9 and 19 show that they are formed "early" on the folding pathway of β_2, at least as compared to all the other folding steps characterized before the use of immunochemistry for studying the folding of β_2 (Blond & Goldberg, 1986). However, it should be noted that these rate constants are still much smaller than those describing the folding of small, one-domain proteins like ribonuclease A or lysozyme [for review, see Baldwin (1975) and Jaenicke (1987)].

The rate constants of association of mAb 19 with the refolding β chains and with native β_2 are practically the same (Blond & Goldberg, 1987); similarly, while we could not determine the rate constant of association of mAb 9 with refolding β chains, we have shown above that it is at least 2 × 10^4 M^{-1} s^{-1}, a value quite close to that found for the association of mAb 9 with native β_2 (2.8 × 10^4 M^{-1} s^{-1}). Also, the affinities of the early immunoreactive intermediates have been shown to be higher than 10^6 M^{-1} for mAb 9 (see above) and 10^7 M^{-1} for mAb 19 (Murry-Brelier & Goldberg, 1988). This shows that the two epitopes that are formed early during the folding process are recognized efficiently by the corresponding antibodies. However, it has been shown elsewhere that different forms of a given antigen can bind to a mAb with similar association rate constants, but with very different affinities that reflect conformational differences between them

(Friguet et al., 1989). The two early epitopes therefore already appear as "native-like" in terms of kinetics, but probably are not native. And indeed, the affinity of the F_1 domain for mAbs 9 and 19 has previously been shown to increase with later steps of the folding process (Friguet et al., 1986).

Native-like epitopes are also recognized by these two mAbs during the folding of the guanidine-unfolded, isolated F_1 fragment. This was not unexpected since isolated F_1 is able to fold into a native-like structure even in the absence of F_2. The step leading to the first observable intermediates in the isolated F_1 fragment had been shown previously to be about 6-fold (at 4 °C) faster than the corresponding step in entire β chains (Blond & Goldberg, 1986). It is noteworthy that the rate constants of appearance of the epitopes to mAbs 9 and 19 are also slightly higher with isolated F_1 fragments than with entire β chains (see Table II). This confirms that the folding of the F_1 domain, while *autonomous* (Högberg-Raibaud & Goldberg, 1977b), is *not independent* since it is slowed down by the presence of the rest of the polypeptide chain.

Native-like epitopes are also present on isolated F_2 fragments very shortly after the initiation of the folding of guanidine-unfolded F_2. Indeed, for the three mAbs against epitopes carried by F_2 (one of them is located in the hinge between the two domains), we could not observe an isomerization that would be rate limiting for the association in the range of mAb concentrations we used (Figure 4). The highest pseudo-first-order rate constants of association we were able to reach in this concentration range were between 2 and 6 s^{-1}, depending on the mAb. Thus, we can conclude that either unfolded F_2 can be recognized by these three antibodies [which seems unlikely, particularly for mAb 93, which has been shown by Friguet et al. (1986) to be conformation dependent] or F_2 regains its native-like immunoreactivity with a rate constant higher than 2–6 s^{-1}. If the latter conclusion were correct, it would mean that the isolated F_2 fragment folds into this immunoreactive conformation in a time comparable to that of the folding of typical small, one-domain proteins. It would be highly interesting to know whether or not this is also true for the F_2 domain integrated in the complete β chain.

The last observation to be discussed here deals with the slow phase of appearance of the fluorescence transfer signal. As already mentioned above, this phase is associated with an isomerization of the β chain which brings the indole ring of tryptophan-177 and the AEDANS linked to cysteine-170 in the native-like relative position that permits the fluorescence transfer from the tryptophan to the AEDANS fluorochromes. This isomerization occurs during the folding of β chains with a rate constant at 12 °C of 0.008 s^{-1}. It occurs, during the folding of β chains or F_1 fragments in the presence of mAb 19, within the already formed antigen/mAb complex, with the same rate constant of 0.008 ± 0.001 s^{-1} (Table II; Blond & Goldberg, 1987; Murry-Brelier & Goldberg, 1988). The same seems to hold true for the folding in the presence of mAb 9. But the rate constant of the isomerization is significantly smaller (i.e., only 0.004 ± 0.002 s^{-1}). Thus mAb 9, once bound to the early immunoreactive intermediates, slows down the isomerization characterized by the slow phase in the appearance of the fluorescence transfer signal and perhaps also some later folding steps. This hindrance is however not observed with the isolated F_1 fragment, which undergoes this isomerization with a rate constant of 0.008 s^{-1} in the presence of mAb 9.

The use of monoclonal antibodies in studies on the protein folding process has provided several valuable pieces of information. It showed that several local structural patterns, native-like enough to be immunoreactive to mAbs specific for the native protein, appear early in the folding process, and independently of one another even inside the same domain. It confirmed that, even for the earliest intermediates thus far observed (those which are recognized by mAb 19), the same steps, with the same kinetics, are involved in the folding of β chains, when they refold from either the entirely unfolded state in guanidine or from the exensively denatured but not totally unfolded state at acidic pH. Finally, it strongly suggests that the isolated F_2 fragment, the renaturation of which had not been followed thus far for lack of a convenient signal, folds quite rapidly as expected for a one-domain polypeptide chain of that size.

In addition to their implications in the understanding of the folding pathway of tryptophan synthase, these results illustrate the power of monoclonal antibodies as tools for investigating the regain of native-like patterns on the surface of a protein during its renaturation. They however also illustrate the drawbacks of the immunochemical approach, which are that native-like does not mean native, that folding steps with half-lives shorter than 100–200 ms are difficult to monitor (very high mAb concentrations needed), and that in some cases the mAb may hinder or affect some of the folding steps which occur after its binding to the antigen. Nevertheless, because of their specificity and because of the sensitivity of the immunochemical assays, mAbs are likely to become a popular tool for detecting and characterizing folding intermediates in experiments involving impure and dilute protein solutions. Thus, attempts to use mAbs for studying the coupling between chain elongation and protein folding have just been started in our laboratory.

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Functional Sites of Glia-Derived Nexin (GDN): Importance of the Site Reacting with the Protease

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ABSTRACT: Glia-derived nexin (GDN) is a 43-kDa serine protease inhibitor with neurite promoting activity in mouse neuroblastoma cells (Guenther et al., 1985). In chick sympathetic neurons, GDN but not hirudin and synthetic peptide inhibitors promoted neurite outgrowth (Zurn et al., 1988). Thus, it was considered that the protease inhibitory activity cannot account for the total biological activity of GDN. We show here that synthetic peptide inhibitors with thrombin specificity mimic GDN at similar concentrations in neuroblastoma cells. Limited proteolysis of GDN with elastase causes a cleavage between sites P_1 and P_2, corresponding to residues Ala-344-Arg-345 of the molecule. The resulting fragments still copurify on heparin-Sepharose, but the protease inhibitor activity of GDN and the GDN neurite promoting activity are lost. The results confirm the necessity of an intact reactive site for the biological activity of GDN.

The function of glial cells is poorly understood although they are believed to play a key role in the differentiation of neurons. Cultured glial cells release a protein which promotes the extension of neurites in both mouse neuroblastoma cells (Monard et al., 1973) and primary cultures of chick sympathetic neurons (Zurn et al., 1988); furthermore, this glia-derived nexin (GDN)[1] affects the migration of granule cells (Lindner et al., 1986). In vivo, GDN is mainly detected in the olfactory system known for its constant degeneration and regeneration of neuronal cells (Reinhard et al., 1988). It is possible that this protein could act as an important mediator between glial cells and neurons.

The glia-derived neurite promoting factor is a 43-kDa protein consisting of a single polypeptide chain (Guenther et al., 1985). Kinetic studies have demonstrated that it is a potent inhibitor of thrombin, trypsin, and, to a lesser extent, urokinase (Stone et al., 1987). Complexes between the inhibitor and these proteases are resistant to SDS treatment and, therefore, can be visualized by SDS-PAGE. Heparin increases the rate at which GDN reacts with thrombin by over 40-fold to 8.9 $\times 10^{-7}$ M^{-1} s^{-1} and decreases the dissociation constant of the complex by over 80-fold to 0.3 pM. Sequence analysis of the rat and human cDNA clones coding for these inhibitors reveals that they belong to the serpin family (Gloor et al., 1986; Sommer et al., 1987). Results published recently show that the primary structure of protease nexin I, a serine protease inhibitor released by human fibroblasts, is identical with human GDN (McGrogan et al., 1988).

GDN contains at least two functional sites: a reactive center which interacts with the protease and a heparin binding site. Data presented here show that, through proteolytic modification close to the reactive center of the inhibitor, its contribution to the overall properties of the native molecule can be elucidated.

Hirudin, an inhibitor of thrombin, has been shown to be as potent as GDN in promoting neurite extension in neuroblastoma cells (Monard et al., 1983). To determine the relevance of protease inhibition for neurite extension, we have tested the effect of other naturally occurring protease inhibitors as well as that of synthetic Arg chloromethyl ketones on their ability to promote neurite extension in neuroblastoma cells. In addition, we show that the integrity of the protease inhibitory site of GDN has to be maintained for neurite promoting activity.

Materials and Methods

All reagents were analytical grade if not otherwise stated. DMEM and MEM powder media were from Gibco, and fetal calf serum was from North American Biological Incorp. Spectra/Por 2 molecular weight cutoff 12 000-14 000 dialysis membranes were from Spectrum Medical Industries Inc. The thrombin chromogenic substrate S-2888 was from Kabi, Sweden. α_2-Macroglobulin and secretory leukocyte proteinase inhibitor were a gift from Dr. Schnebli, Ciba-Geigy, Basel. Antithrombin III, aprotinin, leupeptin, ovomucoid, soybean trypsin inhibitor, and α_1-proteinase inhibitor were from Sigma. The chloromethyl ketones were synthesized as described (Kettner & Shaw, 1981; Walker et al., 1985).

Purification of GDN was carried out as described earlier (Guenther et al., 1985).

Elastase Digestion of GDN. GDN (2 mg in 1 mL) was incubated for up to 160 min at 37 °C in 50 mM Tris-HCl, pH 8 (Tris buffer), containing 400 mM NaCl with porcine elastase (Merck) at a protein to enzyme ratio of 50:1.

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[1] Abbreviations: GDN, glia-derived nexin; DMEM, Dulbecco's modified Eagle's medium; MEM, minimum essential medium Eagle, Tris, tris(hydroxymethyl)aminomethane; EGDN, elastase-cleaved glia-derived nexin; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin; SLPI, secretory leukocyte protease inhibitor.

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