Poole, T., Leach, B. S., & Fish, W. W. (1974) *Anal. Biochem. 60*, 596–607.
Richardson, H. S. (1905) *A Monograph on the Isopods of North America*, pp 682–684, U.S. Government Printing Office, Washington, D.C.
Robinson, H. A., & Ellerton, H. D. (1977) in *Structure and Function of Haemocyanin* (Bannister, J. V., Ed.) pp 55–70, Springer-Verlag, West Berlin.
Schneider, H. J., Markl, J., Schartau, W., & Linzen, B. (1977) *Hoppe-Seyler's Z. Physiol. Chem. 358*, 1133–1141.
Schutter, W. G., Van Bruggen, E. F. J., Bonaventura, J., Bonaventura, C., & Sullivan, B. (1977) in *Structure and Function of Haemocyanin* (Bannister, J. V., Ed.) pp 13–21, Springer-Verlag, West Berlin.
Sevilla, C. (1978) *Comp. Biochem. Physiol. B 60B*, 467–471.
Spackman, H. D., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190–1206.
Sullivan, B., Bonaventura, J., & Bonaventura, C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2558–2562.
Terwilliger, N. B., & Terwilliger, R. C. (1982) *J. Exp. Zool.* (in press).
Terwilliger, N. B., Terwilliger, R. C., Applestein, M., Bonaventura, C., & Bonaventura, J. (1979) *Biochemistry 18*, 102–108.
Terwilliger, R. C., Garlick, R. L., Terwilliger, N. B., & Blair, D. P. (1975) *Biochim. Biophys. Acta 453*, 101–110.
Van Bruggen, E. F. J., Bijlholt, M. M. C., Schutter, W. G., Wichertjes, T., Bonaventura, J., Bonaventura, C., Lamy, J., Lamy, J., LeClerc, M., Schneider, H. J., Markl, J., & Linzen, B. (1980) *FEBS Lett. 116*, 207–210.
Van Holde, K. E., & Van Bruggen, E. F. J. (1971) in *Subunits in Biological Systems* (Timasheff, S. N., & Fasman, G. D., Eds.) pp 1–53, Marcel Dekker, New York.
Van Holde, K. E., & Brenowitz, M. (1981) *Biochemistry 20*, 5232–5239.
Wood, E. J. (1980) *Essays Biochem. 16*, 1–47.

Guanidine Hydrochloride Induced Unfolding of the α Subunit of Tryptophan Synthase and of the Two α Proteolytic Fragments: Evidence for Stepwise Unfolding of the Two α Domains†

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ABSTRACT: The relationship between the domain structure of the α subunit of *Escherichia coli* tryptophan synthase and the mechanism of unfolding of the α subunit is investigated. Previous studies of the unfolding of the α subunit by increasing concentrations of guanidine hydrochloride or urea detected a partially unfolded form of the α subunit at intermediate concentrations of either denaturant. The possibility that this partially unfolded form of the α subunit results from the preferential unfolding of one of the two domains of the α subunit is now investigated. This study utilizes two proteolytic fragments of the α subunit, α-1 and α-2, which have been shown to refold independently and to correspond to two domains of the α subunit. The effects of guanidine hydrochloride concentration on the separate α-1 and α-2 fragments, on the intact α subunit, and on the derivative nicked by trypsin (α') are compared by measuring ellipticity at 222 nm and by measuring the susceptibility of tyrosyl residues to chemical modification. The results show that guanidine hydrochloride induced unfolding of the α subunit results from the stepwise unfolding of the two domains: the α-2 fragment and the corresponding domain in the intact α subunit are unfolded by low concentrations of guanidine hydrochloride; the α-1 fragment and the corresponding domain in the intact α subunit are unfolded by higher concentrations of guanidine hydrochloride.

Although the mechanism of unfolding and refolding of proteins has been extensively studied and reviewed [see Anfinsen (1973), Baldwin (1975, 1978), Creighton (1978), and Wetlaufer (1981)], it is still incompletely understood. The classic approach to determining the pathway of protein unfolding has been to isolate intermediates, to characterize them, and to place them in correct order on the pathway (Baldwin, 1978). This unfolding pathway can be represented as a multistate process:

$$N \rightleftarrows I \rightleftarrows U$$

where N is the native state, U is the unfolded state, and I is one or more unfolding intermediates with a conformation different from N or U. However, in most equilibrium studies of globular proteins, the process is so highly cooperative that intermediates are not detected, and the pathway can be represented by a two-state process:

$$N \rightleftarrows U$$

Since a number of large, single-chain globular proteins appear to contain two or more largely independent regions of structure termed domains, it has been independently proposed by two groups that these domains represent the products of independent folding processes, which begin with independent nucleation steps (Goldberg, 1969; Wetlaufer, 1973). Since several of these multidomain proteins have been shown to unfold by multistate processes, these proteins have been useful for characterizing unfolding intermediates (see Results and Discussion).

One important approach to the study of protein folding has been to isolate protein fragments prepared by limited pro-

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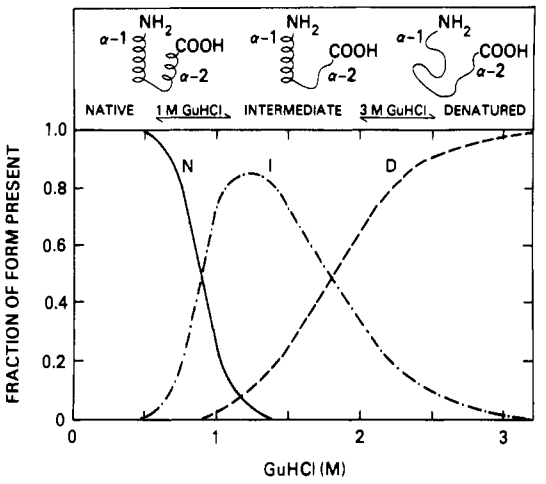

FIGURE 1: Effect of Gdn·HCl concentration on the unfolding of the intact α subunit of tryptophan synthase. (Bottom) The fractions of native (N), intermediate (I), and denatured (D) states at different concentrations of Gdn·HCl were calculated from the circular dichroism data for α in Figure 2A [see Yutani et al. (1979)] at pH 7.0 and 25.8 °C. (Top) The schematic diagram of the denaturation process of the intact tryptophan synthase α subunit is derived from a proposal of Yutani et al. (1980); α-1 and α-2 represent the domains corresponding to the α-1 and α-2 fragments of the tryptophan synthase α subunit prepared by Higgins et al. (1979). The α-2 domain of the intact, native α subunit (N) is shown to unfold at 1 M Gdn·HCl to yield a partially unfolded α intermediate (I); the α-1 domain is shown to unfold at 3 M Gdn·HCl to yield the fully denatured form (D).

teolysis or chemical cleavage and to study the refolding of these fragments. Refolding to a nativelike structure has been demonstrated with fragments of β-galactosidase, lysozyme, serum albumin, penicillinase, and the β_2 subunit of tryptophan synthase [for a review, see Wetlaufer (1981)]. Limited proteolysis of the β_2 subunit of tryptophan synthase (Högberg-Raibaud & Goldberg, 1977a,b) led to the isolation of two unfolded proteolytic fragments, F-1 and F-2, and to the demonstration that these fragments can refold independently. The isolation of these peptide fragments corresponding to independently folding domains in the intact β chain allowed Zetina & Goldberg (1980) to compare the guanidine hydrochloride (Gdn·HCl)[1] induced unfolding of the intact β chain with that of the separately refolded proteolytic fragments F-1 and F-2 (see Results and Discussion).

In this work[2] we use a similar approach to investigate the relationship between the domain structure of the α subunit of *Escherichia coli* tryptophan synthase and the mechanism of the unfolding of this protein. Proteolytic fragments of the α subunit, α-1 and α-2, have previously been isolated in the unfolded state and shown to refold independently (Miles & Higgins, 1978; Higgins et al., 1979); the independently folded fragments are devoid of activity but reassociate upon mixing to yield active nicked enzyme, termed α′. Previous studies by Yutani et al. (1979) of Gdn·HCl-induced unfolding of the intact α subunit gave evidence for a multistate unfolding process in which a partially unfolded α intermediate (I) could be detected maximally at about 1 M Gdn·HCl (see Figure 1). The fractions of the native (N), intermediate (I), and denatured (D) forms in Figure 1 were calculated from both circular dichroism data (see the curve for the α subunit in Figure 2A) and from fluorescence data. Subsequent studies by Matthews & Crisanti (1981) and by Crisanti & Matthews (1981) on the urea-induced unfolding of the α subunit have also provided evidence for a multistate process. The demonstration that the near- and far-UV circular dichroism spectra of the α subunit in 1 M Gdn·HCl were similar to corresponding spectra reported by Higgins et al. (1979) for the folded α-1 fragment in the absence of Gdn·HCl led Yutani et al. (1980) to propose that in the partially unfolded α intermediate, one domain, which corresponds to the α-1 fragment, remains folded but that the other domain, which corresponds to the α-2 fragment, is unfolded (Figure 1, top).

In this work we have tested this proposal by comparing the effects of Gdn·HCl concentration on the unfolding properties of the intact α subunit, on the nicked α derivative (α′), and on the separately refolded α-1 and α-2 fragments. The extent of unfolding has been determined by measuring ellipticity at 222 nm and by measuring the number of tyrosyl residues susceptible to chemical modification. The results are consistent with the model for unfolding shown in Figure 1 (top).

Materials and Methods

Materials. Acetylimidazole was from Sigma. Gdn·HCl (ultrapure reagent) was from Bethesda Research Laboratories or from Nakarai Chemicals (Kyoto, Japan). Urea (ultrapure reagent) was from Bethesda Research Laboratories.

Enzymes. The $\alpha_2\beta_2$ complex and the α subunit of tryptophan synthase (EC 4.2.1.20) from *E. coli* (strain W3110 *trpR⁻ cysB⁻ Δtrp* LD102 *trpB⁺ trpA⁺*/F′ *colVB cysB⁺ Δtrp* LD102 *trpB⁺ trpA⁺*), as well as the nicked α subunit (termed α′) and the α-1 and α-2 fragments, were prepared and assayed as described by Higgins et al. (1979). In some cases the α-1 fragment was further purified after removal of urea by gel elution on a 0.9 × 54 cm column of Sephadex G-100SF in 0.05 M *N,N*-bis(2-hydroxyethyl)glycine buffer, pH 7.8, containing 1.0 mM EDTA, 1.0 mM dithiothreitol, and 0.1 mM pyridoxal phosphate; about 0.2 molar equiv of holo β_2 subunit was added before gel elution of the α-1 fragment in order to form a complex with any contaminating α subunit or α′.

Circular Dichroism Measurements. Circular dichroism was measured with a Jasco J-500 recording spectropolarimeter equipped with a data processor for circular dichroism (Model DP-501). The circular dichroism at 222 nm was measured on protein solutions 0.01–0.033 mg/mL in a cell having a 10-mm light path. Measurements were made after reaching a constant value, on incubation at 25 °C for 30 min in solutions (pH 7.0) containing 0.01 M potassium phosphate buffer, 0.1 mM EDTA, 0.1 mM dithioerythritol, and the indicated concentrations of Gdn·HCl; no further changes in ellipticity values were observed after incubation at various concentrations of Gdn·HCl for 20–24 h.

Chemical Modification of Tyrosyl Residues with Acetylimidazole. Protein solutions (0.03 mM in 0.1–0.2 mL of 0.1 M potassium phosphate, pH 7.5, containing the indicated concentration of Gdn·HCl or urea) were treated with 20 mM acetylimidazole (or other concentration where indicated) for 1 h at 24 °C, followed by treatment with 0.01 M β-mercaptoethanol. For assays of activity of the α-1 fragment, α-1 and excess α-2 fragments were preincubated in the assay mixture at 37 °C for 5 min before addition of excess β_2 subunit. Solutions were dialyzed for 16 h in a microdialysis system (Bethesda Research Laboratories) by continuously pumping 0.1 M potassium phosphate, pH 7.5, containing 3 M Gdn·HCl through the system at 100 mL/h; the total volume of the buffer being recycled was 1 L. Any protein that precipitated was removed by centrifugation. Absorption spectra were measured, with a microcell and a microcell attachment in a Cary 118

[1] Abbreviations: Gdn·HCl, guanidine hydrochloride (GuHCl in figures); EDTA, ethylenediaminetetraacetic acid.

[2] A preliminary report of the chemical modification results was presented at a meeting of the American Society of Biological Chemists, May 31–June 4, 1981 (Miles, 1981).

spectrophotometer, before and 1 h after treatment with 0.1 M hydroxylamine at 24 °C; hydroxylamine removes the acetyl group from the tyrosine hydroxyl. The extent of tyrosine modification was determined from the increase in absorbance at 276 nm after treatment with hydroxylamine by using a molar difference absorbance of 1160 at 276 nm (Riordan & Vallee, 1972). Protein concentrations were determined from the absorbance at 276 nm after hydroxylamine treatment by using extinction coefficients reported by Higgins et al. (1979). The absolute values for the number of tyrosyl residues determined in duplicate experiments (see, for example, Figure 4C) showed a variation of up to one residue, probably due to experimental errors in measuring spectra on small volumes and making base-line corrections; relative values within a single experiment showed less variation.

Results and Discussion

Circular Dichroism Studies. Circular dichroism spectra of the α subunit, of the nicked α subunit (α'), and of the α-1 and α-2 fragments (not shown) at pH 7.0 and 25 °C in the near- and far-ultraviolet were similar to those reported by Higgins et al. (1979). The negative circular dichroism values at 222 nm of α' and of both α-1 and α-2 fragments decreased upon denaturation by Gdn·HCl; the sum of the far-ultraviolet circular dichroism spectra of the α-1 and α-2 fragments in 3.2 M Gdn·HCl and the corresponding spectrum of α' in 3.2 M Gdn·HCl were similar to the spectrum reported by Yutani et al. (1980) for the completely unfolded α subunit. Thus the denaturation or conformational change of the α subunit, α', and the α-1 and α-2 fragments caused by various concentrations of Gdn·HCl was followed by measurement of molar ellipticity at 222 nm (Figure 2A).

The Gdn·HCl denaturation curve for the α subunit is biphasic, suggesting the presence of one stable intermediate state in a denaturation process that goes through two unfolding steps or transitions (Yutani et al., 1979). The midpoints of these two unfolding transitions can be seen to be at 0.9 and at 1.8 M Gdn·HCl from a plot of the calculated concentrations of the native, intermediate, and denatured states at different Gdn·HCl concentrations (Figure 1). The curve for α', which is also biphasic, is essentially identical with the curve for the α subunit at 0 M Gdn·HCl and at concentrations of Gdn·HCl above 1.4 M but is shifted to lower concentrations of Gdn·HCl below 1.4 M. Thus, the second unfolding transition is the same for the α subunit and α' (midpoint at 1.8 M Gdn·HCl), but the first unfolding transition has a lower midpoint for α' (about 0.5 M Gdn·HCl) than for the α subunit (0.9 M Gdn·HCl).

The Gdn·HCl denaturation curves for the α-1 and α-2 fragments (Figure 2A) appear to be monophasic. The ellipticity at 222 nm of the α-1 fragment shows only a small change between 0 and 1.4 M Gdn·HCl; above 1.4 M Gdn·HCl the changes in the ellipticity of the α-1 fragment are similar to those observed in the intact α fragment above 1.4 M Gdn·HCl. Since the second unfolding transition observed with the intact α subunit between 1.4 and 3 M Gdn·HCl is both qualitatively and quantitatively similar to the unfolding transition observed with the α-1 fragment in this range of denaturant concentration, this second unfolding transition of the intact α subunit is probably the unfolding of the domain corresponding to the α-1 fragment. Thus the α-1 fragment appears to be a good model for studying the unfolding of the corresponding domain in the intact α subunit.

As previously shown by Higgins et al. (1979), the added sum of the molar ellipticities at 222 nm of the α-1 and α-2 fragments in the absence of Gdn·HCl is considerably lower than the molar ellipticity at 222 nm of the α subunit or α' (see Figure 2A at 0 M Gdn·HCl). Higgins et al. (1979) suggested that this might be due to the presence of some inactive α' or some α' that had been nicked in more than one location and that yielded some fragments after unfolding in urea that did not refold properly. Since the molar ellipticity at 222 nm of the α-2 fragment in 0 M Gdn·HCl is low and is much less than the calculated difference between the molar ellipticities of the intact α subunit and the α-1 fragment (see calculated curve for $\alpha - \alpha$-1 in Figure 2A), the α-2 fragment either is not fully refolded in 0 M Gdn·HCl or else is a mixture of folded and unfolded forms. Since most of the Gdn·HCl-induced unfolding of the α-2 fragment occurs at concentrations of Gdn·HCl below

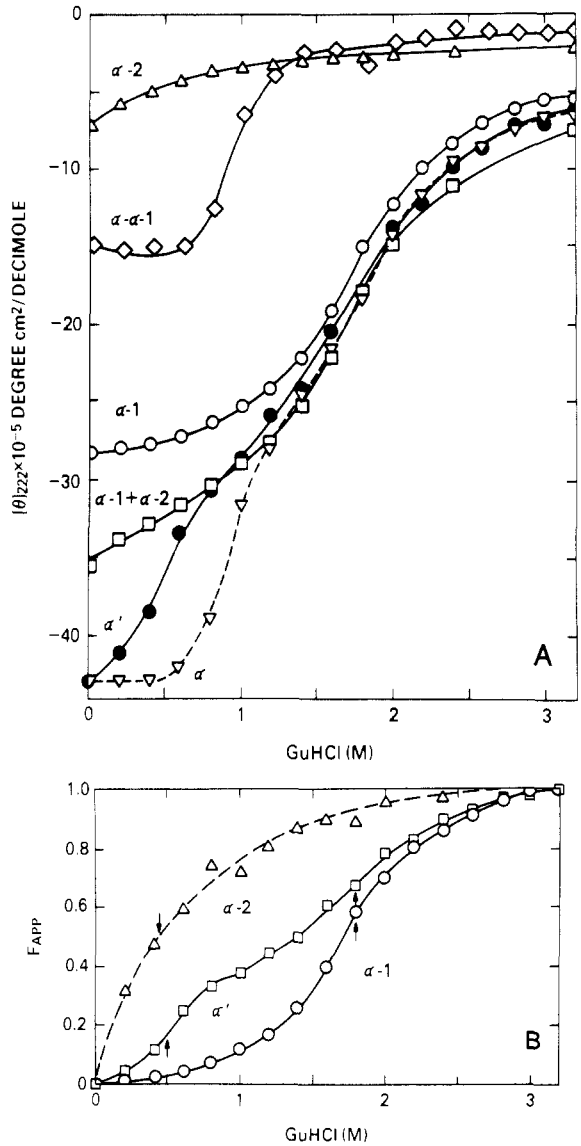

FIGURE 2: Effect of Gdn·HCl concentration on molar ellipticity at 222 nm of the α-1 and α-2 fragments (A) and on the apparent fraction of change of ellipticity (B). (A) Ellipticities at 222 nm of α' (●), α-1 fragment (○), and α-2 fragment (△) were recorded as described under Materials and Methods in a cell with a 10-mm light path. The calculated sums of the data for α-1 fragment and α-2 fragment (α-1 + α-2) are shown by (□). The curve for the α subunit (▽) is taken from Yutani et al. (1979). The calculated difference of α minus α-1 is shown by (◇). (B) The apparent fraction of change of ellipticity at 222 nm was calculated according to the equation $F_{app} = (\theta_N - \theta)/(\theta_N - \theta_D)$ where θ represents the ellipticity at a given concentration of Gdn·HCl and θ_N and θ_D are the ellipticities in the absence and presence of 3.2 M Gdn·HCl, respectively. The effect of Gdn·HCl concentration on the ellipticities of the native and denatured states was not considered because it was within experimental errors and negligible for this analysis. α-1 fragment (○); α-2 fragment (△); α' (□). The arrows point to the estimated midpoint of each transition.

1.4 M and since the midpoint of the observed unfolding transition is at about 0.45 M Gdn·HCl, close to the midpoint of the first unfolding transition observed with α′ (about 0.5 M Gdn·HCl) (see Figure 2B), the first unfolding transition observed with α′ is probably the unfolding of the domain corresponding to the α-2 fragment. Since the midpoints of the unfolding transition for the α-2 fragment and for the first unfolding transition for α′ are closely similar, the preparation of the α-2 fragment probably contains a mixture of properly folded and of unfolded forms, rather than a single, partially folded form. Although it would be preferable for good quantitation to carry out these experiments with a fully folded preparation of the α-2 fragment, the present observation that the midpoints of the unfolding transitions of the α-2 and α-1 fragments correspond closely to the first and second unfolding transitions of α′ gives good support to the proposal that the corresponding α-2 and α-1 domains of α′ unfold in a stepwise process. By analogy, the biphasic unfolding of the intact α subunit proceeds by the stepwise unfolding of the α-2 and α-1 domains in the intact α subunit.

The observation that the midpoint of the first transition of α′ and the corresponding transition in the α-2 fragment are shifted to lower concentrations of Gdn·HCl than the midpoint of the first transition of the α subunit suggests that the α-2 domain is stabilized by its covalent attachment to the α-1 domain in the intact α subunit. Since the midpoint for the unfolding of the α-1 fragment is the same as the midpoint for the second unfolding transition in the α subunit and α′, the α-1 domain is not stabilized by its covalent attachment to the α-2 domain in the intact α subunit.

Chemical Modification of Tyrosyl Residues. The α subunit contains seven tyrosyl residues, six located in the region of the sequence (residues 1–188) contained in the α-1 fragment and one located in the region of the sequence (residues 189–268) contained in the α-2 fragment (Higgins et al., 1979). Solvent perturbation experiments indicate that two to three tyrosyl residues are exposed in the folded α-1 fragment and that the single tyrosyl residue in the α-2 fragment is exposed (Higgins et al., 1979).

Acetylation of tyrosyl residues by acetylimidazole has been used to determine the number of tyrosyl residues in proteins that are "exposed" or susceptible to chemical modification (Riordan & Vallee, 1972). We have used this method to determine whether different numbers of tyrosyl residues are susceptible to modification at different stages of unfolding of the α subunit, α′, and the α-1 fragment. Figure 3 shows the effect of acetylimidazole concentration upon the extent of modification of the α subunit in the absence of denaturant and in 1 and 3 M Gdn·HCl. The results indicate that in 0 and 1 M Gdn·HCl, a stable equilibrium is approached such that there is little further increase in the number of residues reacted as the reagent concentration is increased from 20 to 30 mM. Since all seven tyrosyl residues react with 15–30 mM acetylimidazole in 3 M Gdn·HCl, conditions under which the protein is completely unfolded, the reagent concentration chosen for subsequent studies (20 mM) is sufficient to react with all fully exposed tyrosyl residues under these conditions. A maximum extent of reaction is achieved at each reagent concentration in less than the 60-min incubation period used, since the reagent itself hydrolyzes under these conditions ($t_{1/2}$ is about 15 min).

The effects of Gdn·HCl concentration on the susceptibility of tyrosyl residues in the α subunit, α′, and the α-1 fragment to reaction with acetylimidazole are shown in parts A, B, and C of Figure 4. About one tyrosyl residue in the α subunit

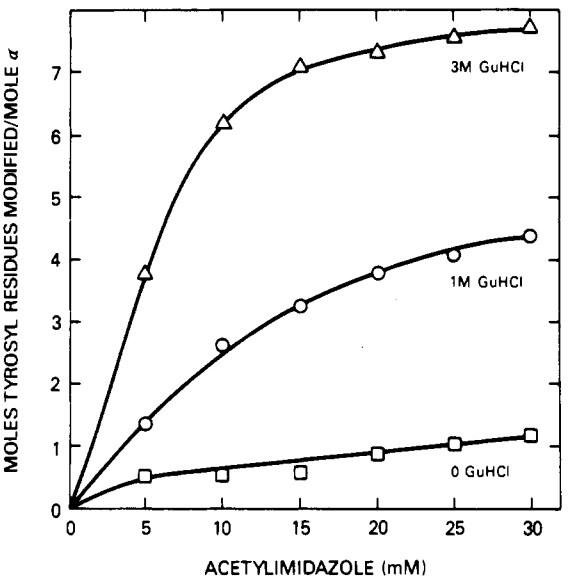

FIGURE 3: Effect of acetylimidazole concentration on modification of the α subunit at different concentrations of Gdn·HCl. Experimental techniques are described under Materials and Methods. The α subunit was treated in 0 (□), 1 (○), and 3 M Gdn·HCl (△) in 0.1 M potassium phosphate buffer at pH 7.5 at the indicated concentration of acetylimidazole for 60 min at 23 °C. Some protein precipitation occurred in 1 M Gdn·HCl but not in 0 or 3 M Gdn·HCl; the precipitate was dissolved during dialysis against 3 M Gdn·HCl.

or α′ is modified at 0 M Gdn·HCl; about three to four residues are modified at 1 M Gdn·HCl; and all seven residues are modified at 3 M Gdn·HCl. Thus two to three tyrosyl residues in the α subunit and α′ become susceptible to modification in each of the two concentration ranges in which unfolding transitions have been identified from circular dichroism studies (see arrows in Figure 4 marked 1 and 2 indicating the midpoints of the two unfolding transitions). The key finding (Figure 4C) is that the α-1 fragment has a plateau level of two to three tyrosyl residues susceptible to modification in the 0–1 M Gdn·HCl concentration range; in the 1–3 M Gdn·HCl concentration range, three additional tyrosyl residues in the α-1 fragment become susceptible to modification. Thus the α-1 fragment does not unfold in the 0–1 M Gdn·HCl concentration range but does unfold at higher Gdn·HCl concentrations. The first step of unfolding of the α subunit and α′ in the 0–1 M Gdn·HCl concentration range allows modification of the same number of tyrosyl residues (about four) that can be modified in the folded α-1 fragment (about three) plus the folded or unfolded α-2 fragment (one). This finding implies that the first step of the unfolding of the α subunit and α′ results in the exposure of two tyrosyl residues of the still folded α-1 domain, which are protected in the native α subunit or α′ by interaction with the folded α-2 domain.

The effects of tyrosyl modification at different Gdn·HCl concentrations on the activities of the α subunit, α′, and the α-1 fragment are also shown in Figure 4 and in Figure 5. Whereas the modification of the one tyrosyl residue of the α subunit that is susceptible to modification in the absence of Gdn·HCl has no effect on the activity, the modification of the two additional tyrosyl residues that become susceptible to modification between 0 and 1 M Gdn·HCl causes full inactivation. Modification of α′ has similar effects on activity. Modification of the α-1 fragment in the 0–1 M Gdn·HCl concentration range causes extensive inactivation. These results show that two or three of the three tyrosyl residues of the α-1 fragment that are susceptible to modification in the absence of denaturant are important for enzyme activity and strongly suggest that these are the same tyrosyl residues that

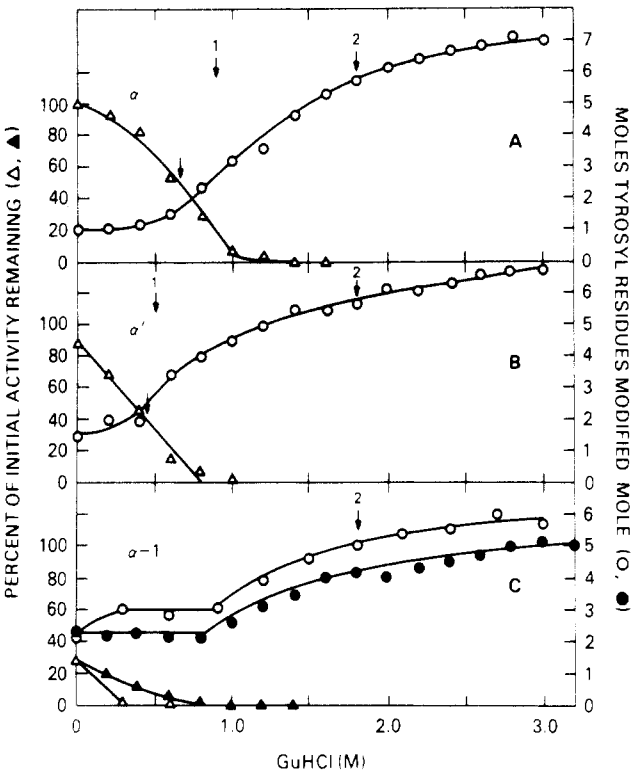

FIGURE 4: Effect of Gdn·HCl concentration on modification by acetylimidazole. (A) α subunit; (B) nicked α subunit (α'); (C) α-1 fragment. Conditions of modification, assay of activity (Δ), determination of the number of tyrosyl residues modified (O), and experimental errors are described under Materials and Methods. In (C), filled symbols represent a second experiment done 3 months after the first experiment on a different preparation of the α-1 fragment with more data points. The activity is expressed as the percent of the untreated control; treatment of the α-1 fragment with acetylimidazole in 0 M Gdn·HCl modified two to three tyrosyl residues with a consequent loss of 75% of the activity. Unlabeled arrows indicate the midpoints of the inactivation curves. Arrows labeled 1 and 2 in (A) indicate the midpoints of the two unfolding transitions of the α subunit determined by circular dichroism measurements (see Figure 1 and the text); analogous arrows in (B) indicate the midpoints of the two unfolding transitions of α' (see Figure 2B and the text). The arrow labeled 2 in (C) indicates the midpoint of the second unfolding transition of the α subunit (see Figure 1 and the text).

become susceptible to modification in the α subunit and α' in the presence of 1 M Gdn·HCl. The Gdn·HCl concentration dependence of inactivation by acetylation of tyrosyl residues should thus reflect an unfolding transition that leads to the exposure of these tyrosyl residues that are important for activity. Whereas the midpoint of the inactivation curve for the α subunit (0.66 M Gdn·HCl) (Figure 4A) is significantly lower than the midpoint of the first unfolding transition observed by circular dichroism measurements (0.9 M Gdn·HCl) (Figure 1), the midpoint of the inactivation curve for α' (0.46 M Gdn·HCl) (Figure 4B) is closer to the midpoint of the unfolding transition of the α-2 fragment (0.45 M Gdn·HCl) and to the midpoint of the first unfolding transition of α' (0.5 M Gdn·HCl) (Figure 2B). Although the differences between the midpoints observed by the two methods could reflect the presence of more than two unfolding transitions or of different types of unfolding processes, it seems more likely that the two methods detect the same first unfolding transition but that the chemically modified α subunit is less stable in Gdn·HCl and undergoes unfolding at somewhat lower concentrations of Gdn·HCl. In fact, considerable precipitation and irreversible inactivation of the α subunit in the 0.4–1.2 M Gdn·HCl concentration range were observed, although the native α subunit is completely soluble in this concentration range. To

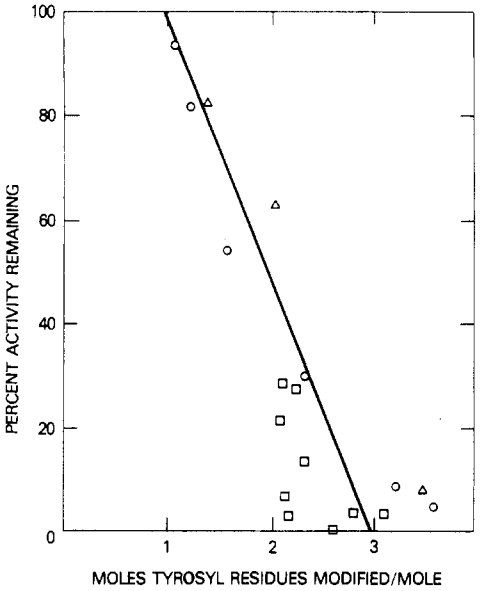

FIGURE 5: Effect of tyrosine modification by acetylimidazole on the tryptophan synthase activity of the α subunit, α', and the α-1 fragment. Proteins were modified and assayed as described in Figure 4 and under Materials and Methods. α subunit (O); α' (Δ); α-1 fragment (□).

test whether this precipitation might interfere with the quantitation of the modification of tyrosyl residues, the experiment shown in Figure 4A was repeated in a range of concentrations of urea from 0 to 7 M (data not shown). Under these conditions, no precipitation of the modified α subunit was observed, but the extents of modification of tyrosyl residues obtained were similar to those in Figure 4A. The midpoint of the inactivation curve for the α subunit was at 2 M urea, whereas the midpoint of the first unfolding transition reported by Matthews & Crisanti (1981) was at 2.6 M urea. The observation in this experiment (data not shown) that the activity of the α subunit modified in the presence of 0–3.5 M urea could be largely restored by treatment of hydroxylamine, followed by dialysis, indicates that inactivation is mainly due to acetylation of tyrosyl residues and is not due to the acetylation of lysyl residues, a reaction not reversed by hydroxylamine.

Characterization of the Pathway of Unfolding and of the Partially Unfolded Form of the α Subunit. Plots of the Gdn·HCl concentration dependence of the ellipticity at 222 nm of the α subunit (Figure 2A) and of the fluorescence of the α subunit are biphasic, suggesting the presence of a partially unfolded form of the α subunit in the unfolding process (Yutani et al., 1979). The data have been fitted to a three-state unfolding process (N ⇄ I ⇄ D) (Figure 1; Yutani et al., 1979). Since the α intermediate form in 1 M Gdn·HCl (I) has a lower ellipticity and greater tyrosine fluorescence than the native form (N), the intermediate presumably results from some decrease in the ordered structure of the α subunit and from a change in the environment of some tyrosyl residues. Since the near-ultraviolet circular dichroism spectrum of the α subunit intermediate (I) in 1 M Gdn·HCl is similar to that of the folded proteolytic fragment α-1 (Higgins et al., 1979), Yutani et al. (1980) proposed that the α intermediate (I) contains one folded domain with a structure similar to that of the folded α-1 fragment and a second domain, unfolded, with a structure similar to that of the unfolded α-2 fragment.

Our results comparing the effects of Gdn·HCl concentration on the ellipticity of the α-1 and α-2 fragments on the nicked α subunit (α'), and on the intact α subunit (Figure 2), support this proposal by showing that the Gdn·HCl concentration

ranges at which the first and second unfolding steps of α' occur correspond to the Gdn·HCl concentration ranges at which the α-1 and α-2 fragments unfold. Since the midpoint of the first unfolding transition of the intact α subunit (0.9 M Gdn·HCl) is significantly higher than the corresponding midpoint for α' (0.5 M Gdn·HCl) and the midpoint for the α-2 fragment (0.45 M Gdn·HCl), the α-2 fragment appears to be stabilized to Gdn·HCl-induced unfolding by covalent attachment to the α-1 fragment in the intact chain (see above).

Although measurements of ellipticity at 222 nm give gross information on total ordered structure, chemical modification measurements can give additional, specific information about the susceptibility of particular residues that can help in characterizing partially unfolded forms of proteins. Since two to three tyrosyl residues in the α-1 fragment can be modified in either 0 or 1 M Gdn·HCl (Figure 4), the α-1 fragment does not unfold in this Gdn·HCl concentration range; this confirms the ellipticity results (Figure 2). Our finding that the partially unfolded form of the α subunit in 1 M Gdn·HCl contains four tyrosyl residues that can be modified (Figure 4A) shows that this intermediate form has the same chemical properties as does the folded α-1 fragment plus the folded or unfolded α-2 fragment. Since the α-1 fragment and the partially unfolded α intermediate (I) in 1 M Gdn·HCl have two more tyrosyl residues susceptible to modification than does the native, intact α subunit (N) in 0 M Gdn·HCl, these residues become exposed in the first unfolding step (N $\rightleftarrows$ I); these residues may be shielded in the domain interaction site in the folded α subunit. The first Gdn·HCl-induced unfolding step has also been reported to result in exposing two sulfhydryl residues, which can be modified in the folded α-1 fragment but which cannot be modified in the α subunit or α' in the absence of denaturant (Miles, 1981; Higgins et al., 1979). Three additional tyrosyl residues become susceptible to modification when the α-1 fragment is unfolded in 3 M Gdn·HCl or when the intact α subunit is further unfolded in the 1–3 M Gdn·HCl concentration range. These results are consistent with the proposal that the second step of unfolding of the intact α subunit (I $\rightleftarrows$ D) involves the unfolding of the domain corresponding to the α-1 fragment.

Our results are consistent with recent estimates (Crisanti & Matthews, 1981) of the number of tyrosyl residues exposed (3.2) when a folded intermediate form I_2 is converted to a form I, which has a less compact structure and all tyrosyl residues exposed to solvent. These recent studies of Matthews & Crisanti (1981) and Crisanti & Matthews (1981) give additional evidence from kinetic experiments and from urea-gradient gel electrophoresis experiments that the partially unfolded α intermediate observed in equilibrium studies is a true intermediate on the pathway of unfolding and refolding of the intact α subunit.

Comparison of Proteins That Unfold by a Multistate Process. Multistate behavior has been detected in equilibrium studies of the unfolding of a few other proteins, including carbonic anhydrase (Wong & Tanford, 1973), penicillinase (Robson & Pain, 1976a,b; Carrey & Pain, 1978; Creighton & Pain, 1980), α-lactalbumin (Kuwajima et al., 1976; Nozaka et al., 1978), and the β_2 subunit of tryptophan synthase (Zetina & Goldberg, 1980). Zetina & Goldberg (1980) have characterized the unfolding pathway of the β_2 subunit of tryptophan synthase by comparing the Gdn·HCl-induced unfolding of the intact β_2 subunit, a nicked β_2 derivative, and the separately refolded proteolytic fragments F_1 and F_2. Since the Gdn·HCl concentration dependence curves for the fluorescence, ellipticity at 220 nm, and $s_{20,w}$ of the β_2 subunit are not coincident, unfolding is probably multiphasic. The concentration of Gdn·HCl (0.4 M) that causes 50% dissociation of the nicked β_2 subunit is much lower than the concentration of Gdn·HCl (0.75 M) that causes 50% unfolding of the F_1 fragment and the concentration (1.1 M) that causes 50% unfolding of the F_2 fragment. Thus the pathway of unfolding of the nicked β_2 subunit as the concentration of Gdn·HCl is increased involves dissociation of the two fragments, followed by the stepwise unfolding of the F_1 fragment and then of the F_2 fragment. Zetina & Goldberg conclude that Gdn·HCl weakens preferentially the interdomain interactions in the nicked β_2 subunit and thus allows the F_1 and F_2 fragments to show independent unfolding transitions. Gdn·HCl probably weakens the interdomain interactions in the intact β_2 subunit as well as in the nicked β_2 subunit since the fluorescence transition of the F_1 fragment is the same in the intact β_2 subunit, in the nicked β_2 subunit, and in the isolated F_1 fragment.

These findings for the β_2 subunit are similar to observations with the α subunit reported here. In both systems the multistate unfolding transition induced by Gdn·HCl may result from a weakening of interdomain interactions, which allows independent, stepwise unfolding of the two domains.

The Gdn·HCl-induced unfolding of the α and β_2 subunits of tryptophan synthase differs somewhat from the Gdn·HCl-induced unfolding of two other proteins that show multistate behavior: penicillinase and α-lactalbumin. Carrey & Pain (1978) have concluded that the partially unfolded form of penicillinase observed in 0.8 M Gdn·HCl results from separation of two or more domains without appreciable change in secondary structure; the first stage of unfolding also results in full exposure of all the tyrosyl residues to solvent. The first stage in the unfolding of α-lactalbumin by Gdn·HCl also results in the exposure of the aromatic residues, which contribute to Cotton effects at 270 and 296 nm, while α-helical regions of the polypeptide chain appear to unfold in the second stage (Kuwajima et al., 1976).

In contrast, the first step in the unfolding of the α subunit results in exposure to solvent of only about half of the tyrosyl residues as judged by near-ultraviolet circular dichroism spectra (Yutani et al., 1980), by absorbance at 286 nm (Crisanti & Matthews, 1981), and by susceptibility to chemical modification (this work) and also results in some changes in secondary structure.

In conclusion, we have found that the unfolding of each of the proteolytic fragments of the α subunit serves as a model for the unfolding of the corresponding domain in the intact α subunit. Comparative studies of the unfolding of the fragments and of the intact α subunit show that the pathway of Gdn·HCl-induced unfolding of the α subunit involves the stepwise unfolding of the α-2 domain followed by that of the α-1 domain. The combined use of these proteolytic fragments, circular dichroism measurements, and chemical modification techniques has made it possible to obtain a clearer picture and more detailed characterization of a partially unfolded intermediate form of the α subunit than would have been possible by using physical techniques alone or by studying the intact protein alone. Extensions of these studies to label specific residues in the known sequence of the α subunit at different stages of unfolding should increase our knowledge of the topography of partially unfolded forms and of the residues involved in domain interaction sites.

References

Anfinsen, C. B. (1973) *Science (Washington, D.C.) 181*, 223–230.

Baldwin, R. L. (1975) *Annu. Rev. Biochem. 44*, 453–475.
Baldwin, R. L. (1978) *Trends Biochem. Sci. 3*, 66–68.
Carrey, E. A., & Pain, R. H. (1978) *Biochim. Biophys. Acta 533*, 12–22.
Creighton, T. E. (1978) *Prog. Biophys. Mol. Biol. 33*, 231–297.
Creighton, T. E., & Pain, R. H. (1980) *J. Mol. Biol. 137*, 431–436.
Crisanti, M. M., & Matthews, C. R. (1981) *Biochemistry 20*, 2700–2706.
Goldberg, M. E. (1969) *J. Mol. Biol. 46*, 441–446.
Higgins, W., Fairwell, T., & Miles, E. W. (1979) *Biochemistry 18*, 4827–4835.
Högberg-Raibaud, A., & Goldberg, M. E. (1977a) *Proc. Natl. Acad. Sci. U.S.A. 74*, 442–446.
Högberg-Raibaud, A., & Goldberg, M. E. (1977b) *Biochemistry 16*, 4014–4020.
Kuwajima, K., Nitta, K., Yoneyama, M., & Sugai, S. (1976) *J. Mol. Biol. 106*, 359–373.
Matthews, C. R., & Crisanti, M. M. (1981) *Biochemistry 20*, 784–792.
Miles, E. W. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1838.
Miles, E. W., & Higgins, W. (1978) *J. Biol. Chem. 253*, 6266–6269.
Nozaka, M., Kuwajima, K., Nitta, K., & Sugai, S. (1978) *Biochemistry 17*, 3753–3758.
Riordan, J. F., & Vallee, B. L. (1972) *Methods Enzymol. 25*, 500–506.
Robson, B., & Pain, R. H. (1976a) *Biochem. J. 155*, 325–330.
Robson, B., & Pain, R. H. (1976b) *Biochem. J. 155*, 331–344.
Wetlaufer, D. B. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 697–701.
Wetlaufer, D. B. (1981) *Adv. Protein Chem. 34*, 61–92.
Wong, K.-P., & Tanford, C. (1973) *J. Biol. Chem. 248*, 8518–8523.
Yutani, K., Ogasahara, K., Suzuki, M., & Sugino, Y. (1979) *J. Biochem. (Tokyo) 85*, 915–921.
Yutani, K., Ogasahara, K., & Sugino, Y. (1980) *J. Mol. Biol. 144*, 455–465.
Zetina, C. R., & Goldberg, M. E. (1980) *J. Mol. Biol. 137*, 401–414.

Role of Aromatic Residues in the Structure–Function Relationship of α-Bungarotoxin†

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ABSTRACT: The conformation of α-bungarotoxin and its cyanogen bromide cleaved and nitrated derivatives was studied by circular dichroism (CD). Native toxin contains no helices but some β forms and possibly β turns. Its ordered conformation is little affected when the peptide bond between Met-27 and Trp-28 is cleaved; however, the CD due to Trp-28 is abolished. The CNBr-cleaved derivative retains its immunoaffinity toward anti-toxin sera but loses its neurotoxicity toward the acetylcholine receptor. On the basis of both CD and fluorescence spectra, Trp-28 is probably stabilized by a short-range interaction with the carboxylate group of Asp-30. The ordered conformation of the toxin is also unaltered when one of the two tyrosine residues, identified as Tyr-54, is nitrated with tetranitromethane. This Tyr(NO_2)-54 derivative possesses both immunoaffinity and neurotoxicity. However, the toxin is denatured and loses its activities when the other tyrosine residue, Tyr-24, is also nitrated in 6 M guanidine hydrochloride, even after the denaturant is removed. Spectrophotometric titration of the toxin indicates that Tyr-54 has a normal apparent dissociation constant (pK_a = 9.7) and Tyr-24 ionizes at pH above 11.2. Both tyrosine residues are in a polar environment, but Tyr-24 is not readily accessible to reagents and is stabilized by long-range interactions, probably involving Glu-41.

Postsynaptic toxins (α-toxins) of snake venoms can be divided into two groups: short (type I) toxins contain 60–62 amino acid residues with four disulfide bonds and long (type II) ones 70–74 residues with five disulfide linkages (Lee, 1972; Tu, 1973; Yang, 1974; Karlsson, 1979). For instance, cobra neurotoxin of *Naja naja atra* (Taiwan), erabutoxin of *Laticauda semifasciata* (Okinawa), and toxin b of *Laticauda semifasciata* (Philippine) belong to type I, whereas α-cobratoxin of *Naja naja siamensis* and α-BuTX[1] of *Bungarus multicinctus* are type II toxins. The short toxin molecules consist of four cyclic polypeptides (loops) which show a high degree of sequence homology with the four major loops of the long toxin molecules, which have an extra minor loop. Because the five disulfide bonds are not fully established, α-BuTX can be tentatively assigned to have the following five loops (Figure 1): (1) residues 3–23 with a single charged group at Glu-20, (2) residues 16–44, which contain most of the invariant residues that recognize the acetylcholine receptor, (3) residues 48–59, (4) residues 60–65, and (5) residues 29–33, often termed as loop 2a which is inside loop 2. The role of this extra loop 2a in the long toxins has not been fully understood, al-

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[1] Abbreviations: α-BuTX, α-bungarotoxin; CB-I and CB-II, cyanogen bromide cleaved components I and II; NP-I and NP-II, mono- and dinitrotyrosyl derivatives; CD, circular dichroism; Tris, tris(hydroxymethyl)aminomethane.

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