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Unfolding Pathway of Myoglobin. Evidence for a Multistate Process†

Ettore Bismuto, Giovanni Colonna,* and Gaetano Irace

ABSTRACT: The free energy of unfolding of horse myoglobin has been calculated from the denaturation pattern induced by guanidine hydrochloride as well as by acid. The ΔG^{H_2O}, i.e., the value in the absence of denaturant obtained by using the two-state transition model, was found to be 25% lower than that determined from the acid denaturation pattern, i.e., 12.0 kcal/mol, although the extent of protein denaturation produced by acid was much lower. The amount of helical structure surviving the acid-induced conformational change was estimated to be 50% of that present in the native protein, and it could be destroyed only after exposure of myoglobin samples kept at pH 3.0 to concentrated guanidine. From the guanidine denaturation pattern at acidic pH, a further variation of free energy of unfolding of 5.5 kcal/mol could be calculated, thus indicating that the overall free energy of unfolding determined from the two consecutive processes corresponds to 17.5 kcal/mol. The discrepancy between the two sets of data, i.e., guanidine unfolding at neutral pH and acid unfolding followed by addition of denaturant, has been considered to depend on the general assumption that the guanidine unfolding of myoglobin is a two-state process in the transition region. According to the recent experimental evidence showing the occurrence of at least two molecular events during the guanidine unfolding of apomyoglobin [Colonna, G., Balestrieri, C., Bismuto, E., Servillo, L., & Irace, G. (1982) *Biochemistry 21*, 212–215], the guanidine denaturation pattern of myoglobin was analyzed in terms of two independent steps. The analysis indicated an overall free energy of unfolding, i.e., ΔG^{H_2O}, of 16.0 kcal/mol whereas the free energy changes associated with the unfolding of each structural unit were 10.0 and 6.0 kcal/mol depending on the degree of structural organization of the other unit; more precisely, the higher value corresponds to the free energy of unfolding of each structural unit in the native protein and the lower value to the free energy of unfolding of the same unit when the other has been unfolded.

The correlation between coding sequences (exons) of DNA and functional elements within the protein molecule has stimulated an increasing interest toward the hemoglobin–myoglobin family of respiratory proteins. In fact, the knowledge of both the gene sequence and the protein structure makes some members of this class of homologous proteins ideal candidates for these studies. Globin genes so far cloned and sequenced consist of five segments, three exons separated by two introns (Nishioka & Leder, 1979; Konkel et al., 1978, 1979). The three coding sequences correspond to amino acid residues 1–31, 32–99, and 100–141 and 1–30, 31–104, and 105–146 for the α- and β-globin genes, respectively. A structural as well as functional role for the three protein segments encoded by exons has been recently proposed by Go (1981). However, in contrast to the immunoglobulins where a precise exon–function correlation has been discovered (Sakano et al., 1979; Early et al., 1979), the junctions between functional elements in globins occur within long α-helical segments. This observation would suggest the absence of a clear demarcation into functional domains. On the other hand, the X-ray analysis (Wetlaufer, 1973), the diagonal plot of Kuntz (1975), and the more recent surface area measurements of Wodak & Janin (1981) have revealed the presence in the globin molecule of two structural "halves" the function of which is to trap the heme molecule.

This conclusion agrees well with the result of the folding simulation process reported by Ptitsyn & Rashin (1975) and with the experimental evidence recently obtained showing the occurrence of two independent molecular transitions when apomyoglobin undergoes acid and/or guanidine unfolding (Balestrieri et al., 1976; Colonna et al., 1978, 1982; Irace et al., 1981).

We have now reexamined the acid- and guanidine-induced unfolding of myoglobin in order to establish whether the results obtained on apomyoglobin might be extrapolated to the whole protein. The results reported in this paper show that the denaturation pattern of myoglobin is consistent with the oc-

† From the Istituto di Chimica Biologica, Cattedra di Chimica e Propedeutica Biochimica, 1ª Facoltà di Medicina e Chirurgia, Università di Napoli, 80138 Napoli, Italy. *Received August 10, 1982; revised manuscript received April 26, 1983.*

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currence of at least two molecular events representing the unfolding of independent molecular domains.

Materials and Methods

Myoglobin. Horse myoglobin was purchased from Sigma; the protein was used after a run on Sephadex G-75 (2.5 × 100 cm) equilibrated with 0.05 M phosphate buffer, pH 7.0. All preparations of myoglobin were metmyoglobin and will be referred to as myoglobin. The homogeneity of the preparations was controlled by disc gel electrophoresis at pH 8.6 on 7% polyacrylamide, as reported by Davis (1964). Protein concentrations were determined spectrophotometrically at 409 nm in 0.1 M phosphate buffer, pH 7.0, by using the extinction coefficient reported by Harrison & Blout (1965).

Chemicals and Solutions. All common chemicals were reagent grade and were purchased from the British Drug House. Ultrapure guanidine hydrochloride (Gdn·HCl)[1] was obtained from Schwarz/Mann.

The denaturation equilibria were measured following the Soret absorbance and the CD activity at 222 nm. Acid titrations were performed by careful addition of small amounts of concentrated HCl to buffered solutions (0.05 M phosphate, 0.01 M acetate, and 0.15 M KCl) from an Agla syringe while the solutions were stirred magnetically. In denaturation experiments, the protein was added to buffered solutions of Gdn·HCl; 0.15 M KCl was present in all solutions. The Soret absorption and the CD activity at 222 nm were then followed in time until an apparent equilibrium was reached (several to 12 h). The small amount of irreversible acid denaturation was corrected by extrapolating the absorbance and CD values to zero time in the manner described by Acampora & Hermans (1967).

Spectral Measurements. CD spectra in the spectral range 200–250 nm were determined on a Cary Model 61 spectropolarimeter, equipped with a temperature-controlled cell holder. Cells of 0.1 cm were used for measurements in the far-ultraviolet. The molar ellipticity, $[\theta]$, in units of deg cm^2/dmol was calculated by using a value of 115 as the mean residue molecular weight.

Absorption spectra were recorded with a Perkin-Elmer Model 575 and/or a Cary 219 C double-beam spectrophotometer equipped with a temperature-controlled cell holder.

Results

Guanidine Unfolding at Neutral pH. The Gdn·HCl unfolding of horse myoglobin at neutral pH has been studied by following the effect of increasing denaturant concentration on the CD activity of the peptide chromophore at 222 nm. In the absence of denaturant, the CD spectrum of horse myoglobin shows two minima centered at 208 and 222 nm, which are typical of a polypeptide in the α-helical conformation. The amount of α-helix content, that is, about 75% at pH 7.0 as estimated by using poly(L-lysine) as a model (Greenfield & Fasman, 1969), decreases following exposure to denaturant or acid. Figure 1 shows the dependence of the CD activity at 222 nm of horse myoglobin on Gdn·HCl concentration at pH 7.0 and 25 °C. The transition midpoint (defined as the concentration required to effect 50% of the total change) occurs at 1.7 M Gdn·HCl. The reversibility of Gdn·HCl unfolding was assessed by diluting samples in concentrated denaturant with phosphate buffer; the dilutions covered the entire denaturation region.

[1] Abbreviations: Gdn·HCl, guanidine hydrochloride; CD, circular dichroism; ANS, 8-anilino-1-naphthalenesulfonate.

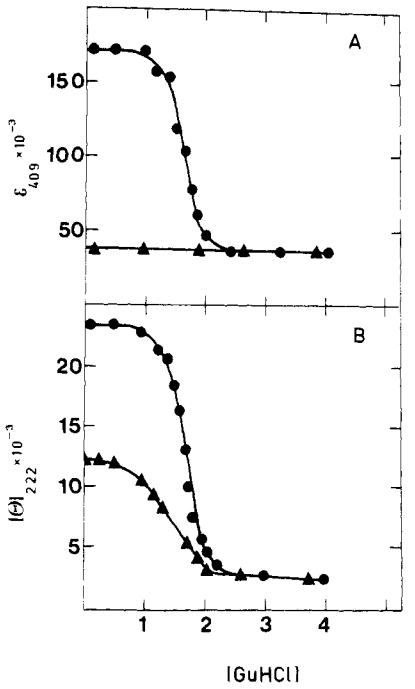


FIGURE 1: Effect of Gdn·HCl concentration on the Soret absorption (A) and the mean residue ellipticity at 222 nm (B) of horse myoglobin at pH 7.0 (●) and at pH 3.5 (▲). All solutions contained 0.05 M phosphate, 0.01 M acetate, and 0.15 M KCl. Temperature was kept at 25 °C. The continuous line (●) shown in part B is theoretical and based on solution of eq 5.

The free energy of unfolding at each concentration of denaturant was calculated by using the following equation:

$$\Delta G = -RT \ln [(X_n - X_i)/(X_i - X_d)] \quad (1)$$

where X_i is the numerical value of the structure-sensitive parameter at the ith denaturant concentration; X_n and X_d are the numerical values of the same parameter relative to the native (n) and denatured (d) states, respectively.

The free energy of unfolding in the absence of denaturant (ΔG^{H_2O}) has been calculated by two independent methods, i.e., the ligand binding model and the Tanford model (Tanford, 1970). The former assumes that the denaturation is due to the binding of the denaturant to the protein. This assumption leads to the following equation:

$$\Delta G = \Delta G^{H_2O} - \Delta nRT \ln (1 + Ka_{\pm}) \quad (2)$$

where K is the average binding constant, $a_{\pm}$ the activity of the denaturant, and Δn the difference in the number of denaturant binding sites between the denatured and the native forms of the protein. According to the suggestion recently made by Pace & Vanderburg (1979), we have used a value of 0.6 for K in our computations.

The other method is based on the accessible solubility differences of protein components in water and in the denaturant and is summarized in the following equation:

$$\Delta G = \Delta G^{H_2O} + \sum_{i=1}^{n} (\Delta\alpha)_i (\Delta G_t)_i \quad (3a)$$

where $(\Delta\alpha)_i$ indicates the difference in the degree of exposure of the ith group in the unfolded protein and of the same group in the native protein and $(\Delta G_t)_i$ is the transfer free energy from water to the particular denaturant concentration. Transfer free energies for amino acids and several small peptides have been determined from solubility measurements in Gdn·HCl (Nozaki & Tanford, 1963, 1970; Robinson & Jencks, 1965). We have used the values reported by Pace & Vanderburg

(1979) except for the polar amino acids, the values of which were taken from Schrier & Schrier (1976).

In the numerical procedure used to obtain ΔG^{H_2O}, eq 3a was modified in order to provide a mean difference in the degree of exposure between the native and denatured protein; i.e., eq 3a becomes

$$\Delta G = \Delta G^{H_2O} + \Delta\alpha \sum_{i=1}^{n} (\Delta G_t)_i \qquad (3b)$$

The ΔG^{H_2O} values obtained by using the two different methods of analysis were quite similar, i.e., 9.7 and 9.2 kcal/mol by using the ligand binding model and the Tanford model, respectively. Δn and $\Delta\alpha$ were 37 and 0.24, respectively. The small discrepancy between our calculations and the ΔG^{H_2O} reported by Puett (1973), i.e., 11 kcal/mol, is probably due to the different values of K used in the numerical computation. According to Pace & Vanderburg (1979), we have used K = 0.6 whereas a value of 1.2 was used by Puett (1973).

Knowledge of the tridimensional structure of myoglobin allows one to predict the mean solvent accessibility difference between the native and denatured protein (Lee & Richards, 1971); the result of this analysis shows that the $\Delta\alpha$ obtained from eq 3b, i.e., 0.24, is lower than that calculated from X-ray data, which is 0.4. If $\Delta\alpha$ is assumed to be equal to 0.4, the ΔG^{H_2O} calculated from eq 3b is 18.5 kcal/mol, which corresponds to a midpoint higher than 2.5 M Gdn·HCl, in contrast with the value experimentally observed (1.7 M Gdn·HCl). The discrepancy between $\Delta\alpha$ obtained from denaturation studies and $\Delta\alpha$ predicted from X-ray analysis could be due to the fact that the latter value is a static measurement which does not take into account protein flexibility. This may result in a value which is somewhat higher than that experimentally observed. Finally, it has to be considered that eq 3b is valid for a two-state process in the transition region; if it is assumed that guanidine unfolding does not occur as a single-step process, the ΔG^{H_2O} as well as $\Delta\alpha$ obtained from the denaturation pattern is to be considered as an apparent value. Figure 1 shows that the transition curves obtained by following the Soret absorbance and the far-ultraviolet CD activity as a function of increasing Gdn·HCl concentration at neutral pH are closely related in occurrence and extent. This would support the idea that myoglobin unfolding occurs as a single-step process. However, much experimental evidence has recently been presented that the guanidine unfolding of apomyoglobin does not occur as a single-step process, but it reflects the unfolding of two independent structural regions (Balestrieri et al., 1976; Irace et al., 1981; Colonna et al., 1982).

Acid Unfolding. Acid unfolding of horse myoglobin in the absence of Gdn·HCl results in a sharp decrease of ϵ_{409} between pH 5.0 and 4.0 (Figure 2A). Further exposure of horse myoglobin to increasing Gdn·HCl concentration at pH 3.5 does not produce any further change in the Soret absorption (Figure 1A).

A quite different pattern has been observed by following the acid pH dependence of the mean residue ellipticity at 222 nm in the absence of Gdn·HCl. In fact, dropping the pH from 5.0 to 4.0 produces a strong decrease of negative ellipticity of approximately 10000 deg cm^2/dmol, which accounts for about 50% loss in helical structure (Figure 2B). The residual optical activity of the peptide chromophore at pH 3.5 is reduced only after exposure of the protein to increasing Gdn·HCl concentration (Figure 1B). The acid pH titrations performed in the presence of various denaturant concentrations, i.e., 1.0 and 2.0 M Gdn·HCl, show that the minimal value of negative ellipticity is reached only when the denaturant concentration approaches 2.0 M (Figure 2B). The acid denaturations have been observed

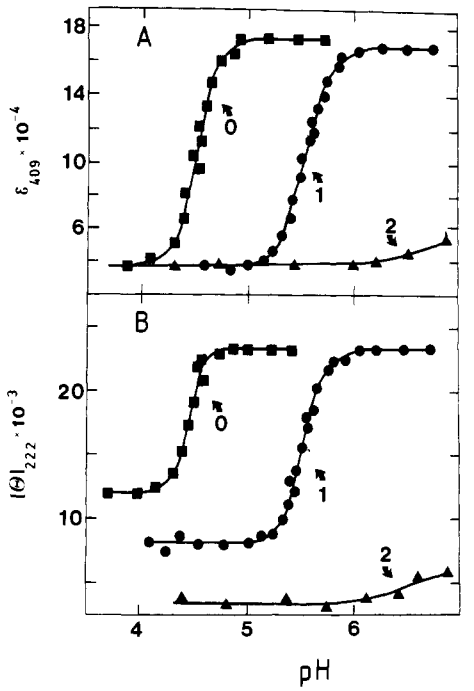


FIGURE 2: Acid dependence of the Soret absorption (A) and the mean residue ellipticity (B) of horse myoglobin at the indicated concentrations of Gdn·HCl. The experimental conditions are those described in Figure 1.

to be promptly reversible providing that the protein solutions do not remain at low pH for extended periods (not more than 30 min).

The acid pH dependence of CD activity at 222 nm of horse myoglobin resembles rather closely that observed for mammalian apomyoglobins (Colonna et al., 1978; Irace et al., 1981). It has been reported that mammalian apomyoglobins as well as that from bluefin tuna lose about 50% of their helical content when the pH is lowered from neutral to acidic. The conformational state of apomyoglobin at pH 3.5 does not correspond to that predicted for a fully unfolded polypeptide (Kirby & Steiner, 1970; Colonna et al., 1978). In fact, the tryptophanyl emission is centered around 335 nm (Irace et al., 1981), a value which indicates that the tryptophanyl residues are still incompletely exposed to aqueous solvent. Moreover, the tryptophanyl fluorescence polarization observed at acidic pH, i.e., 0.10, is significantly higher than that observed for low molecular weight model compounds in water or for fully denatured proteins. These observations suggest that a large amount of organized structure is still present at acidic pH. Similar conclusions can be reasonably drawn also for myoglobin based on the experimental evidence that 50% of the helical structure is still present at pH 3.5, although the Soret absorbance approaches that of free hemin in water. This result has been also observed for the acid-induced molecular transition of apomyoglobin which makes the apoprotein unable to bind hemin or ANS (Colonna et al., 1982). Therefore, the Soret absorption seems not to be the appropriate structure-sensitive parameter as far as the acid pH transition is concerned.

The acid transition curve obtained from CD measurements in the absence of guanidine was analyzed in order to obtain the $\Delta G°$ of the corresponding molecular transition by using the following equation:

$$\Delta G = \Delta G° - RT\Delta r \ln (1 + a_{H^+}/K_d) \qquad (4)$$

where a_{H^+} is the hydrogen ion activity necessary to produce 50% of acid unfolding, Δr the difference in the number of

ionizable groups that are titratable in the acid-unfolded protein and in the native protein, and K_d the proton dissociation constant of the unfolded myoglobin. According to the suggestion made by Puett (1973), we have used a value of 5.9 for pK_d in our computations. The $\Delta G°$ and Δr values which best fitted the experimental data were 12.0 kcal/mol and 6.1, respectively.

Acid denaturation of myoglobin is known to result from histidyl groups that are masked in the native protein. The number of titratable groups that we have found to be responsible for the acid denaturation, i.e., 6.0, agrees well with earlier acid denaturation studies (Acampora & Hermans, 1967; Hermans & Acampora, 1967; Puett, 1973) and with the results of potentiometric titrations (Hartzell et al., 1968; Edmundson, 1965). It is interesting to observe that the $\Delta G°$ value calculated from the acid denaturation pattern is 2.3–2.8 kcal/mol larger than the ΔG^{H_2O} obtained from guanidine unfolding at neutral pH, although the extent of protein denaturation appears to be much lower at acidic pH. In fact, the CD spectrum observed at pH 3.5 is not that expected for a randomly coiled polypeptide in aqueous solution. This condition is reached only when the protein is further exposed to increasing denaturant concentration.

Guanidine Unfolding at Acidic pH. The dependence of the mean residue ellipticity at 222 nm on the Gdn·HCl concentration at pH 3.0 (Figure 1B) has been analyzed according to eq 2 and 3b in order to obtain the thermodynamic parameters. The same value of $\Delta G°$ was obtained by using the two different methods of analysis, i.e., $\Delta G° = 5.5$ kcal/mol. Δn and $\Delta\alpha$ were 27 and 0.16, respectively. It is interesting to observe that Δn is lower than that calculated at neutral pH, thus indicating that a certain amount of guanidine binding sites become exposed to the solvent at pH 3.0. Moreover, the mean residue difference observed in the degree of exposure at pH 3.0 is lower than that calculated at neutral pH, as expected if a certain extent of denaturation takes place when the pH is lowered. More recently, it has been reported that the acid-induced molecular transition of apomyoglobin unfolds the heme binding site of the molecule, but not the N-terminal region as indicated by several physicochemical parameters (Balestrieri et al., 1976; Irace et al., 1981; Colonna et al., 1982). The acid denaturation pattern of myoglobin shown in Figure 2 is consistent with that observed for apomyoglobin since the unfolding of the heme binding site is not related to the overall unfolding of the protein molecule.

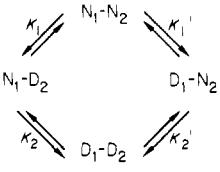
Discussion

The idea that the apomyoglobin structure results from the interaction of two structural units representing the product of independent folding processes (Ptitsyn & Rashin, 1975; Wetlaufer, 1973; Kuntz, 1975) has been recently verified by showing the occurrence of two subsequent molecular transitions (Balestrieri et al., 1976; Irace et al., 1981; Colonna et al., 1982). Such an occurrence has not been reported yet for myoglobin, the denaturation pattern of which has been always interpreted as a two-state transition process (Hermans & Acampora, 1967; Shen & Hermans, 1972; Puett, 1973). The data presented in this paper support the idea that the conformation of myoglobin at acidic pH is far from being that corresponding to a randomly coiled polypeptide. This state seems to be reached only in concentrated guanidine solution. In this respect, the behavior of the myoglobin molecule toward acids is similar to that observed for apomyoglobin, i.e., unfolding of the heme binding site but the presence of a large amount of organized structure in the N-terminal region of the molecule (Irace et al., 1981; Colonna et al., 1982).

A marked difference is observed in the denaturation pattern induced by guanidine; in fact, the unfolding of the heme binding site of apomyoglobin occurs as separate process preceding the unfolding of the remainder of the molecule. The two phenomena seem to be concomitant for myoglobin as documented by the simultaneous variations in the Soret absorption and in the far-ultraviolet CD activity. A reasonable explanation is that the presence of the heme makes the whole protein structure so tightly connected that it behaves as a single cooperative unit. This observation is consistent with the finding of Wodak & Janin (1981), who have recently evidenced that the separation of the two halves of the β chain of hemoglobin as well as myoglobin is much less pronounced when the heme is attached to His-92.

The most interesting feature of the thermodynamic analysis is that the $\Delta G°$ of unfolding determined from the acid denaturation pattern ($\Delta G° = 12.0$ kcal/mol) is higher than that determined from guanidine unfolding at neutral pH ($\Delta G^{H_2O} = 9.2$–9.7 kcal/mol) although the extent of denaturation produced by acid seems to be much lower. Moreover, the overall free energy of unfolding calculated by adding the $\Delta G°$ associated with the acid-induced transition and that associated with the further unfolding produced by guanidine at acidic pH, i.e., $\Delta G^{H_2O} = 12.0 + 5.5 = 17.5$ kcal/mol, was larger than that obtained from guanidine unfolding at neutral pH, i.e., $\Delta G^{H_2O} = 9.2$–9.7 kcal/mol. This result is quite surprising since the conformational states reached in concentrated guanidine at neutral and acidic pH are essentially the same as far as their secondary structure is concerned. Moreover, it has been reported that the pKs of ionizable groups of proteins dissolved in 6.0 M Gdn·HCl are similar to those of the low molecular weight model compounds in the same solvent, thus indicating the absence of any interactions among ionizable groups in the Gdn·HCl-denatured state (Nozaki & Tanford, 1967a,b); in fact, if such interactions occurred, they would result in anomalous pK values.

On the basis of the above considerations, we have analyzed the guanidine unfolding at neutral pH by assuming the occurrence of two independent molecular transitions which affect two structural units. We will call the native protein N_1-N_2 where N_1 and N_2 indicate the two globular structural units, the function of which is to trap the heme molecule, and assume the following denaturation pattern:



where N_1-D_2 and D_1-N_2 indicate intermediates in which one of the two structural units has been unfolded. Both N_1-D_2 and D_1-N_2 are not able to bind the heme since the heme binding site results from the interaction between the two folded units. Finally, D_1-D_2 indicates the fully unfolded protein state reached in concentrated guanidine solution. K_1 and K_2 are the equilibrium constants relative to the unfolding of the two globular units. We have assumed $K_1 = K_1'$ and $K_2 = K_2'$ since the mean solvent accessibility difference between the native and unfolded states as well as the overall transfer free energy from water to guanidine is essentially the same for the two structural units as defined by Wodak & Janin (1981); i.e., $\Delta\alpha$ is equal to 0.4 for both segments, and $\sum_i^n(\Delta G_t)_i$ is equal to 19.2 and 19.6 kcal/mol for segments 1–79 and 80–146, respectively. The dependence of CD activity on guanidine concentration at neutral pH (Figure 1B) was considered as resulting from

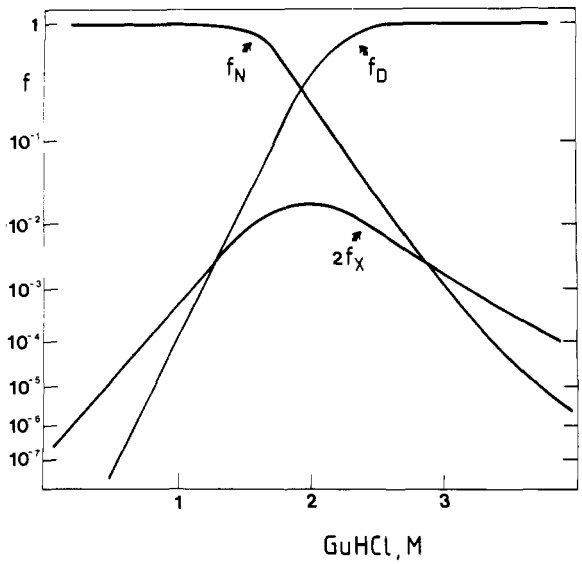


FIGURE 3: Dependence of the mole fraction of native (N), intermediate (X), and fully unfolded (D) forms of horse myoglobin on the Gdn·HCl concentration.

the two independent processes; therefore, the mean residue ellipticity experimentally observed at each denaturant concentration is given by

$$[\theta_{222}]_{obsd} = [\theta_{222}]_N f_N + 2[\theta_{222}]_X f_X + [\theta_{222}]_D f_D$$

where $[\theta_{222}]_N$, $[\theta_{222}]_X$, and $[\theta_{222}]_D$ represent the mean residue ellipticities of the native, intermediate, and denatured proteins, respectively, and f_N, f_X, and f_D represent the mole fraction of each molecular species. f_N, f_X, and f_D are related to K_1 and K_2 by the following equations:

$$f_N = \frac{1}{1 + 2K_1K_2 + K_1} \qquad 2f_X = \frac{2K_1K_2}{1 + 2K_1K_2 + K_1}$$

$$f_D = \frac{K_1}{1 + 2K_1K_2 + K_1}$$

The thermodynamic parameters were calculated by using the following expression derived from eq 2:

$$\Delta G = \Delta G_1 + \Delta G_2 = \Delta G_1^{H_2O} + \Delta G_2^{H_2O} - (\Delta n_1 + \Delta n_2)RT \ln (1 + Ka_{\pm}) \quad (5)$$

where $\Delta G_1^{H_2O}$ and $\Delta G_2^{H_2O}$ are the free energies of unfolding in the absence of denaturant of each structural unit in the native protein, i.e., N_1-N_2, and in the intermediate structure, i.e., N_1-D_2 or D_1-N_2. Δn_1 and Δn_2 are the differences between the number of denaturant binding sites of the native and denatured units, K is the binding constant of the denaturant to the protein, and $a_{\pm}$ is the mean activity of the denaturant.

The values of $\Delta G_1^{H_2O}$ and $\Delta G_2^{H_2O}$ which gave the best fit to the experimental data (Figure 1) were 10.0 and 6.0 kcal/mol, respectively. Δn_1 and Δn_2 were 30 and 32, respectively. The value of $[\theta_{222}]_X$ was similar to that found at acidic pH, thus suggesting that the secondary structure of the intermediate(s) may be quite similar to that of the acid-unfolded myoglobin.

Figure 3 shows the dependence of f_X, f_N, and f_D on Gdn·HCl concentration. The mole fractions of the intermediates, i.e., N_1-D_2 and D_1-N_2, are negligible compared to those of the native and denatured forms. This probably reflects the structural similarities between the two halves which therefore exhibit similar conformational stabilities.

The thermodynamic analysis we have carried out on the guanidine denaturation pattern at neutral pH appears to be consistent with the information provided by the acid denaturation pattern, i.e., the occurrence of two molecular events leading to the unfolding of different molecular districts. The observation that $\Delta G_1^{H_2O}$ is higher than $\Delta G_2^{H_2O}$ is reasonable since it includes the free-energy variations relative to the heme domain associations and the interdomain associations.

The development of immunological approaches to measurements of conformational equilibria (Furie et al., 1975) led to the determination of the standard free energy of unfolding of some localized regions of sperm whale myoglobin (Hurrell et al., 1976). The $\Delta G°$ values of unfolding of the N- (1–55) and C-terminal (132–153) regions were 3.9 and 3.3 kcal/mol, respectively, whereas the $\Delta G°$ of the segment 66–76—a segment of the E helix in which 5 of the 11 residues are inaccessible to solvent (Perutz et al., 1965)—was determined to be at least 8.7 kcal/mol. In this respect, it appears surprising that the overall free energy of unfolding of sperm whale myoglobin determined by Puett (1973), i.e., 13.6, from the guanidine denaturation pattern at neutral pH is lower than that determined for limited segments of the molecule. This confirms that the thermodynamic analysis of the guanidine denaturation pattern at neutral pH cannot be based on the assumption that the reaction is simply a two-state process but it has to be considered as the sum of at least two molecular events.

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Mechanism of Action of Thrombin on Fibrinogen. Kinetic Evidence for Involvement of Aspartic Acid at Position P_{10}†

Henry C. Marsh, Jr.,‡ Yvonne C. Meinwald, Theodore W. Thannhauser, and Harold A. Scheraga*

ABSTRACT: The following peptide was synthesized by classical methods in solution: Ac-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-$NHCH_3$ (F-8). The Michaelis–Menten parameters for the hydrolysis of the Arg–Gly bond in F-8 by thrombin were determined to be $k_{cat} = 31 \times 10^{-11}$ M [(NIH unit/L) s]$^{-1}$ and $K_M = 310 \times 10^{-6}$ M. Comparison of these values with those determined previously for native fibrinogen and for a series of similar synthetic peptides, together with information about the amino acid sequences of this portion of the Aα chain of abnormal fibrinogens, suggests an important role for Asp at position P_{10}. Differences in the Michaelis–Menten parameters between F-8 and the 51-residue N-terminal CNBr fragment of the Aα chain of fibrinogen correspond to only 1–2 kcal/mol in binding affinity.

From the observation that the amino acid sequence in a portion of the fibrinogens of many species is strongly conserved, Blombäck (1967) had suggested that Phe at position P_9[1] of the Aα chain of fibrinogen is essential for normal thrombin action. This suggestion was confirmed by subsequent kinetic experiments involving a series of synthetic peptide substrates (Van Nispen et al., 1977; Meinwald et al., 1980; Marsh et al., 1982). To explain the large effect of a single amino acid residue nine residues distant in the linear sequence from the site of enzyme action, it was proposed that the intervening residues might accommodate a feature such as a β-bend that would allow the Phe at position P_9 to be brought into close spatial proximity to the Arg–Gly bond that is hydrolyzed. The existence of a β-bend is supported by the high reactivity of D-Phe-Val-Arg-*p*-nitroanilide (but not the corresponding L-Phe compound) toward thrombin (Claeson et al., 1977) and by NMR observations (Rae & Scheraga, 1979) that indicate that the D-Phe residue is folded back over the Val residue but that this arrangement is not found in the L-Phe peptide.

While peptides containing Phe at P_9 (but not Asp at P_{10}) are substantially better substrates than those lacking this residue, they are poorer substrates of thrombin than even CNBr Aα, primarily because of differences in binding affinities reflected in the values of K_M. On the basis of immunochemical studies, Nagy et al. (1982) concluded that it is possible that these substrates contain all the residues that interact directly at the active site of thrombin yet lack residues that provide the long-range interactions necessary to stabilize the native conformation required for normal binding at the active site.

Direct involvement of Asp at P_{10} can be inferred from the abnormal rate of release of fibrinopeptide A from fibrinogen Lille, in which Asn replaces Asp at P_{10} (Morris et al., 1981). Furthermore, an acid residue (Asp or Glu) is present at position P_{10} in most fibrinogen species (Dayhoff, 1972, 1973, 1976). The only exceptions among the 56 reported species are Thr (for rabbit, kangaroo, and lizard), Ser (for wombat), and Gly (for slow loris).

Recognizing the importance of Asp (or Glu) at position P_{10} and its possible effect in improving the binding affinity (i.e., value of K_M) for a Phe(P_9)-containing synthetic peptide substrate, we have synthesized the peptide Ac-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-$NHCH_3$ (F-8); this sequence is identical with that of this portion of

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[1] Abbreviations: Ac, acetyl; AcONP, 4-nitrophenyl acetate; Boc, *tert*-butyloxycarbonyl; CNBr Aα, the N-terminal CNBr fragment of the Aα chain of fibrinogen; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMF, *N*,*N*′-dimethylformamide; HOAc, acetic acid; HOBT, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance; OBu^t, *tert*-butyloxy; TFA, trifluoroacetic acid; TLC, thin-layer chromatography. The abbreviations used for the amino acid residues and the notation of peptides are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (1972). The positions of residues in peptide substrates are described by the nomenclature of Schechter & Berger (1967) wherein residues on the N-terminal side of the Arg–Gly bond are designated as P_1, P_2, etc. and those on the C-terminal side are designated as P_1', P_2', etc. (see Table I).

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