Folding Domains as Functional Tools in Allosteric Systems: A Heme-Dependent Domain in Hemoglobin β Subunits†

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ABSTRACT: We have investigated the denaturation by guanidine hydrochloride (Gdn·HCl), temperature, and pH of hemoglobin β subunits and of the peptides β(1–146), β(56–146), and β(1–55). The last peptide was insensitive to all of the three agents. In the other polypeptides denaturation by Gdn·HCl and temperature showed the presence of several structural domains characterized by different stabilities. Analyses of the data obtained in Gdn·HCl indicated the presence in β subunits of an α-helical domain involving some 40 amino acids whose free energy of denaturation is only 2000 cal. This domain is heme dependent, and removal of heme in apo-β(1–146) abolishes the presence of the domain as a structural entity. Acid denaturation reveals in β subunits, apo-β(1–146), and β(56–146) the presence of buried histidines with very similar characteristics, indicating a similar tertiary structure in the three polypeptides. This suggests that removal of the heme produces an unfolding of β subunits, involving preferentially the 1–55 portion of the chain. This portion of the polypeptide contributes substantially to the formation of the $\alpha_1\beta_2$ interface in hemoglobin. The low stability of this domain implies a very small contribution to the stability of the system as a whole. Instead it makes it very sensitive to conformational attitudes of the heme, suggesting a role in the mechanism of ligand binding cooperativity and subunits interactions in hemoglobin.

The functional conformational changes of hemoglobin have been amply documented by crystallographic analyses (Perutz, 1979; Baldwin & Chothia, 1979) and explored by computer simulations (Gelin & Karplus, 1977; Kuntz, 1975). These studies have shown that concerted displacements of large groups of residues are at the basis of the R–T transition in hemoglobin. It appears that allosteric behavior is linked to the presence of structural domains capable of rearranging their position within the system.

Results emerging from several laboratories clearly indicate that in hemoglobin and in myoglobin there are structural domains with different resistance to denaturation (Puett, 1973; Puett & Hammonds, 1973; Wetlaufer, 1973; Colonna et al., 1982). These domains have been interpreted as centers of independent folding of the proteins, possibly connected with the exon–intron expression of the genes.

In systems where allosteric behavior is produced by special rearrangements of structural domains, it is possible that some of these domains have a functional significance. For example, it is well-known that removal of heme in hemoglobin and its subunits produces unfolding of the proteins. This may represent a contribution of the heme to the stability of the native structure of the system. It may also indicate a special interaction of the heme with a structural domain that amplifies and disseminates throughout the subunits information about the conformational attidues of the heme, as modified by the presence of ligands.

In order to clarify this proposition, we have studied the comparative denaturation of β subunits, apo-β(1–146), β-(56–146), and β(1–55) by Gdn·HCl,[1] temperature, and pH. The data show that in this system there is an α-helical domain with very low stability, whose conformation is regulated by the heme. Probably this domain is localized in the initial portion of the polypeptide chain. Its correlation with the heme and its role in forming the $\alpha_1\beta_2$ interface in hemoglobin support the hypothesis of a functional relevance of the domain.

Materials and Methods

Hemoglobin β subunits and the polypeptides β(1–55), β-(56–146), and apo-β(1–146) were prepared as previously described (Fronticelli-Bucci & Bucci, 1975; Fronticelli & Gold, 1976). In these preparations all of the available cysteines at β93 and β112 are carboxamidomethylated, so to prevent formation of intra- and intermolecular disulfide bonds.

The state of aggregation of these polypeptides is shown in Table I where data are collected from experiments of sedimentation velocity and equilibrium. Some of the data have been already published from this laboratory (Fronticelli-Bucci & Bucci, 1975; Fronticelli & Gold, 1976; Oton et al., 1981).

The heme-free polypeptides are self-associating systems whose average molecular weight is concentration dependent. The data presented in Table I indicate that at the concentrations used in spectropolarimetric experiments, 0.04–0.09 mg/mL, the heme-free peptides were essentially monomeric. For apo-β(1–146), the weight-average molecular weight at 0.07 mg/mL was between that of a monomer and that of a dimer. It should be remembered that weight-average molecular weights are biased in favor of the larger molecular species. Table I also shows that carboxamidomethylated β subunits were monomeric under all conditions.

Protein concentration was measured spectrophotometrically for carbon monoxy β subunits by using $\epsilon = 14000\ M^{-1}\ cm^{-1}$ at 540 nm. For the heme-free peptides quantitative amino acid analysis was used. Alternatively, for apo-β(1–146), spectrophotometric measurements were performed by using $E = 1.0$ mL mg^{-1} cm^{-1} at 280 nm, in 0.1 M NaOH.

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[1] Abbreviations: apo-β(1–146), heme-free derivative of the β subunits of hemoglobin; β(1–55), polypeptide including the first 55 residues of hemoglobin; β(56–145), polypeptide including the indicated residues of the β subunits of hemoglobin; β subunits, hemoglobin β subunits; Gdn·HCl, guanidine hydrochloride.

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Table 1: Sedimentation Data[a]

polypeptide	buffer	concn (mg/mL)	M_w from aa comp	M_w	expected monomer $s_{20,w}$	measured $s_{20,w}$
apo-β(1–146)	0.04 M borate, pH 8.9	0.07	16 000	25 300		
β(56–146)	0.04 M borate, pH 8.0	0.09	10 000	13 900		
β(1–55)	0.04 M borate, pH 8.9	0.30	6 000	7 200		
β subunits	0.04 M borate, pH 9.5	3.10	16 500		1.80	1.76

[a] Weight-average molecular weights (M_w) were estimated by sedimentation equilibrium measurements performed as previously described (Oton et al., 1981) at temperatures between 7 and 10 °C. Sedimentation velocity measurements were performed at 18 °C, using the schlieren optics. A Beckman Model E analytical ultracentrifuge was used in these experiments. All proteins and polypeptides were carboxamidomethylated at the available β93 and β112 cysteines. For the heme-free peptides the data reflect the minimum concentrations detectable in our experiments.

Titrations with Gdn·HCl were performed by diluting an 8 M solution of Gdn·HCl in 0.04 M borate buffer at pH 8.6 with the appropriate amount of polypeptide in the same buffer. The solutions were left overnight at 2–4 °C in order to reach equilibrium before the measurements. Spectra taken after 18 and 36 h were indistinguishable.

Denaturation with pH was achieved by using 0.04 M borate buffers in the alkaline and 0.02 M phosphate buffers in the neutral range of pH. Below pH 5.0 the solutions of phosphate buffers were adjusted to pH with 0.1–0.2 M HCl. Measurements were performed 2 h after a constant pH was reached in the solutions; for the next several hours we could not detect any drift with time in the CD spectra. When solutions of β subunits were titrated below pH 5.0, the pH drifted to about 3.3. Therefore, for this protein data could not be collected in this pH interval.

Temperature denaturations were performed in 0.04 M borate buffer at pH 8.6 either by following the optical properties of polypeptide solutions whose temperature was continuously increased at the rate of 1–2 °C/min or by raising the temperature in steps of 5 °C and waiting 20 min before taking a full spectrum at each temperature. The two procedures gave identical results. The two kinds of data will not be identified in the rest of this presentation. Water-jacketed cuvettes were used, and temperature was monitored by using a blank-jacketed cuvette filled with buffer, heated by water emerging from the sample cuvette. The probe of a digital thermometer (Digitek 5810) was immersed in the middle of the blank cuvette. Controls indicated that the temperature in the two cuvettes was the same at all temperatures.

The relative amounts of secondary structure of the various polypeptides was estimated as proposed by Chen et al. (1974) as already described (Fronticelli-Bucci & Bucci, 1975).

Spectral grade Gdn·HCl was obtained from Heico Inc. All other reagents were reagent grade or better. The suppliers were Fisher, Aldrich, and Sigma.

A Cary 14 spectrophotometer and a Jasco-20 spectropolarimeter were used in the various experiments.

Theory

Treatment of the Data for Multistep Denaturation in Gdn·HCl. Following the treatment developed by Aune & Tanford (1969) for a two-state denaturation process based on the assumption that Gdn·HCl binds to denaturing peptides, we can write

$$K_{app} = \frac{D}{N} = K^0 \prod_i \left(\frac{1 + K_{d,i}a}{1 + K_{n,i}a} \right) \quad (1)$$

where K_{app} is the denatured (D) to native (N) equilibrium constant of the peptide, K^0 is the equilibrium constant in the absence of denaturant, $K_{d,i}$ and $K_{n,i}$ are the affinity constants of each group i for Gdn·HCl in the unfolded and folded peptide, respectively, and a is the activity in solution of Gdn·HCl.

The groups for which $K_d = K_n$ disappear from eq 1, which remains defined only by those "critical" groups for which $K_d \neq K_n$. Also, the relatively low values of the affinity constants allow us to rewrite eq 1 as

$$K_{app} = K^0 \left(\frac{1 + \bar{K}_d a}{1 + \bar{K}_n a} \right)^m \quad (2)$$

where m is the number of critical groups and $\bar{K}_d$ and $\bar{K}_n$ are the average affinity of the groups for the denaturant in the unfolded and native peptide, respectively.

When intermediates are present and the denaturation is not a two-state process, we have

$$K_{app} = \frac{\sum_i D_i}{N} \quad (3)$$

where D_i is the concentrations of the ith form of denaturation. Each of the D_i's is in equilibrium with all of the other forms, including N; therefore several equilibrium constants can be defined by

$$K^s_{app} = K^s \left(\frac{D_s}{N + \sum_{i \neq s} D_i} \right) \quad (4)$$

This equation applies to the case where a multistep denaturation shows the presence of a predominant specie being titrated at each step. Thus eq 2 becomes

$$K^s_{app} = K^s \left(\frac{1 + \bar{K}_d a}{1 + \bar{K}_n a} \right)^m \quad (5)$$

where K^s is an average equilibrium constant for the transformation of D_s into all of the other species. When the denaturation process is resolved into steps involving different ranges of denaturant concentration, the meaning of K^s becomes uncertain because it includes a denaturant activity difficult to evaluate. The approximate free energy change produced by the titration of the D_s specie can be estimated from

$$\Delta G_s = -mRT \ln \frac{\bar{K}_d}{\bar{K}_n} \quad (6)$$

and the total energy of denaturation up to the step s is the sum

$$\Delta G_t = \sum_s \Delta G_s \quad (7)$$

Results

Denaturation by Gdn·HCl. Figure 1 shows the denaturation in Gdn·HCl of β subunits, β(1–55), β(56–146), and apo-β-(1–146). The titration with the denaturant was followed, recording the CD spectrum between 250 and 220 nm. For β subunits the presence of heme allowed also the monitoring

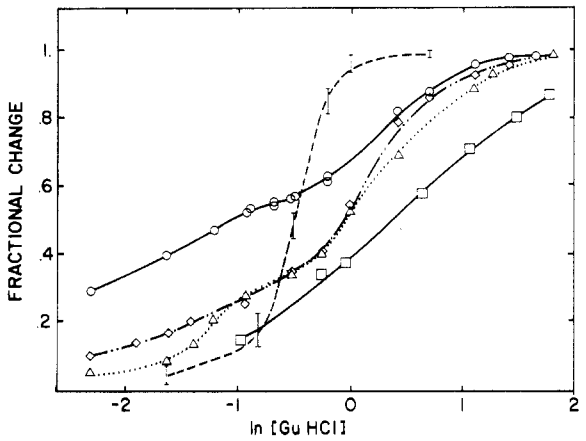

FIGURE 1: Denaturation in Gdn·HCl followed by CD at 222 nm of (○) β subunits, (◇) β(1–146), (△) β(56–146), and (□) β(1–55). The vertical bars indicate the variability of the data obtained for the denaturation of the heme pocket of the β subunits monitored by CD at 420 and 270 nm and by OD at 420 nm. Protein concentration was in the range 0.04–0.08 mg/mL, for CD measurements at 222 nm, and near 0.075 mg/mL, for OD measurements in the Soret region. In 0.04 M borate buffer at pH 8.6, 23 °C.

of the titration either by recording the CD spectrum between 300 and 250 nm, or by recording the OD and CD spectra between 450 and 330 nm. The data obtained by measurements in the near-UV and Soret regions of the spectrum were indistinguishable.

In this way the variation of the secondary structure was followed by CD in the far-UV region of the spectrum, while the conformation of the heme pocket, which is representative of the tertiary structure, was monitored by OD in the Soret region and by CD in the Soret and near-UV regions of the spectrum.

In the far-UV region of the spectrum the spectropolarimetric titrations of the heme-free polypeptides were completely reversible, as proven by dilution or dialysis of the samples. For β subunits the titrations in the far-UV region of the spectrum were reversible only from the first unfolding step.

Titration of the heme pocket of β subunits followed by CD and OD in the near-UV and Soret regions of the spectrum were reversible up to at least 80% of the unfolding. The spectra taken at various steps of the titrations showed the presence of isosbestic points, suggesting a limited heterogeneity of the molecular species produced by the denaturant.

Figure 1 shows that the denaturation of the secondary structure of β subunits, apo-β(1–146), and β(56–146) occurred in two distinct steps. The relative amplitude of the first step was largest in β subunits. For β subunits a third denaturation step was represented by the unfolding of the heme pocket which fell in the middle between the two steps of unfolding of the secondary structure. This implies that the species titrated in the final step of the curves at high Gdn·HCl concentrations did not contain the structure of the heme pocket.

Equation 5 was used to fit the data as shown in Figure 2. The activity of Gdn·HCl was calculated from its molar concentration as suggested by Aune & Tanford (1969).

Table II lists the values of the various parameters at each step of the denaturation. For β subunits an intermediate Soret step is there included after the first step observable in Gdn·HCl. Instead the data obtained for the denaturation at high Gdn·HCl concentration are omitted, because the denaturation was not reversible.

Crystallographic analyses (Fermi, 1975) and spectropolarimetric data (see Figures 9 and 10) concur in indicating the absence of β conformation in the structure of hemoglobin.

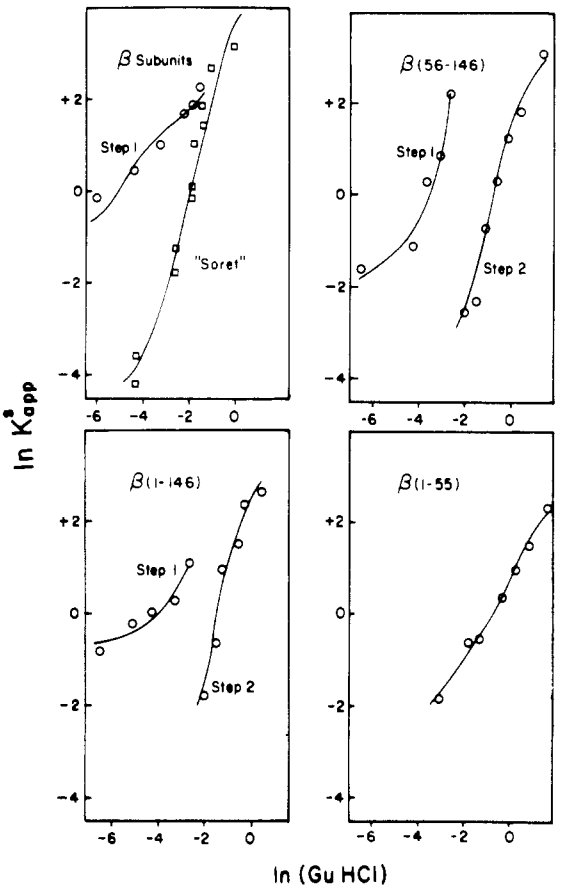

FIGURE 2: Computer simulations of the denaturation steps of the various polypeptides. Equation 5 was used with the iterative procedure developed by Marquardt (1963). The continuous lines were obtained with the parameters listed in Table II.

Table II: Denaturation of Apo-β Subunits, β(1–146), β(56–146), and β(1–55) by Gdn·HCl[a]

polypeptide	m	$\bar{K}_n$ (M)	$\bar{K}_d/\bar{K}_n$	ΔG_s (kcal)	ΔG_t (kcal)
β subunits					
step 1	5.2	33.00	1.9	−1.9	−1.9
Soret	2.8	0.70	54.0	−6.5	−8.4
apo-β(1–146)					
step 1	7.9	3.1	2.3	−3.8	−3.8
step 2	3.2	1.0	105.0	−8.6	−12.4
β(56–146)					
step 1	8.6	4.0	3.9	−6.9	−6.9
step 2	2.9	0.9	116.0	−8.0	−14.9
β(1–55)					
step 1	1.4	0.18	47.0	−3.1	−3.1

[a] The various parameters were evaluated by using eq 5 and the iterative procedure of Marquardt (1963).

Therefore the first step of unfolding of the secondary structure of β subunits, shown in Figure 1, involved a domain including about 40% of the total helical content of the protein. As shown in Table II this domain was characterized by a relatively high affinity for the denaturant of the critical groups in the native form of the peptide. Table II shows that this domain had a stability of only 1900 cal.

Figures 9 and 10 show that removal of heme produced about a 40% decrease of the helical content of the system, and Figure 1 shows that the structural domain which unfolded at low Gdn·HCl concentrations in β subunits is almost absent in apo-β(1–146) and in β(56–146). This suggests that the helical structure stabilized by the presence of heme is the domain which is first denatured in β subunits.

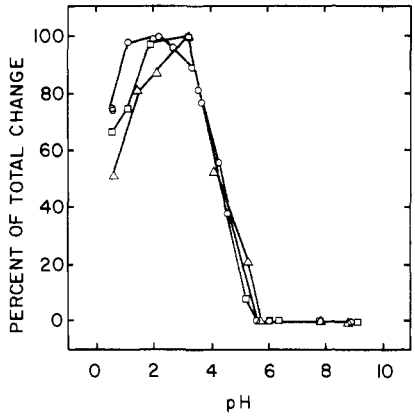

FIGURE 3: Denaturation with pH of (□) β subunit, (△) apo-β(1–146), and (○) β(56–146). Denaturation of the β subunits was followed spectrophotometrically at 420 nm. For the other two polypeptides it was monitored by CD at 222 nm. Protein concentration was 0.077 mg/mL for OD measurements and in the range 0.05–0.09 mg/mL for CD measurements. In either 0.04 M borate or 0.02 M phosphate buffers, at 23 °C.

Notably removal of the first 55 residues in β(56–146) did not modify much the helical content of the polypeptide (Figures 9 and 10). Also, denaturation in Gdn·HCl was very similar in β(56–146) and apo-β(1–146) (Figure 1).

The behavior of β(1–55) is also of interest. As reported by Fronticelli & Gold (1976) the peptide contains only 10% helical structure and 5% β conformation. Nevertheless it denatured only at high concentrations of Gdn·HCl. Analysis of the denaturation parameters, shown in Table II, indicates that the energy of denaturation of the polypeptide was low, near 3000 cal. This seems to exclude the presence of a stable folded structure, and the resistance of the polypeptide to Gdn·HCl finds its explanation in the low affinity for the denaturant of the critical groups. This suggests that the residual structure of the polypeptide in the absence of Gdn·HCl is produced by the special geometry of a few residues. Notably in β(1–55) there are two prolines, which produce bends in polypeptides and have a low affinity for Gdn·HCl (Pace, 1975). The average number of critical groups in β(1–55) is 1.4 (Table II).

Denaturation by pH. The denaturation with pH was followed spectrophotometrically at 420 nm for β subunits and spectropolarimetrically at 222 nm for apo-β(1–46), β(1–55), and β(56–146).

As mentioned above, in the transition region the titration of β subunits was time dependent, so that only a few points at the two extremes of the transition could be collected. Also, there were distinct changes of the isosbestic points in the absorption spectra, and the titrations were irreversible. Changes in the valence of iron at acid pH probably contributed to these phenomena.

The titrations of apo-β(1–146) and of β(56–146) were reversible and superimposable. The few data points obtained for the β subunits indicated that the transition occurred in the same range of pH. This is shown in Figure 3.

Figure 3 also shows that the titrations of the peptides were in part reversed below pH 3.0. This suggests that at high concentrations of inorganic acids the polypeptides bind negative ions, quenching their positive charges and inhibiting the expansion of the molecules and the unfolding due to electrostatic repulsion. This helped in reforming some secondary structure in the systems. Whether this represented a recovery of the original structure or the formation of a new folding is impossible to tell.

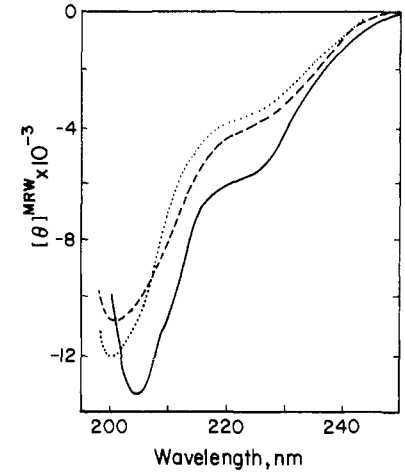

FIGURE 4: Circular dichroism spectra of the residual structures at the maximum of pH denaturation of (—) β subunits at pH 3.3, (···) apo-β(1–146) at pH 3.3, and (- - -) β(56–146) at pH 2.5 at 23 °C.

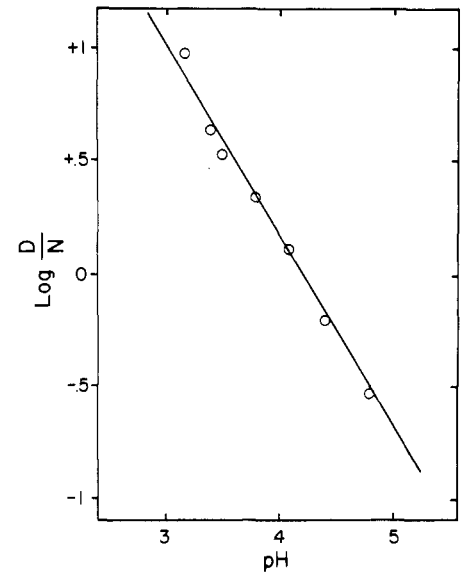

FIGURE 5: Hill plot of the data shown in Figure 3. The slope of the line is 0.86. The intercept on the pH axis is very near 4.2.

Figure 4 shows the CD spectra of acid-denatured β subunits, apo-β(1–146), and β(56–146). The spectra were taken at the pH of maximum denaturation (between 2.5 and 3.5 as shown in Figure 3) and indicated that the unfolding was not complete. Figure 9 shows the relative amounts of secondary and nonordered structures at the pH of maximum denaturation.

The pK of the groups involved in the denaturation was evaluated by making a Hill plot of all the data available in the region of the main conformational transition, between pH 3 and 5. The plot is shown in Figure 5, and the intercept on the abscissa gives a pK = 4.2 for the groups involved. The plot is linear with a slope of 0.86. This value of the slope, slightly less than 1.0, may result from small pK variations of the groups in the various polypeptides and from electrostatic interactions. The data suggest a homogeneous class of ionizable groups to be responsible for the denaturation of the polypeptides.

Admittedly, because of pH drifts, the denaturation curve of β subunits, as shown in Figure 3, is ill-defined. Still the data show either that it was simultaneous to that of the secondary structure of apo-β(1–146) and β(56–146) or that it occurred at higher pH values.

This observation contains most relevant and unexpected

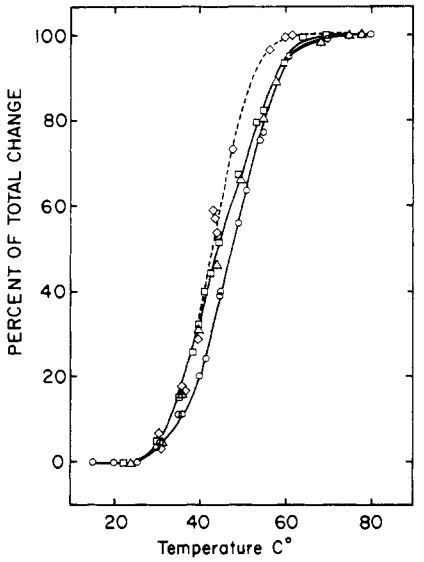

FIGURE 6: Temperature denaturation of (○) β subunits, (□) apo-β-(1–146), and (△) β(56–146) monitored by CD at 222 nm. (◇) Denaturation of β subunits monitored by OD at 420 nm and by CD at 420 nm; at 260 nm, the three different procedures are not identified in the graph. Protein concentration was 0.082 mg/mL for CD measurements at 222 nm and 0.050 mg/mL for the OD and CD measurements. In 0.04 M borate buffer at pH 8.6.

information. In fact, it implies that the ionization characteristics of the critical groups were very similar in β subunits, apo-β(1–146), and β(56–146). It has to be stressed that the ionization characteristics of the critical groups are an expression of the tertiary structure of polypeptides and that their pK shifts upon unfolding are defined by the stability of the structures. Therefore it appears that the tertiary structures of the heme-free peptides β(1–146) and β(56–146) were similar to that of β subunits and at least equally stable. If the denaturation curve for β subunits was displaced at higher pH values, it would in fact indicate a lower stability of the chains. It must be stressed that this reasoning remains valid whatever is the type of groups titrated in Figure 3, which produce the unfolding of the polypeptides.

All carboxyl groups of native β subunits are titratable (Bucci et al., 1968; Scholberg et al., 1980); therefore the critical groups are very likely the buried histidines whose protonation produces the acid denaturation of hemoglobin systems (Steinhardt et al., 1962; Steinhardt & Hiremath, 1967; Puett, 1973).

It should be stressed that in β(56–146) only the histidine at β2 is missing, which is titratable in the native structure of β subunits.

The number of buried histidines in β subunits is two (Bucci et al., 1968; Scholberg et al., 1980). Assuming that in their unburied condition these residues have a pK near 6, their apparent pK = 4.2, estimated from the denaturation curves, produces a free energy change upon unfolding of approximately 6000 cal. This value is smaller than the total energy of denaturation obtained for the denaturation in Gdn·HCl of any of the three polypeptides. It is consistent with the presence of residual structures in the acid-denatured polypeptides, which were unfolded only by Gdn·HCl. The stability to pH of the three polypeptides compared well with that of the intermediate Soret step of β subunits in Gdn·HCl, listed in Table II.

The residual structures of all polypeptides could be unfolded by addition of Gdn·HCl, and not by temperature.

The CD spectrum of β(1–55) was totally insensitive to pH in the range used for the other polypeptides, as already reported (Fronticelli & Gold, 1976).

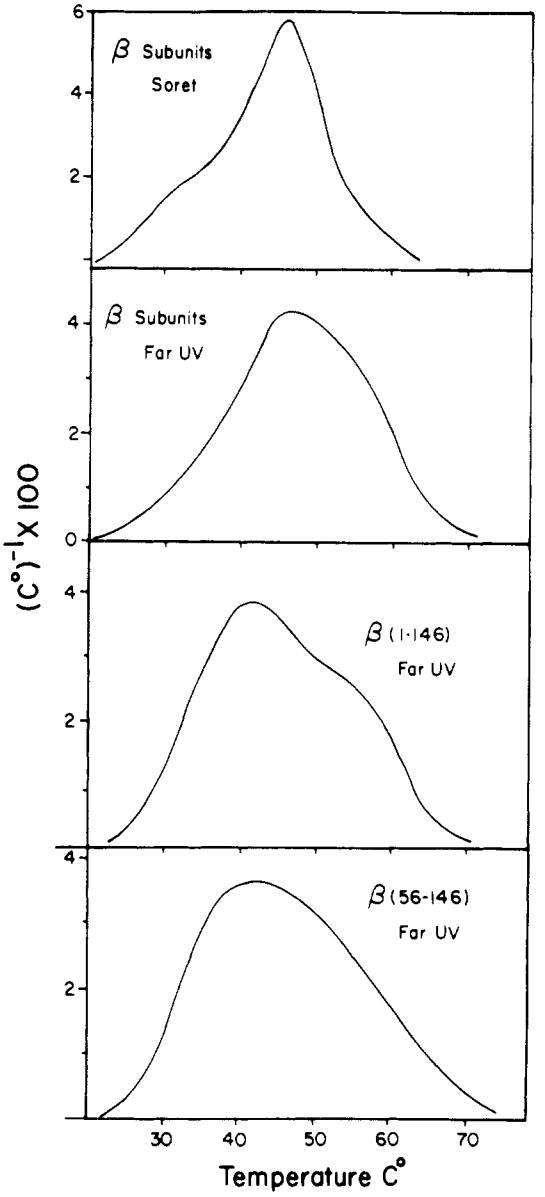

FIGURE 7: Derivative curves computed from the titrations in Figure 6.

Denaturation by Temperature. Figure 6 shows the temperature denaturation curves obtained for β subunits, β(1–146), and β(56–146) in the UV, near-UV, and Soret regions of the spectra. The denaturation was reversible for the heme-free peptides, while for β subunits it was so only for the initial stages of the unfolding. The curves obtained for apo-β(1–146) and β(56–146) were in practice superimposable, suggesting a similar structure in the two polypeptides.

Figure 7 shows the derivative curves computed from the titrations in Figure 6. Their marked asymmetry and presence of shoulders can be taken as evidence for the presence of structural domains with different stabilities.

The maximum unfolding obtained with temperature in β subunits, apo-β(1–146) and β(56–146) left a residual structure whose content of α helix and β conformation is shown in Figure 9. As an example, Figure 8 shows the denaturation of β(56–146), its further unfolding with Gdn·HCl, and its reversibility between 22 and 75 °C.

The unfolding of the heme pocket of β subunits occurred at a lower temperature than the unfolding of the secondary structure. Therefore also in this case the residual structure

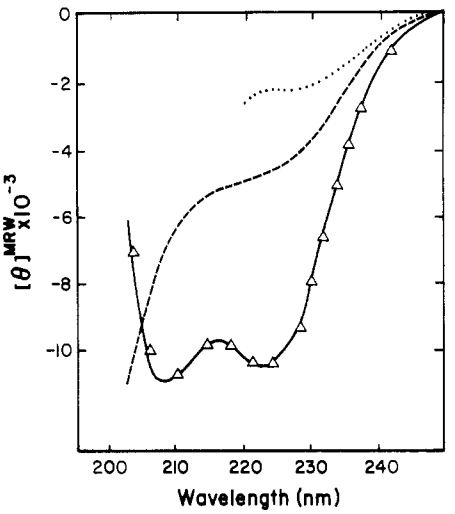

FIGURE 8: Circular dichroism spectra of β(56–146) (—) at 23 °C, (---) at 75 °C, and (···) at 60 °C in 6 M Gdn·HCl. The open triangles show the reversibility of the denaturation from 75 °C.

did not contain the conformation of the heme pocket. The residual structures could be unfolded by Gdn·HCl and not by acid pH.

The CD spectrum in the far-UV region of β(1–55) was totally insensitive to temperature variations in the range investigated for the other polypeptides.

Discussion

A variety of structural domains characterized by different stabilities is evident in all the denaturation processes here investigated.

The presence of residual structures after pH and temperature denaturations has been reported also for other systems (Aune et al., 1967). As mentioned above the residual structures here obtained contained little tertiary structure. This may be consistent with the hypothesis of Levinthal (1968) that the early stages of protein folding are dominated by short-range interactions resulting in secondary structures.

Of the various structural domains apparent in this investigation, the heme-dependent domain was the most amenable to characterization and most interesting.

As shown in Figures 9 and 10, removal of heme produced a loss of about 40% of the original α-helical content of the system. Comparison of the denaturation in Gdn·HCl of β subunits and β(1–146) (Figure 1) proves that this structural domain was largely included in the very initial step of denaturation of β subunits and was stabilized by a mere 1900 cal.

A stability of 1900 cal appears very low considering that the domain in question was essentially α helical and included some 40 amino acids. It may be argued that heterogeneity of the denatured species lowers the values of the estimated free energy changes (Pace, 1975). Still the free energy change was low because of the low Gdn·HCl concentrations at which denaturation occurred.

This entire domain seemed to have the stability of only a few hydrogen bonds. It may be explained only by rapid fluctuations in solution of the domain in and out of its helices. This is consistent with results obtained by Englander & Rolfe (1973) and by the hypothesis of Cooper (1976) on the conformational fluctuation of proteins in solution.

Additional notions on the function and possible localization of this structural domain arise from the following observations.

In all of the media tested the denaturation curves of apo-β(1–146) and β(56–146) were practically superimposable.

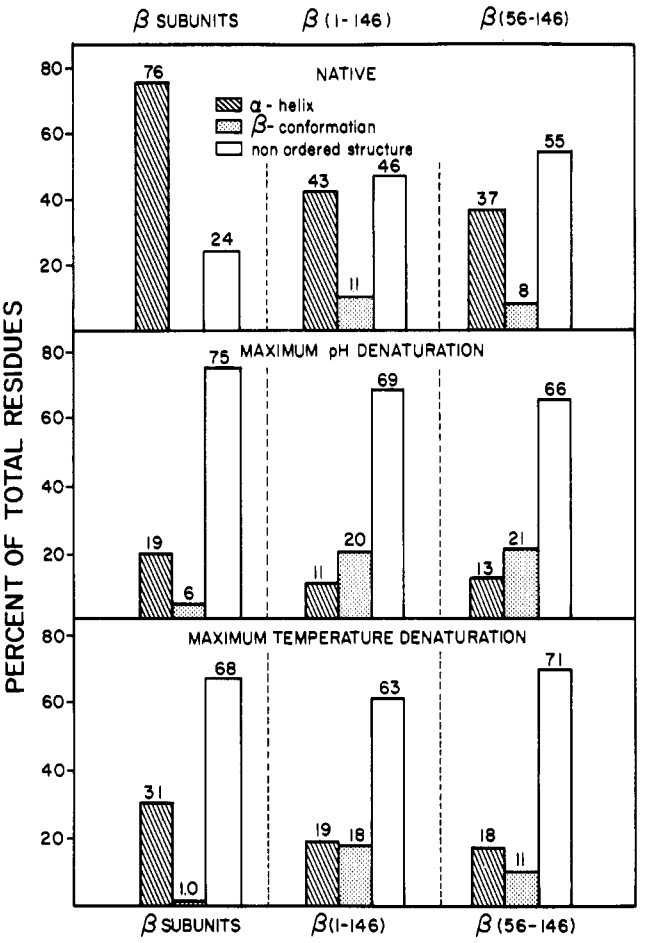

FIGURE 9: Relative percentage of α helix, β conformation, and nonordered structure in β subunits, apo-β(1–146), and β(56–146).

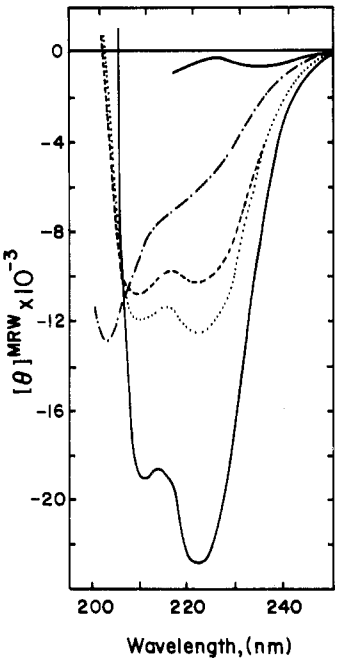

FIGURE 10: Circular dichroism spectra of (—) β subunits, (···) apo-β(1–146), (---) β(56–146), and (-·-) β(1–55). Upper continuous line, any of the four polypeptides in 8 M Gdn·HCl.

Also, the presence of buried histidines with similar characteristics indicated that they had a tertiary structure similar to and as stable as that of β subunits. This can be explained only by assuming that the first 55 amino acids of apo-β(1–146)

are part of an independent domain of structure and suggests that removal of heme produce an unfolding involving preferentially this domain.

A localized destabilization of β subunits upon removal of heme is consistent with data showing little modification of molecular distances in apo-β(1–146) (Oton et al., 1981) and in apohemoglobin (Kowalczyk & Bucci, 1982) as compared to those measurable in the model of crystalline hemoglobin.

The presence of an independent center of folding in the initial portion of β subunits is also consistent with model analyses of Wetlaufer (1973) and with the observation that the first exon of β subunits involves the first 30 residues (Konkel et al., 1979). Recent results in the field (Jensen et al., 1981) may also suggest that a second exon can be construed involving the 31–68 portion of the polypeptide chain.

As shown by eq 7, the low stability of the heme-dependent domain implies a limited contribution to the stability of the system as a whole. It suggests a functional relevance of the domain. In fact the low stability makes it very sensitive to small impulses, like repositioning of the heme when it binds ligands (Perutz, 1979; Gelin & Karplus, 1977; Baldwin & Chothia, 1979). The interaction of the heme with this domain may represent a mechanism for disseminating throughout the subunits information in regard to the state of ligation of the heme.

It is worth considering possible correlations between this heme-dependent domain and the allosteric domains detected in experiments of tritium exchange (Lien et al., 1980; Malin & Englander, 1980) and by probing the dissociability of the hemoglobin tetramer (Pettigrew et al., 1982).

In both cases the structural domains appeared highly localized. The heme-dependent domain is also localized, although in our case it appears to be larger than those studied in the laboratories of Englander and Ackers (Lien et al., 1980; Malin & Englander, 1980; Pettigrew et al., 1982).

It is worth stressing that the localization claimed by both authors involves multiple loci. The extensive contacts of heme with many different parts of the protein molecule may suggest that the heme-dependent domain includes several of the domains explored by tritium exchange and hemoglobin dissociation. It is interesting to note that most of the domains evident in tritium exchange experiments had an allosteric free energy near 2000 cal, irrespective of the size of the domains.

The heme-dependent domain here investigated may provide communication between the $\alpha_1\beta_2$ interface and the heme. In fact the first 50 residues of β subunits participate to the formation of the $\alpha_1\beta_2$ interface with 7 out of the 12 residues contributed by β subunits. They include the many contacts produced by the tryptophan at β37 and by the arginine at β40. Destabilization of this portion of the polypeptide chain is consistent with the dissociation of apohemoglobin into $\alpha_1\beta_1$ dimers.

The presence of structural domains with different stabilities in myoglobin (Colonna et al., 1982; Irace et al., 1981; Puett, 1973) may be taken as a suggestion that centers of independent folding in the system have evolved into allosteric functional domains in hemoglobin subunits. Their stability is low, making them sensitive to low energy impulses. Still the folding correlation within the domains allows them to transform minuscule events in the 100-dalton range into conformational changes involving masses several thousand times larger.

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