Lajzerowicz-Bonneteau, J. (1976) in *Spin Labeling: Theory and Applications* (Berliner, L. J., Ed.) pp 239–249, Academic Press, New York.
Latruffe, N., Brenner, S. C., & Fleischer, S. (1980) *Biochemistry 19*, 5285–5290.
Leigh, J. S., Jr. (1970) *J. Chem. Phys. 52*, 2608–2612.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Maurer, A., McIntyre, J. O., Churchill, S., & Fleischer, S. (1985) *J. Biol. Chem. 260*, 1661–1669.
McIntyre, J. O., Bock, H.-G. O., & Fleischer, S. (1978) *Biochim. Biophys. Acta 513*, 255–267.
McIntyre, J. O., Wang, C.-T., & Fleischer, S. (1979) *J. Biol. Chem. 254*, 5199–5207.
McIntyre, J. O., Churchill, P., Maurer, A., Berensky, C., Jung, C. Y., & Fleischer, S. (1983) *J. Biol. Chem. 258*, 953–959.
McIntyre, J. O., Fleer, E. A. M., & Fleischer, S. (1984a) *Biochemistry 23*, 5135–5141.
McIntyre, J. O., Robinson, B. H., & Fleischer, S. (1984b) *Biophys. J. 45*, 238a.
Mehlhorn, R. J., & Keith, A. D. (1972) in *Membrane Molecular Biology* (Fox, C. F., & Keith, A. D., Eds.) pp 192–227, Sinauer Associates, Stanford, CT.
Nielsen, N. C., Zahler, W. L., & Fleischer, S. (1973) *J. Biol. Chem. 248*, 2556–2562.
Pake, G. E. (1962) *Paramagnetic Resonance*, pp 12–13, W. A. Benjamin, New York.
Pearson, R. H., & Pascher, I. (1979) *Nature (London) 281*, 499–501.
Poole, C. P., Jr. (1983) *Electron Spin Reonance: A Comprehensive Treatise on Experimental Techniques*, Wiley-Interscience, New York.
Robinson, B. H., & Dalton, L. R. (1980) *J. Chem. Phys. 72*, 1312–1324.
Rossman, M. G., Liljas, A., Branden, C.-I., & Banaszak, L. J. (1975) in *The Enzymes* (Boyer, P. D., Ed.) pp 61–102, Academic Press, New York.
Rouser, G., Kritchevsky, G., & Yamamoto, A. (1967) in *Lipid Chromatographic Analysis* (Marinetti, G. V., Ed.) Vol. 1, pp 99–162, Marcel Dekker, New York.
Seelig, J., & Seelig, A. (1980) *Q. Rev. Biophys. 13*, 19–61.
Seigneuret, M., Davoust, J., Herve, P., & Devaux, P. F. (1981) *Biochimie 63*, 867–870.
Taylor, J. S., Leigh, J. S., Jr., & Cohn, M. (1969) *Proc. Natl. Acad. Sci. U.S.A. 64*, 219–226.
Taylor, M. G., & Smith, I. C. P. (1981) *Chem. Phys. Lipids 28*, 119–136.
Thomas, D. D., Dalton, L. R., & Hyde, J. S. (1976) *J. Chem. Phys. 65*, 3006–3024.
Wagner, S., Keith, A., & Snipes, W. (1980) *Biochim. Biophys. Acta 600*, 367–375.
Weinstein, S., Wallace, B. A., Morrow, J. S., & Veatch, W. R. (1980) *J. Mol. Biol. 143*, 1–19.

Unfolding Pathway of Myoglobin: Effect of Denaturants on Solvent Accessibility to Tyrosyl Residues Detected by Second-Derivative Spectroscopy

Raffaele Ragone, Giovanni Colonna, Ettore Bismuto, and Gaetano Irace*

Cattedra di Chimica e Propedeutica Biochimica, Istituto di Chimica e Chimica Biologica, I[a] Facoltà di Medicina e Chirurgia, Università di Napoli, 80138 Napoli, Italy

Received April 21, 1986; Revised Manuscript Received September 30, 1986

ABSTRACT: The effects of denaturants on the solvent accessibility to tyrosyl residues of apomyoglobin have been examined by means of second-derivative spectroscopy in the near-ultraviolet. Three apomyoglobins, i.e., sperm whale, horse, and tuna, were selected because of the different distribution of tyrosyl residues in their primary structure. The results are consistent with the occurrence of two independent consecutive events in the guanidine-induced denaturation pattern of apomyoglobin. The first event, which is responsible for the lack of the ability to bind the heme, has been proved to involve conformational changes in both the domains, i.e., segments 1–79 and 80–153, identified in the myoglobin molecule. However, the conformational changes are not of the same type. In fact, the solvent accessibility to tyrosine HC2 is increased probably because of a partial unfolding of the 80–153 domain. Conversely, the solvent accessibility to tyrosine B2 is decreased, thus indicating that a refolding occurs in some region of the N-terminal moiety (1–79 domain) of the molecule.

The absorption of tyrosyl residues in proteins is known to be largely masked by the stronger absorption of tryptophanyl residues (Wetlaufer, 1962). Althouth it has been proved that second-derivative spectra may contribute to the resolution of overlapping bands, the mutual interference between the two chromophores could not be eliminated at all by this technique (Balestrieri et al., 1978a, 1980). More recently, the overlap between the absorption bands of tyrosine and tryptophan has been utilized for detecting the degree of exposure of tyrosyl residues in proteins as well as for the simultaneous determination of the two residues (Ragone et al., 1984; Servillo et al., 1982).

The data reported in this paper show the effects of two denaturants, i.e., guanidine hydrochloride and acid, on the second-derivative spectra of three apomyoglobins, i.e., tuna, horse, and sperm whale, selected because of the different distribution of tyrosyl residues in their primary structure. This finding made it possible to analyze structural changes oc-

* Address correspondence to this author.

0006-2960/87/0426-2130$01.50/0

curring in different molecular areas by determining the degree of solvent accessibility to tyrosyl residues under different experimental conditions.

Acids are known to unfold the heme binding site but not the N-terminal region of the molecule, whose structure seems to become more compact at acidic pH (Colonna et al., 1982).

The guanidine-induced denaturation pattern at neutral pH has been found to involve, at low denaturant concentration, a molecular intermediate, whose properties resemble those possessed by the acid-denatured form of the protein (Irace et al., 1986). The characterization of the two partially unfolded forms indicated that acids disorganize the 80–153 domain (Wodak & Janin, 1981) to a greater extent than that induced by low guanidine concentration (Irace et al., 1986). On the contrary, the structure of the 1–79 domain (Wodak & Janin, 1981) appears to be quite similar in the two molecular forms.

The results reported in this paper further confirm the occurrence of two independent consecutive events in the guanidine-induced denaturation pattern of apomyoglobin. The first event, which is responsible for the lack of the ability to bind the heme, has been proved to produce conformational changes involving the HC segment and the B helix.

Materials and Methods

Sperm whale and horse heart myoglobins were purchased from Sigma Chemical Co. Bluefin tuna myoglobin was prepared according to the methods previously described (Balestrieri et al., 1973, 1978b). Apomyoglobins were prepared by the 2-butanone method described by Teale (1959). In all cases no significant contamination of the apoprotein by myoglobin was observed in the Soret region of the spectrum. The molar absorptivity at 280 nm of sperm whale, horse, and tuna apomyoglobin was calculated from the tryptophanyl and tyrosyl content, according to Wetlaufer (1962). In every case the absorption was not less than 0.4 OD at 280 nm.

All common chemicals were reagent-grade and were purchased from British Drug Houses. Ultrapure Gdn-HCl[1] was obtained from Schwarz/Mann Laboratories.

The solvent accessibility to tyrosyl residues was determined as described by Ragone et al. (1984). Second-derivative spectra were performed on a Perkin-Elmer Model 575 spectrophotometer equipped with an electronic derivative accessory (Hitachi 200-0507 derivative spectrum unit), with the instrumental conditions described elsewhere (Balestrieri et al., 1980).

Acid titrations were performed by addition of small amounts of concentrated HCl to buffered solutions from an Agla syringe while the solutions were stirred magnetically. Measurements of pH were carried out by using a Radiometer Model 26 pH meter. In denaturation experiments, the apoproteins were added to buffered solutions of Gdn-HCl in the presence of 0.1 M KCl. The second-derivative measurements were then followed in time until an apparent equilibrium was reached.

Results

The mutual interference between the second-derivative bands of tyrosine and tryptophan, evaluated in terms of ratio between two peak-to-peak distances, has been found dependent on the polarity of the medium in which tyrosyl residues are embedded (Ragone et al., 1984, 1985).

[1] Abbreviations: Gdn-HCl, guanidine hydrochloride; ANS, 1-anilino-8-naphthalenesulfonic acid; 1,5-AEDANS, *N*-[(acetylamino)-ethyl]-5-naphthylamine-1-sulfonic acid; TNS, 2-*p*-toluidinyl-6-naphthalenesulfonic acid.

Table I: Tyrosyl Distribution in Apomyoglobins of Different Origin

apomyoglobin	tyrosyl distribution			
sperm whale		G-4 (103)	H-23 (146)	HC-2 (151)
horse		G-4 (103)	H-23 (146)	
bluefin tuna	B-2 (21)		H-23 (146)	

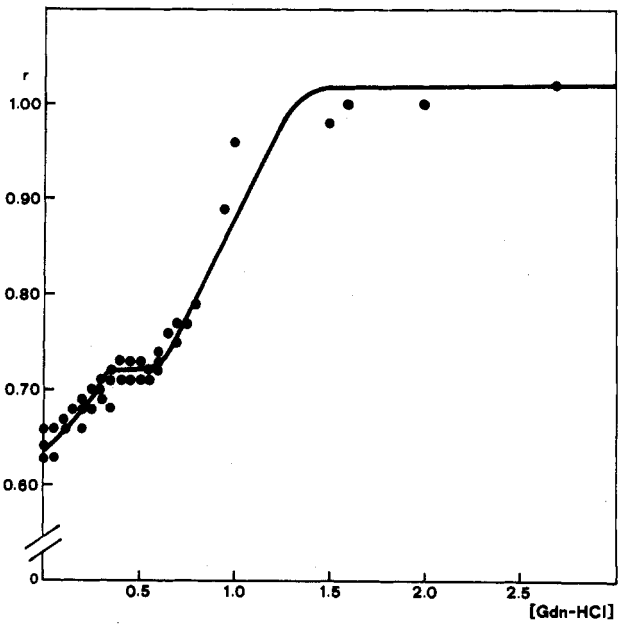

FIGURE 1: Effect of increasing concentration of Gdn-HCl on derivative ratio of sperm whale apomyoglobin. Protein concentration was 4×10^{-5} M; solvent was 0.05 M phosphate–0.15 M KCl, pH 7.0, 25 °C.

We have examined the effect produced by increasing concentrations of Gdn-HCl on the derivative ratio of three apomyoglobins that possess tyrosyl residues differently located in their covalent structure (Dayhoff, 1972; Watts et al., 1980). Table I shows the position of phenolic chromophores in the three examined proteins.

Denaturation Pattern of Sperm Whale Apomyoglobin. The denaturation pattern of sperm whale apomyoglobin induced by Gdn-HCl at neutral pH is shown in Figure 1. The value of the derivative ratio r in the absence of denaturant provides an average exposure of the tyrosyl residues to the solvent of about 38%. The derivative ratio increases, following a complex nonsigmoidal pattern, when guanidine is added to the protein. This pattern reveals the occurrence of at least two consecutive molecular events (Servillo et al., 1980).

The conformational change observed at low denaturant concentration may either involve the overall protein molecule or reflect a microenvironmental variation of a specific tyrosyl residue. However, the most important observation is that the conformational state generated by low denaturant concentration may undergo further structural changes. Recently, Irace et al. (1986) have demonstrated that partially unfolded conformations of apomyoglobin are generated when the heme binding site is destroyed by acid or low guanidine concentration. The main structural feature of these molecular states is the presence of a residual secondary as well as tertiary structure, which can be destroyed by exposing the protein to a high concentration of denaturant (Irace et al., 1986). The results reported in this section confirm the occurrence of consecutive molecular events involved in the guanidine-induced unfolding of apomyoglobin. Moreover, the increase of the derivative ratio observed at low denaturant concentration indicates that the disarrayment of the heme binding site is concomitant with a conformational change affecting the tyrosyl microenvironments. Since the tyrosyl residues of sperm whale

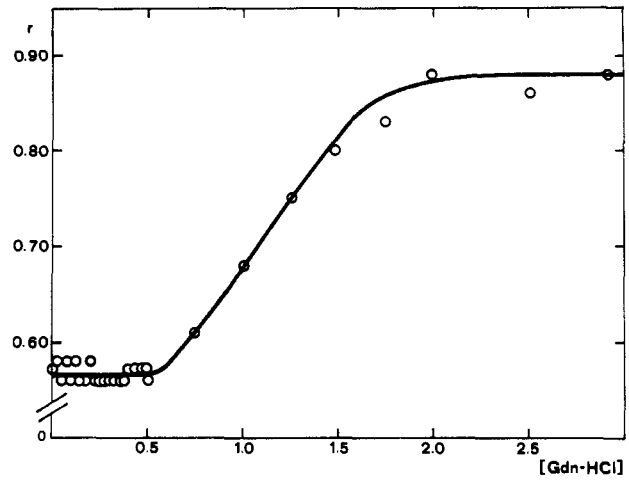

FIGURE 2: Dependence of derivative ratio of horse apomyoglobin on Gdn-HCl concentration at neutral pH. Protein concentration was 4×10^{-5} M; solvent was 0.05 M phosphate–0.15 M KCl, pH 7.0, 25 °C.

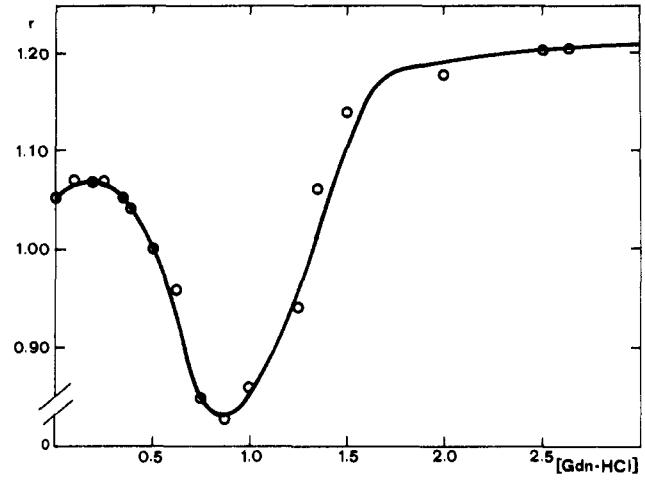

FIGURE 3: Dependence of derivative ratio of tuna apomyoglobin on Gdn-HCl concentration at neutral pH. Protein concentration was 4×10^{-5} M; solvent was 0.05 M phosphate–0.15 M KCl, pH 7.0, 25 °C.

myoglobin are located in the 80–153 domain, the derivative ratio may be used for discriminating molecular changes occurring in this molecular district.

Denaturation Pattern of Horse Apomyoglobin. Horse apomyoglobin contains two tyrosyl residues both located in the 80–153 domain at positions G4 and H23. X-ray diffraction studies (Takano, 1977) performed on sperm whale myoglobin have revealed that tyrosine HC2 projects its phenolic group into the solvent. Therefore, the lack of this residue in horse myoglobin would result in a decrease of the average exposure of phenolic chromophores to the solvent. By use of the derivative method of Ragone et al. (1984), the average exposure of tyrosyl residues of horse apomyoglobin has been calculated to be 22% in the absence of denaturant at neutral pH.

The effect of increasing guanidine concentration on the derivative ratio of horse apomyoglobin is reported in Figure 2. No variation of the derivative ratio takes place between 0 and 0.5 M guanidine. This result would lead to the conclusion that the denaturation pattern of this protein is different from that observed for sperm whale apomyoglobin. However, Irace et al. (1986) have shown that horse apomyoglobin undergoes a denaturation pattern similar to that observed for sperm whale apomyoglobin, consisting of at least two consecutive molecular events. The first event, which takes place at low denaturant concentration, i.e., 0–0.6 M Gdn-HCl, destroys the heme binding site as documented by the lack of the ability to bind ANS (Colonna et al., 1982).

The difference between the denaturation patterns reported in Figures 1 and 2 may be understood if one assumes that the conformational change observed at low denaturant concentration involves the HC segment of the molecule. In fact, variations of the derivative ratio reflect changes only in the degree of solvent accessibility to tyrosyl residues. Since the HC segment of horse apomyoglobin does not contain any tyrosyl residue, the second-derivative method employed in our analysis is not able to detect structural changes affecting this molecular region.

Denaturation Pattern of Tuna Apomyoglobin. The distribution of tyrosyl residues in tuna apomyoglobin is different from that of sperm whale and horse apomyoglobin. In fact, the latter two proteins contain all of the tyrosyl residues in the 80–153 domain of the molecule whereas the former possesses one residue in the 1–79 domain, i.e., Tyr B2, and the other in the 80–153 domain, i.e., Tyr H23. Therefore, the dependence of the derivative ratio on denaturant concentration may provide information on changes occurring in the two distinct domains that contribute to the overall structure of the myoglobin molecule.

Figure 3 shows the effect of increasing guanidine concentration on the derivative ratio of tuna apomyoglobin. In the absence of denaturant the average exposure of tyrosyl residues in this protein is 82%. The larger solvent accessibility to tyrosyl residues observed for tuna apomyoglobin may be related to the low α-helical content found for the protein and to the more open conformation compared to that of sperm whale (Fosmiri & Brown, 1976; Bismuto et al., 1985). Moreover, solvent perturbation by 20% ethylene glycol indicated that the two tyrosyl residues of tuna globin are almost fully exposed to the solvent. The average exposure of tyrosyl residues decreases from 82% to 58% when the guanidine concentration is increased from 0 to 0.8 M. At higher concentrations of denaturant, the derivative ratio increases, thus indicating that the tyrosyl residues become exposed to solvent. Comparing the denaturation pattern and the tyrosyl distribution of the three examined apomyoglobins, one may easily conclude that the decrease of the average tyrosyl exposure observed for tuna apomyoglobin at low guanidine concentration is mainly due to the phenolic group in position B2. In fact, the denaturation patterns of sperm whale and horse apomyoglobin, in which this residue is missed, do not reveal any decrease of solvent accessibility to tyrosyl residues. Since the conformational state reached at low guanidine concentration resembles that reached at acidic pH (Irace et al. 1986), we have analyzed the effect of acid on the derivative ratio of tuna apomyoglobin in order to observe whether the variation of this parameter followed the same pattern. Figure 4 shows that acidification of tuna apomyoglobin from pH 7.0 to pH 4.0 produces a decrease of the derivative ratio to same extent of that produced by low guanidine concentration.

The UV absorption spectrum generated by the acid-induced molecular transition of tuna apomyoglobin (Figure 5) shows two negative peaks centered at 292 and 285 nm, which arise from a blue shift in the absorption spectrum. The negative peak at 292 nm arises from the indole group of the protein (Donovan, 1969). The second, smaller peak is at 285 nm and is about half as intense as the major peak at 292 nm. This peak probably corresponds to the minor peak of tryptophanyl pertubation, the major peak of tyrosyl perturbation being usually observed at slightly higher wavelength (Donovan, 1969). This conclusion is further supported by the fact that

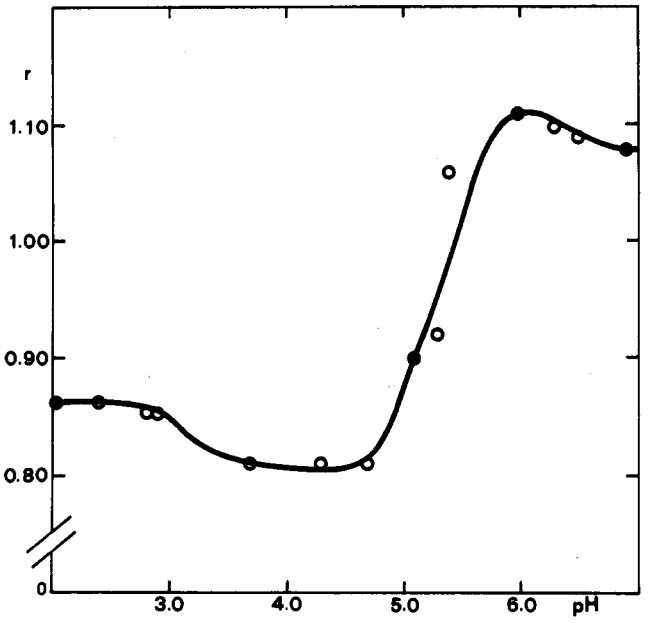

FIGURE 4: Effect of acid on derivative ratio of tuna apomyoglobin. The experimental conditions are those described in Figure 3.

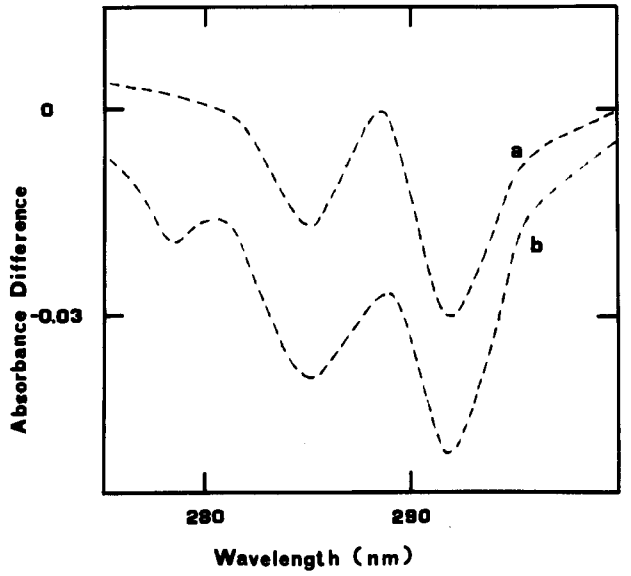

FIGURE 5: Absorbance difference spectra of tuna apomyoglobin induced by acid pH (curve a) and by 6.0 M Gdn-HCl (curve b). Protein concentration was 4×10^{-5} M. Solutions contained 0.05 M phosphate and 0.15 M KCl.

an isosbestic point occurs around 280 nm where it is supposed to find the minor peak of tyrosyl perturbation.

Therefore, the UV difference absorption spectrum generated by the acid-induced molecular transition as well as by 1.0 M Gdn-HCl at neutral pH does not show any negative contribution arising from a blue shift in the tyrosyl absorption due to an increase in the polarity of the chromophore environment. On the other hand, a red shift in the tyrosyl absorption would be expected on the basis of the results obtained by second derivative. Probably, this effect is masked by the strong negative band at 285 nm due to the increased polarity of the tryptophanyl environment. The negative tyrosyl contribution at 278 nm is observed when the protein becomes fully unfolded in the presence of 6.0 M Gdn-HCl.

Discussion

It has been suggested by several authors (Rossmann & Argos, 1975; Argos & Rossmann, 1979; Creighton, 1985) that all myoglobins possess the same basic folding. On the basis of this assumption, a comparison among myoglobins containing a different distribution of aromatic chromophores may contribute to obtain information on different molecular sites of the protein matrix. We have investigated the state of tyrosyl residues in apomyoglobins, purified from phylogenetically distant species, under different experimental conditions.

The extent of solvent accessibility to tyrosyl residues has been investigated by using the method recently developed by Ragone et al. (1984). This method, based on the mutual overlap between the second-derivative bands of the aromatic amino acid side chains of tyrosine and tryptophan, provides average exposure values of the phenolic groups to the solvent consistent with those obtained from X-ray diffraction studies (Lee & Richards, 1971).

The results reported in this paper confirm and extend the general picture developed in the last decade from denaturation studies. Acids are able to destroy the heme binding site of the molecule, but the presence of well-defined elements of secondary and tertiary structure suggests that the acid-denatured form of myoglobin as well as apomyoglobin is not that corresponding to a random-coiled polypeptide. The acid-denatured form resembles rather closely that observed at low guanidine concentration, although some differences have been pointed out (Irace et al. 1986). However, the most important feature is that the two molecular states are not able to bind heme or other chromophores like ANS, TNS, etc. (Balestrieri et al., 1976; Colonna et al., 1982; Irace et al., 1986). The unfolding of the heme binding site induced by acid or by low guanidine concentration could be due to either the unfolding of one of the two structural "halves" identified in the myoglobin molecule, i.e., segments 1–79 and 80–153, respectively (Wetlaufer, 1973; Kuntz, 1975; Wodak & Janin, 1981), or the loss of the molecular interaction connecting the two structural units.

The data shown in this paper indicate that the conformational change determining the loss of the ability to bind the heme at acidic pH or at low guanidine concentration involves the two structural domains of the molecule. The increase of solvent accessibility to Tyr HC2 is due to a partial unfolding of the 80–153 domain, which is also responsible for the reduced intensity of the dichroic bands arising from the phenolic chromophores (Irace et al. 1986). The decrease of solvent accessibility to Tyr B2 is not surprising. In fact, it has been reported that the acid-induced unfolding of the heme binding site produces a refolding of some region of the N-terminal moiety as documented by fluorescence studies. By using 1,5-AEDANS covalently bound to Cys A11 of tuna apomyoglobin, Colonna et al. (1982) observed an increase of the fluorescence intensity and a blue shift of the emission maximum on lowering the pH from 6.0 to 4.0. This result was explained in terms of changes occurring in the microenvironment of the bound chromophore, which becomes less polar.

In conclusion, the conformational states observed at acid pH and at low guanidine concentration can be regarded as a stable intermediate in the unfolding–refolding pathway of apomyoglobin. The differences between the two molecular states are probably due to the different intrinsic mechanisms of unfolding induced by the two denaturants.

Acknowledgments

We are indebted to A. Facchiano and F. Facchiano for performing second-derivative spectra.

References

Argos, P., & Rossman, M. G. (1979) *Biochemistry 18*, 4951–4960.

Balestrieri, C., Colonna, G., & Irace, G. (1973) *Comp. Biochem. Physiol., B: Comp. Biochem. 46B*, 667–672.
Balestrieri, C., Colonna, G., Giovane, A., Irace, G., & Servillo, L. (1976) *FEBS Lett. 66*, 60–64.
Balestrieri, C., Colonna, G., Giovane, A., Irace, G., & Servillo, L. (1978a) *Eur. J. Biochem. 90*, 433–440.
Balestrieri, C., Colonna, G., Giovane, A., Irace, G., Servillo, L., & Tota, B. (1978b) *Comp. Biochem. Physiol., B: Comp. Biochem. 60B*, 195–199.
Balestrieri, C., Colonna, G., Giovane, A., Irace, G., & Servillo, L. (1980) *Anal. Biochem. 106*, 49–54.
Bismuto, E., Colonna, G., Savy, F., & Irace G. (1985) *Int. J. Pept. Protein Res. 26*, 195–207.
Colonna, G., Balestrieri, C., Bismuto, E., Servillo, L., & Irace, G. (1982) *Biochemistry 21*, 212–215.
Creighton, T. E. (1985) *J. Phys. Chem. 89*, 2452–2459.
Dayhoff, M. O. (1972) *Atlas of Protein Sequence and Structure*, Vol. 5, National Biomedical Research Foundation, Washington, DC.
Donovan, J. W. (1969) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S. J., Ed.) Part A, pp 101–170, Academic, New York.
Fosmiri, G. J., & Brown, W. D. (1976) *Comp. Biochem. Physiol., B: Comp. Biochem. 55B*, 293–299.
Irace, G., Bismuto, E., Savy, F., & Colonna, G. (1986) *Arch. Biochem. Biophys. 244*, 459–469.
Kuntz, I. D. (1975) *J. Am. Chem. Soc. 97*, 4362–4366.
Lee, B., & Richards, F. M. (1971) *J. Mol. Biol. 55*, 370–400.
Ragone, R., Colonna, G., Balestrieri, C., Servillo, L., & Irace, G. (1984) *Biochemistry 23*, 1871–1875.
Ragone, R., Colonna, G., Servillo, L., & Irace, G. (1985) *Photochem. Photobiol. 42*, 505–508.
Rossmann, M. G., & Argos, P. (1975) *J. Biol. Chem. 250*, 7525–7532.
Servillo, L., Colonna, G., Ragone, R., Irace, G., & Balestrieri, C. (1980) *Ital. J. Biochem. 29*, 449–450.
Servillo, L., Colonna, G., Balestrieri, C., Ragone, R., & Irace, G. (1982) *Anal. Biochem. 126*, 251–257.
Takano, T. (1977) *J. Mol. Biol. 110*, 535–568.
Teale, F. W. J. (1959) *Biochim. Biophys. Acta 35*, 543.
Watts, D. A., Rice, R. H., & Brown, D. B. (1980) *J. Biol. Chem. 255*, 10916–10924.
Wetlaufer, D. B. (1962) *Adv. Protein Chem. 17*, 303–390.
Wetlaufer, D. B. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 697–701.
Wodak, S. J., & Janin, J. (1981) *Biochemistry 20*, 6544–6552.

Secondary Structure Determination in Proteins from Deep (192–223-nm) Ultraviolet Raman Spectroscopy†

Robert A. Copeland and Thomas G. Spiro*
Department of Chemistry, Princeton University, Princeton, New Jersey 08544
Received September 9, 1986; Revised Manuscript Received December 12, 1986

ABSTRACT: Raman intensities obtained with UV laser excitation at 223, 218, 204, 200, and 192 nm are reported for the amide I, II, III, and II′ bands of random-coil polylysine. The excitation profiles show enhancement via the π–π^* electronic transition, at ~190 nm. Enhancement for amide I is weak, however, and most of the intensity can be accounted for by preresonance with a deeper UV transition at ~165 nm. The amide II′ band dominates the spectrum in D_2O, consistent with the suggestion that the main distortion coordinate in the π–π^* excited state is the stretching of the C–N peptide bond. Amide II intensities with 200- and 192-nm excitation are reported for several proteins. The previously reported negative linear correlation with α-helix content (due to Raman hypochromism in the α-helices) is found not to apply to proteins with high β-sheet content when the excitation wavelength is 200 nm. Much higher intensities are seen for these proteins and are attributed to a red shift of the π–π^* absorption for the β-structure. A linear correlation with α-helix content is found for excitation of 192 nm, which corresponds to an isosbestic point of the β-sheet and random-coil absorption bands. Characteristic amide II Raman cross sections are derived for α-helical, β-sheet, and random-coil elements and are used to determine secondary structure for α_1- and β-purothionin, by use of amide II intensities with 200- and 192-nm excitation. The results are in good agreement with a previous determination based on amide I band deconvolution in off-resonance Raman spectra.

The amide linkage is the fundamental structural component of all proteins and polypeptides. Differences in inter-amide hydrogen bonding are the basis for the diversity of protein structure exhibited in nature (Creighton, 1983). This H bonding stabilizes well-ordered (secondary) structures (e.g., α-helix and β-sheet) where the proximity and orientation of adjacent amides lead to significant dipolar interactions (Creighton, 1983). Such interactions influence several spectroscopic probes, which can be used to monitor secondary structure (Cantor & Schimmel, 1980).

Ultraviolet (UV) circular dichroism is perhaps the most widely used method for secondary structure determinations in proteins (Cantor & Schimmel, 1980). Although this technique has proved reliable in many cases, ambiguities can arise from non-amide chiral chromophores within proteins (Campbell & Dwek, 1984; Walton, 1981). Aromatic amino acids, arginine residues, and prosthetic groups can interfere with the circular dichroism determinations. Nonresonance Raman (Frushour & Koenig, 1975; Lippert et al., 1976;

† This work was supported by NIH Grant GM 25158.
* Author to whom correspondence should be addressed.

0006-2960/87/0426-2134$01.50/0