Heathcote, J. G., Bailey, A. J., & Grant, M. E. (1980) *Biochem. J. 190*, 229–237.
Juva, K., & Prockop, D. J. (1966) *Anal. Biochem. 15*, 77–83.
Kefalides, N. A., Alper, R., & Clark, C. C. (1979) *Int. Rev. Cytol. 61*, 167–228.
Kivirikko, K. I., & Prockop, D. J. (1972) *Biochim. Biophys. Acta 258*, 366–379.
Kivirikko, K. I., & Myllylä, R. (1979) *Int. Rev. Connect. Tissue Res. 8*, 23–72.
Kivirikko, K. I., & Myllylä, R. (1980) in *The Enzymology of Post-Translational Modifications of Proteins* (Freedman, R. B., & Hawkins, H. C., Eds.) pp 53–104, Academic Press, London.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lembach, K. J., Branson, R. E., Hewgley, P. P., & Cunningham, L. W. (1977) *Eur. J. Biochem. 72*, 379–383.
Majamaa, K. (1981) *Biochem. J. 196*, 203–206.
Minor, R. R., Clark, C. C., Strause, E. L., Koszalka, T. R., Brent, R. L., & Kefalides, N. A. (1976) *J. Biol. Chem. 251*, 1789–1794.
Myllylä, R. (1981) *Biochim. Biophys. Acta 658*, 299–307.
Myllylä, R., Risteli, L., & Kivirikko, K. I. (1975a) *Eur. J. Biochem. 52*, 401–410.
Myllylä, R., Risteli, L., & Kivirikko, K. I. (1975b) *Eur. J. Biochem. 58*, 517–521.
Myllylä, R., Risteli, L., & Kivirikko, K. I. (1976) *Eur. J. Biochem. 61*, 59–67.
Myllylä, R., Alitalo, K., Vaheri, A., & Kivirikko, K. I. (1981) *Biochem. J. 196*, 683–692.
Oikarinen, A., Anttinen, H., & Kivirikko, K. I. (1976) *Biochem. J. 156*, 545–551.
Oikarinen, A., Anttinen, H., & Kivirikko, K. I. (1977) *Biochem. J. 166*, 357–362.
Prockop, D. J., Berg, R. A., Kivirikko, K. I., & Uitto, J. (1976) in *Biochemistry of Collagen* (Ramachandran, G. N., & Reddi, A. H., Eds.) pp 163–273, Plenum Press, New York.
Prockop, D. J., Kivirikko, K. I., Tuderman, L., & Guzman, N. A. (1979a) *N. Engl. J. Med. 301*, 13–23.
Prockop, D. J., Kivirikko, K. I., Tuderman, L., & Guzman, N. A. (1979b) *N. Engl. J. Med. 301*, 77–85.
Risteli, J., & Kivirikko, K. I. (1976) *Biochem. J. 158*, 361–367.
Risteli, J., Tryggvason, K., & Kivirikko, K. I. (1977) *Eur. J. Biochem. 73*, 485–492.
Risteli, J., Tryggvason, K., & Kivirikko, K. I. (1978) *Anal. Biochem. 84*, 423–431.
Schwartz, C. E., Hellerqvist, C. G., & Cunningham, L. W. (1979) *Biochem. Biophys. Res. Commun. 90*, 240–246.
Tryggvason, K., Majamaa, K., & Kivirikko, K. I. (1979) *Biochem. J. 178*, 127–131.
Tryggvason, K., Gehron Robey, P., & Martin, G. R. (1980) *Biochemistry 19*, 1284–1289.
Tuderman, L., Kuutti, E.-R., & Kivirikko, K. I. (1975) *Eur. J. Biochem. 52*, 9–16.
Turpeenniemi-Hujanen, T. M. (1981) *Biochem. J. 195*, 669–676.
Turpeenniemi-Hujanen, T. M., Puistola, U., Kivirikko, K. I. (1981) *Collagen Relat. Res.: Clin. Exp. 1*, 355–366.
Uitto, J., & Prockop, D. J. (1974) *Biochim. Biophys. Acta 336*, 234–251.
Uitto, V.-J., Uitto, J., Kao, W. W.-Y., & Prockop, D. J. (1978) *Arch. Biochem. Biophys. 185*, 214–221.

Reversed Unfolding–Refolding Process of Cobra Neurotoxin†

Andrzej Gałat,‡ Jacques P. Degelaen,§ C. C. Yang, and Elkan R. Blout*

ABSTRACT: Circular dichroism and nuclear magnetic resonance spectroscopies have been used to study the unfolding process of cobrotoxin upon addition of fluoro alcohols and/or sodium dodecyl sulfate to its aqueous solution. In each final unfolded state, the protein had its disulfide bonds intact. The unfolding process has been found to be reversible in the case of fluoro alcohol/water mixtures, while no such reversibility was found in the case of sodium dodecyl sulfate. However, when hexafluoro-2-propanol is added to the sodium dodecyl sulfate unfolded protein, refolding is induced. The mechanism of unfolding is discussed in terms of the different interactions which govern the protein conformation in solution.

Neurotoxins constitute a group of homologous proteins found in snake venoms. They block transmission of the nerve impulses by binding with high affinity to the acetylcholine receptor of the motor end plate on the postsynaptic membrane (Lee, 1972; Tu, 1973; Yang, 1974). During the last few years, a large number of studies including optical spectroscopy (Chicheportiche et al., 1972; Harada et al., 1976; Chen et al., 1977; Menez et al., 1976; Visser & Louw, 1978), nuclear magnetic resonance (Arseniev et al., 1976; Bystrov et al., 1978; Lauterwein et al., 1977; Fung et al., 1979; Inagaki et al., 1978; Endo et al., 1979), and X-ray crystallography (Low et al., 1976; Tsernoglou & Petsko, 1976, 1977) as well as theoretical predictions of the molecular conformations (Ryden et al., 1973; Eterovic & Ferchmin, 1977) and the use of chemically modified toxins (Seto et al., 1970; Karlsson & Sundelin, 1976; Yang, 1976, 1974) have been made with the aim of understanding the relations between the spatial structures of these proteins and their biological functions.

Cobrotoxin from Taiwan cobra (*Naja naja atra*) venom is a postsynaptic neurotoxin. It consists of a single polypeptide chain of 62 amino acid residues and contains 4 disulfide bridges which divide the molecule into 4 loops. In a recent NMR[1]

† From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115 (A.G., J.P.D., and E.R.B.), and the Institute of Molecular Biology, National Tsing Hua University, Hsinchu, Taiwan 300, Republic of China (C.C.Y.). *Received July 31, 1980; revised manuscript received August 10, 1981.* This work was supported in part by U.S. Public Health Service Grant AM07300. J.P.D. was also supported in part by Fulbright and NATO grants. The high-field NMR experiments were performed at the NMR Facility for Biomolecular Research located at the F. Bitter National Magnet Laboratory, Massachusetts Institute of Technology, Cambridge, MA. The NMR Facility is supported by Grant PR00995 from the Division of Research Resources of the National Institutes of Health.

‡ Present address: Institute of Biology, College of Pedagogics, Zonierska 14, 10-561 Olsztyn, Poland.

§ Present address: General Pathology Unit, International Institute of Cellular and Molecular Pathology, Brussels, Belgium.

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study of this protein, Endo et al. (1979) assigned the signals corresponding to the histidine, aromatic, and methyl protons. By using pH titration and NOE techniques, they were also able to give pertinent information about some microenvironments in this protein. In CD studies Yang et al. (1967, 1968) and Chen et al. (1977) analyzed the spectra in pure aqueous solution with respect to the involvement of β turns, β sheets, and α helices in the structure of the protein. This study also showed that the protein is stable in 7.5 M urea, 1,2-ethanediol, and the mixed solvent 1-propanol–1,2-ethanediol–water (5:1:1 v/v) in the temperature range 5–70 °C and in the pH range 3.2–12.7. The toxin denatures at temperatures higher than 70 °C and in 4.1 M guanidinium chloride.

In our CD and NMR studies, we have shown that addition of fluoro alcohols (hexafluoro-2-propanol and trifluoroethanol) or sodium dodecyl sulfate to an aqueous solution of cobrotoxin induces its unfolding. This unfolding, however, is not complete. The final quasi-unfolding in fluoro alcohol–water mixtures was found to be reversible, while no such reversibility was observed for the $NaDodSO_4$ unfolded protein. However, when hexafluoro-2-propanol is added to the $NaDodSO_4$ unfolded protein, refolding is induced as a result of the expulsion of the $NaDodSO_4$ molecules from the protein.

Thus cobrotoxin and other related snake toxins [see, for example, Tsetlin et al. (1979), Menez et al. (1976), Fox & Tu (1979), and Bailey et al. (1979)] may be suitable models for future investigations of protein unfolding–refolding phenomena [for excellent recent reviews of this subject, see Némethy & Scheraga (1977) and Creighton (1978)].

Experimental Procedures

Materials. Cobrotoxin was prepared from Taiwan cobra (*Naja naja atra*) venom as previously described (Yang, 1965). Prior to each measurement, the solution of the protein was filtered through a Millipore No. HATF 1300 filter. The concentrations of the protein were determined spectrophotometrically by using $E^{\%}_{1cm}$ = 13.7 at λ 280 nm (Chen et al., 1977). CD spectra were measured in deionized water or in 0.05 M phosphate buffer, pH 7 (Fisher lot 775970). NMR spectra were measured in 0.05 M phosphate buffer in D_2O. Hexafluoro-2-propanol (HFIP) was a gift from E. I. du Pont de Nemours Co., Inc.; deuterated HFIP was purchased from Merck Sharp & Dohme Canada Ltd.; TFE was supplied by J. T. Baker Chemical Co.; sodium dodecyl sulfate was supplied by Fisher Scientific Co.; D_2O (99.8 mol %) was obtained from Bio-Rad.

Methods. CD spectra were determined with a Cary 60 dichrograph with a Model 6001 circular dichroism attachment. The dichrometer was calibrated with camphorsulfonic acid, θ = 7840 deg cm^2 dmol^{-1} at λ = 290 nm. The molar absorbance of the samples was adjusted to be within the range 0.5–1.5 absorbance units. CD spectra were measured in silica cuvettes with path lengths varying from 0.5 mm to 2 cm. The spectra were measured at least 2 times on different days on newly prepared solutions and the results averaged. The base line was determined each time for each spectrum. The circular dichroism is reported as the mean residue ellipticity, calculated from the relationship $\theta = hs/(Lc)$, where h is the height of the CD band in fraction of the full scale, s is the sensitivity of the instrument in degrees per full scale, L is the path length of the cuvette in centimeters, and c is the mean residual concentration in moles per liter. The mean residue ellipticity $[\theta]$ is expressed in the units of degree centimeter squared per decimole, using a mean residue weight of 112.1.

For the NMR experiments, the exchangeable protons (amino, hydroxyl, amide, etc.) were replaced by deuterium by incubating the protein in D_2O for 4 h at 25 °C followed by lyophilization. The procedure was repeated twice in order to cause an almost complete exchange. The initial samples were prepared by dissolving the protein to a concentration of 2 mM in 400 μL of 50 mM phosphate buffer in D_2O. Samples at different HFIP/D_2O ratios were obtained by successively adding adequate amounts of HFIP (during the unfolding) and D_2O (during the refolding) to the initial D_2O–phosphate buffer solution of the protein directly in the NMR tube. Samples containing $NaDodSO_4$ were obtained in a similar way by adding successive aliquots of a 5% (w/v) stock solution of $NaDodSO_4$ in D_2O–phosphate buffer to the protein solution in the NMR tube. The NMR spectra were obtained by using a Bruker WH270 spectrometer field frequency locked to the deuterium in the solvent and equipped with a variable temperature accessory. Generally, 4096 data points were collected for a 3000-Hz spectral width. Before Fourier transformation, the free induction decay was multiplied by an exponential function giving a line broadening of 1 Hz. All the experiments were done at 37 °C, and the chemical shifts are referenced to external DSS standard. The pH measurements were made on a Radiometer Model 26 pH meter and were not corrected for the isotope effect.

Results

(*A*) *Conformation of Cobrotoxin in Water.* The CD spectrum of cobrotoxin in water contains five major bands. Three of them at about 288, 282, and 228 nm are unambiguously related to the electronic transitions of aromatic amino acids and disulfide bridges, while the two bands in the far-UV are related to the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions of peptide chromophores (Chen et al., 1977; Woody, 1978). Due to the considerable variability of (ϕ, ψ) angles along the protein chains in β turns and antiparallel β structure and strong contributions of the Cotton effects of aromatic amino acids to the far-UV region, the CD spectrum is atypical compared to other CD spectra. However, despite these limitations, CD spectra are very useful in the investigation of the protein unfolding–refolding phenomenon.

The NMR spectrum of cobrotoxin in 50 mM phosphate buffer (pH 6.8) at 37 °C is shown in Figure 1a. It is essentially similar to the one previously reported by Fung et al. (1979) and Endo et al. (1979). The nonequivalence of the histidine-4 and histidine-32 C_2H protons (peaks 1 and 2), the unusual chemical shifts of the ortho and meta protons of tyrosine-25 (peaks 3 and 4), the presence of several signals in the region located between 6.5 ppm and the water peak (4.6 ppm), and the spreading out of the methyl signals are all features which are related to the native structure of the protein. Most of these signals are related to protons which are located in close vicinity to polar or aromatic groups and which, therefore, undergo substantial chemical shifts as compared to their normal resonance positions in isolated amino acids or in random-coil proteins (Wüthrich, 1976). This is particularly true for the signals located between 6.5 ppm and the water peak. Indeed, this region of the spectrum is essentially blank in the case of a random-coil protein. Therefore, these signals are useful probes for the study of the conformational changes which occur during the unfolding and refolding of the protein.

(*B*) *Conformation of Cobrotoxin in TFE–H_2O Mixture.* The conformation of the neurotoxin is not affected much by

[1] Abbreviations used: CD, circular dichroism; NMR, nuclear magnetic resonance; HFIP, hexafluoro-2-propanol; TFE, 2,2,2-trifluoroethanol; $NaDodSO_4$, sodium dodecyl sulfate; NOE, nuclear Overhauser effect; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate.

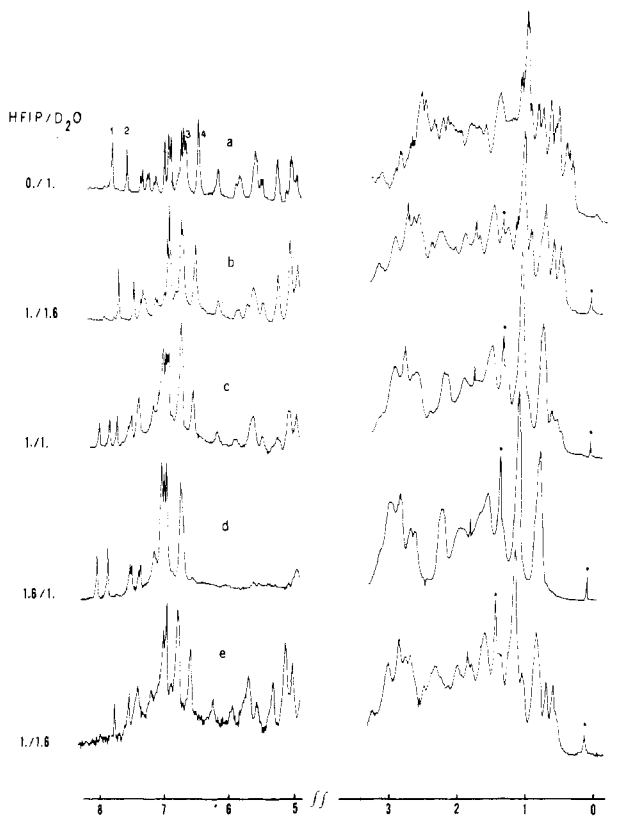

FIGURE 1: 270-MHz ^1H NMR spectra at 37 °C of cobrotoxin at various HFIP/50 mM phosphate buffer (D_2O) ratios: (a) 0:1, pH 6.70, concentration of the protein 2.0 mM; (b) 1:1.6, pH 6.30, concentration of the protein 1.2 mM; (c) 1:1, pH 6.10, concentration of the protein 1.0 mM; (d) 1.6:1, pH 6.00, concentration of the protein 0.8 mM; (e) 1:1.6, pH 6.25, concentration of the protein 0.5 mM. The line positions are given in parts per million from external DSS. NMR peaks labeled (*) are spectrometer-related artifacts.

increasing the TFE concentration in water. For example, CD spectra measured in aqueous solution are very similar to the CD spectra measured in a mixture of H_2O/TFE (1:1 v/v) (see Figure 2A) in the far-UV, suggesting that the main conformation of the neurotoxin remained almost unchanged. In the near-UV region, more pronounced effects were observed (Figure 2B), though the vibronic structure of the tryptophan and tyrosine electronic transitions is clearly visible. An increase of the ratio TFE/H_2O to 5:1 (v/v) influenced the CD spectra to a larger extent. Only a small band at about λ 228 nm is retained, followed by an increase of the intensity of the band around λ 215 nm and a decrease of the band in the vicinity of λ 199 nm. Also the near-UV region is changed, but sharply ordered vibronic structure is still visible, while the molecular ellipticity decreased to $\theta = -130$ deg cm^2 dmol^{-1}. An increase of TFE concentration up to 10:1 (v/v) resulted in the disappearance of the band at about λ 228 nm and a 50% decrease of the intensity of the band centered around λ 199 nm, as compared with the CD spectrum of the neurotoxin in water. The molecular ellipticity of the band at about λ 214 nm increased considerably, from $\theta = -1400$ deg cm^2 dmol^{-1} to $\theta = -3500$ deg cm^2 dmol^{-1}, and the half-bandwidth increased 4 times. In the near-UV region, a flat CD band decreased to $\theta = -90$ deg cm^2 dmol^{-1}. Reversed dilution with water of the 10:1 (TFE/H_2O) solution gave identical CD spectra to those obtained when the concentration of TFE had been elevated. The CD ellipticity values and the positions of the Cotton effects of the neurotoxin in TFE/H_2O mixtures are given in Table I.

(C) Conformation of Cobrotoxin in HFIP–H_2O Mixture. *(1) CD Results.* When the ratio HFIP/H_2O is kept within

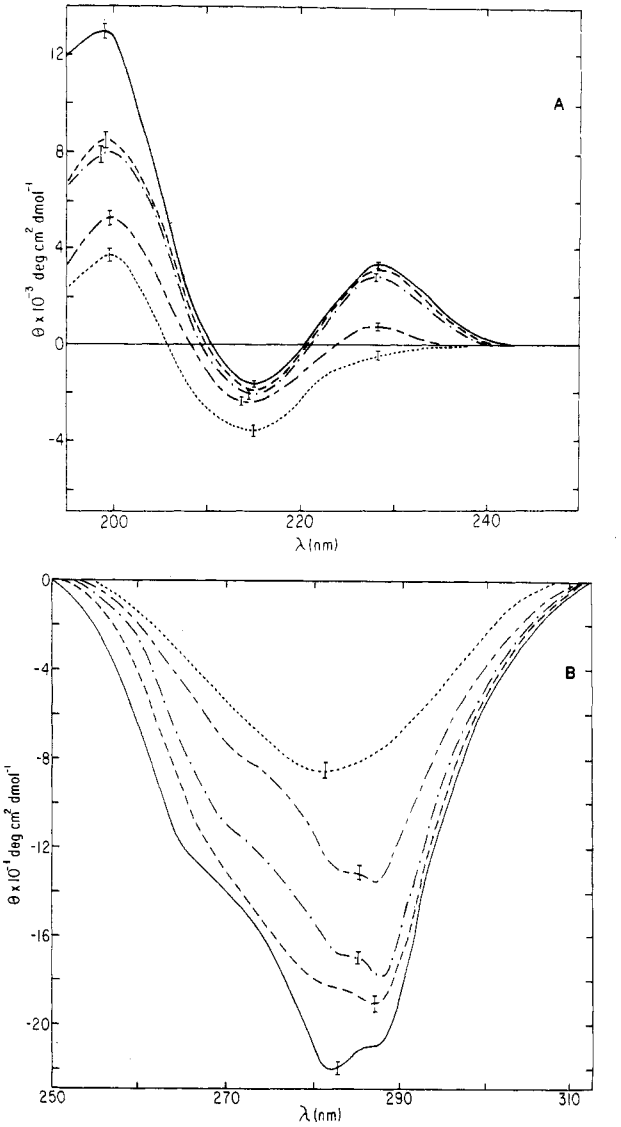

FIGURE 2: (A, B) CD spectra of cobrotoxin (mean residue ellipticity) in TFE/H_2O (v/v) mixture: (—) H_2O; (– – –) 1:5; (–·–) 1:1; (–··–) 5:1; (···) 10:1.

the range 1:10 to 1:2, only small changes were observed in the CD spectra (see Figure 3). When the ratio of HFIP/H_2O reaches 1:1 (v/v), the bands at λ 228 nm and at λ 199 nm disappear, and a broad band with a maximum around λ 207 nm is observed. In the near-UV, a considerable decrease of the intensity of the Cotton effects is also found, while it is still clear that even in this state, the aromatic chromophores are not completely in random environments, since the molecular ellipticity of this set of CD bands is about $\theta = -100$ deg cm^2 dmol^{-1}. Further increase of HFIP concentration up to 2:1 involved the shift of the far-UV band to λ 201 nm, followed by considerable enhancement of its molecular ellipticity to $\theta = -12\,600$ deg cm^2 dmol^{-1}. In addition, a small shoulder at about λ 220 nm appeared. In the near-UV region, the trace of the CD band still remains. Water addition to the 2:1 sample, where only the denatured protein exists, gave a fully reversed conformation closely similar to that of lower concentrations of HFIP in water. The complete CD results of these conformational transitions are given in Table II.

(2) NMR Results. At the beginning of the addition of HFIP, between the HFIP/H_2O ratios 0:1 and 1:1.6, small chemical shift changes are observed in some of the lines. However, the most important changes in the NMR spectrum are observed when the HFIP/D_2O ratio is increased between 1:1.6 and 1.6:1. The spectra at three different ratios are given

Table I: Positions and Ellipticity Values of Cobra Neurotoxin in TFE/H_2O Mixtures[a]

TFE/H_2O ratio	I		II		III		IV		V	
	λ	[θ]	λ	[θ]	λ	[θ]	λ	[θ]	λ	[θ]
0:10	288	−210	282	−220	228	3300	215	−1200	199	12500
1:10	288	−200	282	−180	228	3200	215	−1400	199	11600
1:5	288	−190	282	−180	228	3150	215	−1500	199	8800
1:2	288	−180	282	−170	228	3050	215	−1500	199	8000
1:1	288	−180	282	−170	228	2600	214	−1600	199	7800
2:1	288	−150	282	−170	229	2150	214	−1700	199	7700
5:1	288	−140	282	−130	229	750	214	−2400	199	5200
10:1	288	−80	282	−90			214	−3500	199	4000
reversed										
5:1	288	−130	282	−120	229	750	214	−2500	199	5000
2:1	288	−140	282	−140	229	2450	214	−2100	199	7200
1:1	288	−170	282	−170	228	2800	215	−1800	199	8000
1:2	288	−180	282	−170	228	3300	215	−1600	199	8300

[a] The positions of the Cotton effects are expressed in nanometers, the molecular ellipticity values are expressed in degrees centimeter squared per decimole, and the standard deviation of these values in the near-UV is ±10 deg cm^2 $dmol^{-1}$; for the Cotton effects at about λ 215 and 228 nm, the deviation is ±150 deg cm^2 $dmol^{-1}$, while for the one at about λ 199 nm, the deviation is ±500 deg cm^2 $dmol^{-1}$.

Table II: Positions and Ellipticity Values of Cobra Neurotoxin in HFIP/H_2O Mixtures[a]

HFIP/H_2O ratio	I		II		III		IV		V	
	λ	[θ]	λ	[θ]	λ	[θ]	λ	[θ]	λ	[θ]
0:10	288	−210	282	−220	228	3300	215	−1200	199	12500
1:10	288	−190	282	−200	228	3150	215	−1400	199	10600
1:5	288	−180	282	−190	228	2900	215	−1450	199	9000
1:2	288	−150	282	−170	228	1950	215	−2000	199	6100
1:1	285	−90	280	−110			207	−4800		
2:1			280	−90	221	−4700			201	−12600
5:1			280	−80	221	−4800			201	−12800
reversed										
2:1			280	−90	221	−4600			201	−12500
1:1	285	−80	280	−100			207	−5000		
1:2	288	−140	282	−150	228	1450	215	−2250	199	5700

[a] The positions of the Cotton effects are expressed in nanometers, the molecular ellipticity values are expressed in degrees centimeter squared per decimole, and the standard deviation of these values in the near-UV is ±10 deg cm^2 $dmol^{-1}$; for the Cotton effects at about λ 215 and 228 nm, the deviation is ±150 deg cm^2 $dmol^{-1}$, while for the one at about λ 199 nm, the deviation is ±500 deg cm^2 $dmol^{-1}$.

in Figure 1b–d. At the ratio 1:1.6 (Figure 1b), the spectrum still shows all the features typical of the cobrotoxin in the native form, as seen in pure aqueous solution (Figure 1a). On increasing the HFIP/D_2O ratio, there is a decrease in the intensities of the lines corresponding to the native structure, but at the same time, a new set of signals appears and increases until finally, at the ratio 1.6:1, it is the only one present (Figure 1d). At this ratio, almost all the lines of the spectrum can be related to resonance positions of isolated amino acids in D_2O at pH 7.0 as given in Tables 2–7 in Wüthrich (1976). Another striking feature is the absence of any signal in the region located between 6.5 ppm and the water peak.

The preceding observations suggest that the neurotoxin is in an unfolded state at the 1.6:1 HFIP/D_2O ratio. This unfolding, however, is not complete, as can be seen from the nonequivalence of the C_2H protons of histidine-4 and histidine-32. This is not surprising since the disulfide bridges are probably not much affected in the HFIP titration process. The spectrum shown in Figure 1c taken at the intermediate 1:1 HFIP/D_2O ratio is almost exactly the superposition of the spectra in Figure 1b,d. This indicates that at this ratio the native and unfolded forms of the neurotoxin are present in equimolar concentrations and that the exchange between these two forms is slow on the NMR time scale.

When D_2O is added to the sample containing the unfolded cobrotoxin at the 1.6:1 ratio, refolding is induced. The spectrum obtained at the 1:1.6 HFIP/D_2O ratio after the neurotoxin had been unfolded is shown in Figure 1e and should be compared with the corresponding spectrum in Figure 1b, which was obtained at the same ratio but during the unfolding process. Although the two sets of spectra have different signal to noise ratios due to the different protein concentrations, it is clearly seen that they are exactly identical. Therefore, as far as the NMR structural features are concerned, the unfolding of cobrotoxin in HFIP/D_2O mixtures is essentially reversible.

(D) Influence of $NaDodSO_4$ on Cobrotoxin. (1) CD Results. Sodium dodecyl sulfate even in low molar ratio to the protein disturbs its conformation (see Figure 4). For example, when the ratio of $NaDodSO_4$/cobrotoxin is 1:10, the shape of the CD spectrum is somewhat changed, since the intensity of the band at about λ 228 nm does decrease about 20% and the band at about λ 282 and 288 nm is also slightly decreased. Further increase of $NaDodSO_4$ molar concentration up to the ratio 1:1.5 involves considerable modification of the neurotoxin conformation, but the aromatic CD bands are still visible both in the near-UV as well as in the far-UV regions; at λ 285 nm, $\theta = -90$ deg cm^2 $dmol^{-1}$, and at λ 228 nm, $\theta = 2400$ deg cm^2 $dmol^{-1}$, respectively. When the molar ratio is 1.5:1, all aromatic bands disappear, and a broad band at about λ 212 nm with $\theta = -3350$ deg cm^2 $dmol^{-1}$ is observed. Finally, when the ratio is 3:1, only a denatured state is observed for which the CD parameters are as follows: λ 201 nm and $\theta = -12600$ deg cm^2 $dmol^{-1}$. Further increase of the $NaDodSO_4$ concentration does not influence the conformation of the neurotoxin. An addition of small amounts of HFIP to such a so-

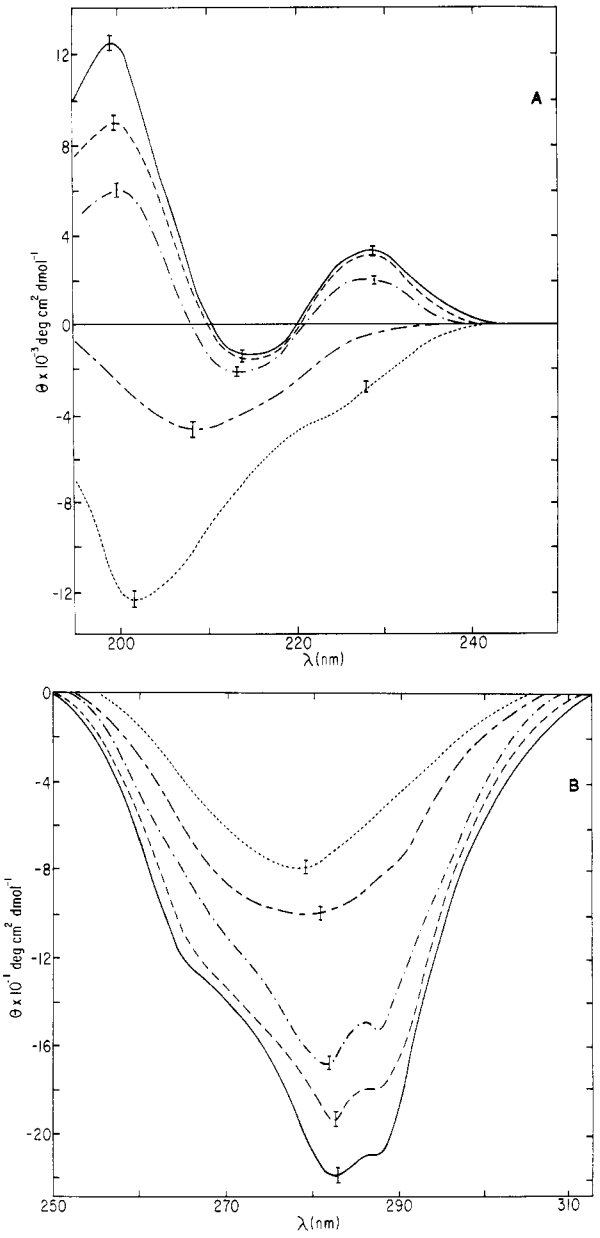

FIGURE 3: (A, B) CD spectra of cobrotoxin (mean residue ellipticity) in HFIP/H_2O mixtures: (—) H_2O; (– – –) 1:5; (–·–) 1:2; (– – –) 1:1; (···) 2:1.

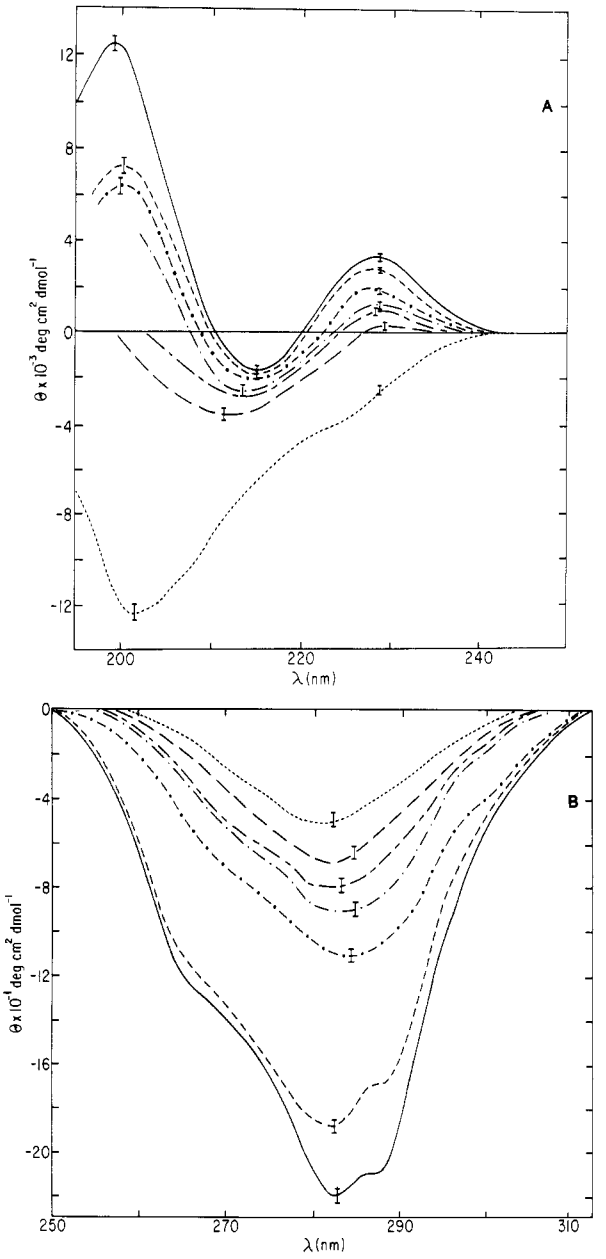

FIGURE 4: (A, B) CD spectra of cobrotoxin (mean residue ellipticity) in water in elevated $NaDodSO_4$ to cobrotoxin molar ratio (cobrotoxin/$NaDodSO_4$, M/M): (—) native in water; (– – –) 10:1; (–•–) 3:1; (–·–) 1.5:1; (– – –) 1:1; (——) 1:1.5; (···) 1:3.

lution in the ratio approximately equal to 1:5 HFIP/H_2O caused reversible transformation of cobrotoxin to its native conformation. The elevation of HFIP concentration in this solution involves the same conformational changes of the neurotoxin as those found in HFIP/H_2O solution without $NaDodSO_4$. The complete results of these conformational transition states are given in Table III.

(*2*) *NMR Results.* When sodium dodecyl sulfate is added to the cobrotoxin in aqueous solution, the typical signals related to the tertiary structure of the protein disappear. Parts A and B of Figure 5 show the lower field parts of the spectra of cobrotoxin without and with $NaDodSO_4$, respectively. These spectra indicate that when 3 mol of $NaDodSO_4$ is added to 1 mol of cobrotoxin, the latter is essentially unfolded. The higher field parts of the spectra are not shown, as most of the information is lost due to the strong overlapping of the $NaDodSO_4$ lines. When HFIP is added to the $NaDodSO_4$-unfolded cobrotoxin, its refolding occurs as seen in Figure 5C. Comparison of this spectrum with that of Figure 5A shows that most of the features of the original native neurotoxin have been recovered. There are, however, differences which may either result from the interaction between HFIP and cobrotoxin or reflect some residual binding of $NaDodSO_4$. Further studies are underway in order to clarify this point. However, it seems that in this case again the refolding is essentially reversible.

Discussion

The CD and NMR spectra of cobrotoxin in aqueous solution are closely similar to, if not identical with, those published in the earlier papers (Yang et al., 1967, 1968; Chen et al., 1977; Fung et al., 1979; Endo et al., 1979). A detailed discussion of the cobrotoxin conformation was recently given by Chen et al. (1977) and Hseu et al. (1977, 1978), who on the basis of Woody's theoretical calculation (1974), the prediction method of Chou & Fasman (1974), and the experimental results of Urry et al. (1974), postulated that the conformation of this neurotoxin mainly contains a mixture of β turns and antiparallel β structure. This suggestion is fully consistent with the X-ray analysis performed on related snake toxins (Tser-

Table III: Positions and Ellipticity Values of Cobra Neurotoxin in the Changes of $NaDodSO_4$/Protein Ratios[a]

$NaDodSO_4$/ protein ratio	I		II		III		IV		V	
	λ	[θ]	λ	[θ]	λ	[θ]	λ	[θ]	λ	[θ]
1:100	288	−210	282	−220	228	3300	215	−1200	199	12500
1:30	288	−180	282	−200	228	2900	215	−1600	199	7500
1:10	288	−170	282	−190	228	2700	215	−1950	199	6900
1:3	285	−110			228	1950	215	−2000	199	6400
1:1.5	285	−90			228	1050	214	−2400		
1:1			282	−80	228	850	214	−2700		
1.5:1			280	−70	229	200	212	−3500		
3:1			280	−50					201	−12600
addn of HFIP in HFIP/H_2O ratio										
5:1	288	−210	282	−210	228	2550	215	−1600		
2:1	288	−170	282	−160	228	1550	214	−2400		
1:1			280	−80			207	−7500		
2:1			280	−50	221	−5200			201	−12500

[a] The positions of the Cotton effects are expressed in nanometers, the molecular ellipticity values are expressed in degrees centimeter squared per decimole, and the standard deviation of these values in the near-UV is ±10 deg cm^2 $dmol^{-1}$; for the Cotton effects at about λ 215 and 228 nm, the deviation is ±150 deg cm^2 $dmol^{-1}$, while for the one at about λ 199 nm, the deviation is ±500 deg cm^2 $dmol^{-1}$.

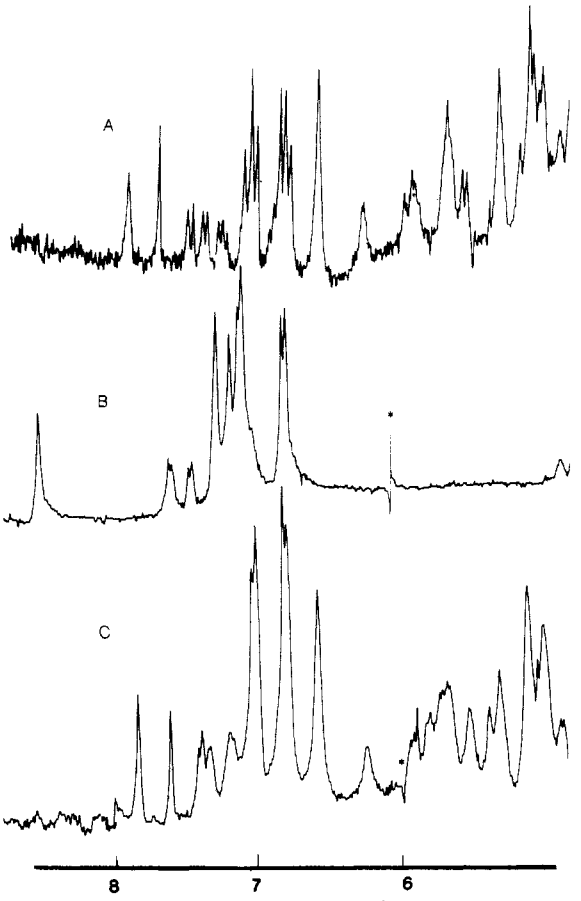

FIGURE 5: Low-field part of the 270-MHz ^1H NMR spectra at 37 °C of cobrotoxin in 400 μL of 50 mM phosphate buffer in D_2O. The concentration of the protein is 2.0 mM. (A) Without $NaDodSO_4$, native form of cobrotoxin in phosphate buffer, pH 6.8; (B) with 0.5% $NaDodSO_4$ (w/v), pH 6.7; (C) the same as in (B) plus 40 μL of HFIP, pH 6.6. The line positions are given in parts per million from external DSS. NMR peaks labeled (*) are spectrometer-related artifacts.

noglou & Petsko, 1976, 1977; Low et al., 1976) and is also confirmed by statistical conformational analysis (Gabel et al., 1976; Tanaka & Scheraga, 1977). The possibility that α-helical segments are present in cobrotoxin may be excluded, since even in HFIP, which is a well-known α-helical supporting solvent (Timasheff, 1970; Parrish & Blout, 1971, 1972), no evidence for any α-helical contribution was found.

However, it is difficult to correlate the observed CD spectra of cobrotoxin with models of β turns or β structures. It is well-known that the aromatic amino acids contribute strongly to the far-UV region, especially in low molecular weight proteins which lack any α helix in their structure. Furthermore, in the case of cobrotoxin, a statistical average of various β forms and β turns occurs, and the observed CD spectra are weighted means of different Cotton effects attributable to particular peptide (φ, ψ) angles of the chain.

Conformation of Cobrotoxin in TFE/H_2O and HFIP/H_2O Mixtures. Comparison of the CD spectra obtained in both mixtures shows that TFE is much less effective than HFIP in inducing conformational changes on the neurotoxin. For instance, consideration of the CD spectra at the 5:1 TFE/H_2O ratio and 1:2 HFIP/H_2O ratio indicates closely similar molecular ellipticity values for the bands at about 199 and 214 nm. This suggests that the neurotoxin conformations are very similar in both mixtures. On the other hand, slightly larger differences are observed on the bands at about 228 nm and the near-UV bands within the range 250–320 nm, which presumably indicates that the aromatic chromophores have been disrupted to different extents. Also at the 10:1 ratio, the CD spectrum of the protein in the TFE/H_2O mixture still shows bands related to the original structure, while in the HFIP/H_2O mixture, there has been a considerable change in the conformation. In the following discussion, we will, therefore, mainly concentrate on the protein's behavior in the HFIP/H_2O mixtures.

Both from the CD and NMR spectra, it can be seen that there are at least two major stages in the modification of the neurotoxin structure upon HFIP addition. During the first stage, in which the HFIP/water ratio is varied between 0:1 and approximately 1:2, some slight but significant changes occur over the entire spectra. The NMR spectra measured at the 0:1 and 1:1.6 HFIP/D_2O ratios show that slight shifts of the line positions have occurred, in particular, in the signals related to the tryptophan and one of the tyrosines. But most importantly, the signals related to the native structure of the neurotoxin have been little affected. The preceding NMR observations suggest that during this first stage of HFIP addition only minor changes have occurred in the native structure of cobrotoxin. It is interesting and important to correlate the changes seen on the aromatic signals in the NMR spectrum

and the changes in the CD spectra. It may be that the decrease of the band at 199 nm and the increase of the band at 214 nm are related to the modification of the aromatic bands as seen at 228 nm and in the near-UV. Therefore, the changes in the former bands may indicate the extent of the contributions of the electronic transitions related to aromatic chromophores to the intensities of the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions of the peptide units. This would then again indicate, based on CD, that the native secondary structure has not been substantially affected in the HFIP/water range from 0:1 to 1:2. In fact, from the NMR spectra which show that only tryptophan and tyrosine-35 have been affected, but not tyrosine-25, it seems that only the exterior surface of the cobrotoxin in direct contact with the solvent has undergone structural modifications.

During the second stage between the HFIP/water ratio 1:2 and 2:1, large conformational changes occur as seen from both the CD and NMR spectra. The most striking feature in this ratio range, as seen from the NMR spectrum in Figure 1c, is the presence of two major conformational states in slow exchange on the NMR time scale. The first conformational state is identical with the one obtained at the HFIP/D_2O ratio 1:1.6 (Figure 1b), and it is very similar, as shown previously, to the native conformation of the protein. The second conformational state is identical with the one obtained at the HFIP/D_2O ratio 1.6:1.0, and it is related to a "quasi"-unfolded form of cobrotoxin. This unfolded form is not modified by further addition of HFIP. Noteworthy is the fact that at the 1:1 HFIP/D_2O ratio there is an equivalent amount of each conformational state in the mixture. The CD curves can be easily explained on the basis of our NMR observations. For instance, it can be seen that the CD curve corresponding to the 1:1 ratio is almost exactly superimposable with the curves obtained by adding the curves corresponding to the 1:2 and 2:1 ratios. This again indicates the presence at the 1:1 ratio of equivalent amounts of two species, one as found at the 1:2 ratio and the other as found at the 2:1 HFIP/H_2O ratio.

Consideration of both CD and NMR spectra at the 1.6:1 HFIP/water ratio or higher indicates that although the major part of cobrotoxin does exist in an unfolded state, some elements of the native structure are still present. The features which support this idea are the nonequivalence of the two histidine C_2H protons in the NMR spectrum and the broadening of the band at 201 nm in the CD spectrum. This is not surprising since we know that the disulfide bridges have not been disrupted during the alcohol addition process and one may, therefore, expect that some patches of structure remain present.

We have also shown that addition of water to the unfolded neurotoxin in the 1:1 HFIP/water mixture induces its refolding. Both the CD and NMR spectra show that the conformations of cobrotoxin at identical ratios during unfolding and refolding are exactly similar. Because of the way our experiments were performed, the concentrations of the neurotoxin at identical ratios during unfolding and refolding are very much different. This indicates that the effect of HFIP on cobrotoxin does not depend upon the concentration of the latter, and it suggests that conformational changes induced by the alcohol are not primarily due to specific interactions with the neurotoxin.

Conformational Changes upon NaDodSO$_4$ Addition. Consideration of the CD spectra shows that there is a regular change in cobrotoxin structure when up to 3 equiv of NaDodSO$_4$ is added to 1 equiv of the neurotoxin. Noteworthy is the fact that the curve corresponding to the 3:1 NaDodSO$_4$/protein ratio is very similar to the curve obtained at high HFIP/water ratios. The preceding observations suggest that, after 3 equiv of NaDodSO$_4$ has been added to the protein, it is in an unfolded state which from the spectroscopic point of view closely resembles that at high HFIP/water ratios. The only difference is detected in the NMR spectrum which shows one unique signal for the two histidines in the NaDodSO$_4$-denatured neurotoxin.

In fact, it may be incorrect to consider the HFIP and NaDodSO$_4$ denatured states as being completely similar. A more real picture is one in which one considers that cobrotoxin can exist in a large number of different conformations in either excess HFIP or NaDodSO$_4$. Rapid exchange between these conformations both on the CD and NMR time scales eventually leads to these identical average spectra. Addition of water to the NaDodSO$_4$-denatured cobrotoxin did not induce refolding, indicating that the interaction of NaDodSO$_4$ with the neurotoxin is strong and specific. On the other hand, addition of HFIP to the NaDodSO$_4$-denatured cobrotoxin induces its refolding to a conformation which is closely related to the native one. Small differences are, however, detected in the NMR spectra (Figure 5A,B), which suggest that these two conformational states—namely, native and refolded—may not be exactly identical. It is obvious from these experiments, however, that addition of HFIP has resulted in the expulsion of the majority of the NaDodSO$_4$ molecules from cobrotoxin.

Mechanism of the Unfolding–Refolding Process of Cobrotoxin. In order to understand the mechanism of disruption of the cobrotoxin molecule as a result of HFIP (or TFE) and NaDodSO$_4$ addition to its aqueous solution, it is essential to consider the different forces which stabilize its native structure in pure aqueous solution. Three major types of interactions are considered to be involved in the stabilization of a folded protein: hydrogen bonding, electrostatic, and hydrophoboic interactions (Anfinsen & Scheraga, 1975). Although it is now generally agreed that the main contribution results from the hydrophobic effect, by which the nonpolar groups of a protein tend to be shielded from the aqueous phase, resulting in their being in the inside of the protein, it is clear that the other two interactions are also important in the final stabilization of the native structure. Therefore, any agent which disrupts the balance between these three types of interactions will induce conformational changes in the protein.

The HFIP molecule is amphipathic as it contains both hydrophobic trifluoromethyl groups and an hydrophilic hydroxyl group. In the above, we have seen that the unfolding of the protein upon HFIP addition does not take place in a single process. At the beginning of the HFIP addition—between the HFIP/water ratios 0:1 and 1:2—small changes occur in both the CD and NMR spectra. Our studies suggest that during this first stage, the secondary structure of the protein is very little, if at all, affected. However, modifications occur in the tertiary structure of the molecule and, in particular, with aromatic groups located at or near the surface of the protein. Upon further addition of HFIP, between the HFIP/water ratios 1:2 and 2:1, there is a dramatic change in the secondary structure of the molecule, and the final conformational state at high alcohol concentrations is of the random type, in which all the groups—nonpolar and polar—are exposed to the solvent.

The preceding observations can be best explained by considering that, as a result of the HFIP addition, the hydrophobic interactions—and to a certain extent the hydrogen-bond interactions—between groups in the protein are replaced by hydrophobic and hydrogen-bond interactions between the peptide groups and the solvent, resulting in a modification of

the conformations and mobilities of these groups, as seen from both the CD and NMR spectra. Of course, one may expect that the effect will take place in regions of the protein which are easily accessible to the solvent. It is, therefore, not surprising that tryptophan-29 and tyrosine-35, which are known to be solvent exposed or accessible, are the first ones to undergo these changes. It is only in the second stage when a sufficient amount of HFIP is added to counterbalance the intramolecular interactions between groups located in the inside of the protein that the major conformational change takes place. At this stage, it is not clear whether or not this second step in the unfolding process takes place as a result of the structural modifications which occur in the first step, resulting in a greater accessibility of the inside of the protein to the solvent. In fact, even in very rigid molecules, the solvent may come in contact with groups located in the inside. This is because proteins undergo fluctuations, which may result in short-lived conformations in which even nonpolar groups may become temporarily exposed to the solvent. Therefore, at some critical point at which the alcohol–water composition is such that the internal hydrophobic interactions can be compensated by hydrophobic interactions between the peptide groups and the solvent, the protein molecule does not refold any more.

At this stage, it is important to ask the question how the HFIP addition may alter the interactions in the protein. Two main possibilities are generally considered. Either HFIP binds preferentially to the protein and accumulates in domains around some peptide units, resulting in a kind of alcohol solubilization of the latter, or the nature of the water and, consequently, of the solvent is modified as a result of the HFIP addition. The first effect was found to be operative in the titration of lysozyme with chloroethanol (Timasheff, 1970). It was shown that a larger portion of alcohol was located near the protein than in the solvent. In this study, we have shown that at identical HFIP/water ratios (for instance, 1:1.6 in Figure 1) during unfolding and refolding, the spectra are exactly identical and independent of the protein concentration, suggesting that the conformational changes observed at the HFIP/water ratios higher than 1:1.6 are due to changes in the solvent properties. However, this does not mean that specific interactions between the alcohol and protein are absent. They may be masked by the nonspecific solvent effect. These specific interactions may also play an important role in the changes which are observed at HFIP/water ratios lower than 1:2. However, at these lower ratios, the spectral changes are too small to allow a distinction between a specific alcohol–protein effect and a nonspecific solvent effect.

The Interaction of $NaDodSO_4$ with the Neurotoxin Is Different from That Observed with HFIP and TFE. We have shown that only three molecules of $NaDodSO_4$ per molecule of protein are needed to fully induce the quasi-unfolded conformation of the protein. This conformation is not affected upon addition of water, suggesting a specific interaction between the denaturant and the protein. It is known that the binding of $NaDodSO_4$ to a protein is able to induce various conformational states depending on the protein (Jirgensons & Capetillo, 1970; Visser & Blout, 1971). In these states, one or more $NaDodSO_4$ molecules bind strongly to the protein, and the hydrophobic tails of $NaDodSO_4$ are partially or completely hidden in the interior of the protein (Yonath et al., 1977). Clearly, it is mainly the replacement of the hydrophobic interactions between groups in the protein by similar interactions between protein groups and the $NaDodSO_4$ hydrophobic tails that induces the conformational changes of the protein.

Addition of a small amount of HFIP to the $NaDodSO_4$-denatured cobrotoxin caused release of the $NaDodSO_4$ from the protein, leading to a reformation of the native protein structure. This effect may be related to a relatively stronger $NaDodSO_4$–HFIP interaction as compared to the $NaDodSO_4$–protein interaction, eventually leading to the formation of $NaDodSO_4$–HFIP clusters in which the hydrophobic tail of $NaDodSO_4$ is buried by the CF_3 groups of HFIP. This would then restrict, partially or totally, any interaction between $NaDodSO_4$ and cobrotoxin. The concentration of HFIP in this case was too low to induce itself any substantial conformational transition of the neurotoxin.

In this report, we have shown that denaturants like TFE, HFIP, and $NaDodSO_4$ induce unfolding of the cobrotoxin molecule. They act by changing the balance between the various interactions which stabilize the protein in pure aqueous solution. However, there are differences in the mechanisms by which these denaturants act. In the case of $NaDodSO_4$, the unfolding results from a specific interaction between the denaturant and the protein, while in the case of the fluoro alcohols, TFE and HFIP, the main unfolding effect results from a change in the solvent properties.

It is indeed well-known that water itself plays an important role in the formation of a native protein structure. When water is in contact with hydrophobic groups, there is a structure of the former which results in an unfavorable decrease in entropy. Therefore, clustering of hydrophobic groups in the inside of a protein generally is a thermodynamically favorable process. Addition of alcohol to the water will induce changes in the properties of the latter and change the thermodynamic balance of the protein–solvent system, leading eventually to the unfolding of the protein. This effect is strictly related with the internal nature of the denaturant, since even almost 90% of concentrated TFE or other alcohol mixtures (Chen et al., 1977) do not affect the structure of cobrotoxin as do HFIP and especially $NaDodSO_4$.

Thus, an exact explanation of protein unfolding–refolding phenomena in vitro caused by different solvents, salts, or temperature primarily requires proper knowledge of the water structure, both about the external shell surrounding protein and in the internal "lattice" of water molecules hidden in the interior of a protein [see, e.g., Franks (1978)]. This problem obviously is intricate, and our understanding is far from complete.

References

Anfinsen, C. B., & Scheraga, H. A. (1975) *Adv. Protein Chem. 29*, 205.

Arseniev, A. S., Balashova, T. A., Utikin, Y. N., Tsetlin, V. I., Bystrov, V. F., Ivanov, V. T., & Ovchinnikov, Yu. A. (1976) *Eur. J. Biochem. 71*, 595.

Bailey, G. S., Lee, J., & Tu, A. T. (1979) *J. Biol. Chem. 254*, 8922.

Bystrov, V. F., Arseniev, A. S., & Gavrilov, Y. D. (1978) *J. Magn. Reson. 30*, 151.

Chen, Y. H., Lo, T. E., & Yang, J. T. (1977) *Biochemistry 16*, 1826.

Chicheportiche, R., Rochat, C., Sampieri, F., & Lazdunski, M. (1972) *Biochemistry 11*, 1681.

Chou, P. Y., & Fasman, G. D. (1974) *Biochemistry 13*, 222.

Creighton, T. E. (1978) *Prog. Biophys. Mol. Biol. 33*, 231.

Endo, T., Inagaki, F., Hayashi, K., & Miyazawa, T. (1979) *Eur. J. Biochem. 102*, 417.

Eterovic, V. A., & Ferchmin, P. A. (1977) *Int. J. Pept. Protein Res. 10*, 245.

Fox, J., & Tu, A. T. (1979) *Arch. Biochem. Biophys. 193*, 407.

Franks, F. (1978) in *Characterization of Protein Conformation and Function* (Franks, F., Ed.) p 37, Symposium Press, London.
Fung, C. M., Chang, C. C., & Gupta, R. K. (1979) *Biochemistry 18*, 457.
Gabel, D., Rasse, D., & Scheraga, H. A. (1976) *Int. J. Pept. Protein Res. 8*, 237.
Harada, I., Takamatsu, T., Shimanouchi, T., Miyazawa, T., & Tamiya, N. (1976) *J. Phys. Chem. 80*, 1153.
Hseu, T. H., Liu, Y. C., Wang, C., Chang, H., Hwang, D. M., & Yang, C. C. (1977) *Biochemistry 16*, 2999.
Hseu, T. H., Chang, H., Hwang, D. M., & Yang, C. C. (1978) *Biochim. Biophys. Acta 537*, 284.
Inagaki, F., Miyazawa, T., Hori, H., & Tamiya, N. (1978) *Eur. J. Biochem. 89*, 433.
Jirgensons, B., & Capetillo, S. (1970) *Biochim. Biophys. Acta 274*, 1.
Karlsson, E., & Sundelin, J. (1976) *Toxicon 14*, 295.
Lauterwein, J., Wüthrich, K., Schweitz, H., Vincent, J.-P., & Lazdunski, M. (1977) *Biochem. Biophys. Res. Commun. 76*, 1071.
Lee, C. Y. (1972) *Annu. Rev. Pharmacol. 12*, 265.
Low, B. W., Preston, H. S., Sato, A., Rosen, L. S., Searle, F. E., Rudko, A. D., & Richardson, J. S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2991.
Menez, A., Bouet, R., Tamiya, N., & Fromageot, P. (1976) *Biochim. Biophys. Acta 453*, 121.
Nèmethy, G., & Scheraga, H. A. (1977) *Q. Rev. Biophys. 19*, 233.
Parrish, J., & Blout, E. R. (1971) *Biopolymers 10*, 1431.
Parrish, J., & Blout, E. R. (1972) *Biopolymers 11*, 1001.
Ryden, L., Gabel, D., & Eaker, D. (1973) *Int. J. Pept. Protein Res. 5*, 261.
Seto, A., Sato, S., & Tamiya, N. (1970) *Biochim. Biophys. Acta 214*, 483.
Tanaka, S., & Scheraga, H. A. (1977) *Macromolecules 10*, 305.
Timasheff, S. N. (1970) *Acc. Chem. Res. 3*, 62.
Tsernoglou, D., & Petsko, G. A. (1976) *FEBS Lett. 68*, 1.
Tsernoglou, D., & Petsko, G. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 971.
Tsetlin, V. I., Arseniev, A. S., Utkin, Y. N., Gurevich, A. S., Senyavina, L. B., Bystrov, V. F., Ivanov, V. T., & Ovchinnikov, Y. A. (1979) *Eur. J. Biochem. 94*, 337.
Tu, A. T. (1973) *Annu. Rev. Biochem. 42*, 337.
Urry, D. W., Long, M. M., Ohnishi, T., & Jacobs, M. (1974) *Biochem. Biophys. Res. Commun. 61*, 1427.
Visser, L., & Blout, E. R. (1971) *Biochemistry 10*, 743.
Visser, L., & Louw, A. I. (1978) *Biochim. Biophys. Acta 533*, 80.
Woody, R. W. (1974) *Pept. Polypeptides Proteins, Proc. Rehovot Symp., 2nd*, 338.
Woody, R. W. (1978) *Biopolymers 17*, 1451.
Wüthrich, K. (1976) *NMR in Biological Research: Peptides and Proteins*, Elsevier/North-Holland, New York.
Yang, C. C. (1965) *J. Biol. Chem. 240*, 1616.
Yang, C. C. (1974) *Toxicon 12*, 1.
Yang, C. C. (1976) *Snake 8*, 57.
Yang, C. C., Chang, C. C., Hamaguchi, K., Ikeda, K., Hayashi, K., & Suzuki, T. (1967) *J. Biochem. (Tokyo) 61*, 272.
Yang, C. C., Chang, C. C., Hayashi, K., Suzuki, T., Ikeda, K., & Hamaguchi, K. (1968) *Biochim. Biophys. Acta 168*, 373.
Yonath, A., Podjarny, A., Honig, B., Sielecki, A., & Traub, W. (1977) *Biochemistry 16*, 1418.

Purification and Characterization of Avian Dopamine β-Hydroxylase†

Robert A. Long, Roger M. Weppelman,* Joyce E. Taylor, Richard L. Tolman, and George Olson

ABSTRACT: Dopamine β-hydroxylase (EC 1.14.17.1) has been purified from the chromaffin granules of avian adrenals. The enzyme has a molecular mass of approximately 320K daltons and consists of four apparently identical subunits joined in pairs by disulfide bonds. Analysis of the products formed from dopamine tritiated in the β position indicated that 1.72 times as much tritium was retained in norepinephrine as was released as water. Ferrocyanide could serve as a reductant, but ascorbate at equal concentrations afforded higher rates. The enzyme had a pH optimum of 5–6 and was activated by either fumarate or acetate, with fumarate being far more effective. Kinetic experiments varying the concentrations of the substrates ascorbate and dopamine and those of the products dehydroascorbate and norepinephrine suggested that the mechanism was un-uni bi-uni ping pong. By this mechanism, the enzyme released dehydroascorbate after being irreversibly reduced by ascorbate and then sequentially bound oxygen and dopamine and released the product norepinephrine. The enzyme was inhibited by high but probably physiological concentrations of the substrate ascorbate and was activated by low concentrations of the product dehydroascorbate. Ascorbate inhibition was noncompetitive with dopamine, and dehydroascorbate activation was due to an increase in the enzyme's affinity for ascorbate with little or no change in its V_{max}. Substrate inhibition by ascorbate and product activation by dehydroascorbate might together ensure that the rate of norepinephrine synthesis in vivo remains relatively unaffected by changes in the ratio of ascorbate to dehydroascorbate within chromaffin granules.

The monoxygenase dopamine β-hydroxylase [3,4-dihydroxyphenylethylamine,ascorbate:oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1], which catalyzes the conversion of dopamine to norepinephrine, is present in the chromaffin granules of adrenal medullary tissue (Levin et al., 1960). To date, this enzyme has been purified from bovine (Friedman & Kaufman, 1965), ovine (Rush & Geffen, 1972), and rat (Grzanna & Coyle, 1976) adrenal medullas as well as from human pheochromocytoma (Stone et al., 1974) and

† From Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065. *Received February 27, 1981.*

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