Circular Dichroism Study of the Unfolding–Refolding of a Cardiotoxin from Taiwan Cobra (*Naja naja atra*) Venom†

Andrzej Gałat,‡,§ Chen-chung Yang,‖ and Elkan R. Blout*,§

Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115, and Institute of Molecular Biology, National Tsing Hua University, Hsinchu, Taiwan 300, Republic of China

Received September 26, 1984; Revised Manuscript Received April 1, 1985

ABSTRACT: Circular dichroism spectroscopy has been used to study the unfolding–refolding process of a cardiotoxin from Taiwan cobra (*Naja naja atra*) venom upon addition of fluoroalcohols or sodium dodecyl sulfate (SDS) to its aqueous solution. In these experiments, the disulfide bridges remained intact. The unfolding process has been found to be reversible both for fluoroalcohols and for SDS unfolding. The reversibility of the unfolding–refolding process of cardiotoxin in aqueous mixtures of fluoroalcohols was dependent on the volume per volume ratio of alcohol to water. SDS did not unfold the secondary structures of cardiotoxin whereas its tertiary structure was affected. If the SDS concentration in aqueous solution exceeded the critical micelle concentration value of SDS, a quasi-refolded state of cardiotoxin was observed. The mechanism of unfolding–refolding is discussed in terms of molecular interactions which might govern the protein conformation in solution.

Cardiotoxin is one of the major protein components present in the venom of Taiwan cobra (*Naja naja atra*). Its sequence was determined by Narita & Lee (1970) and is shown in Figure 1. The cardiotoxin molecule consists of a single peptide chain containing 60 amino acids, and the molecule is divided into four loops by four disulfide bridges. It has been shown that while only one of the three tyrosines is exposed to solvent (Keung et al., 1975), two tyrosines are easily nitrated. Upon nitration, the immunological activity is retained, while reduction of disulfides of the nitrated cardiotoxin results in complete loss of its immunological activity (Keung et al., 1975). Hung & Chen (1977) reported that cardiotoxin's conformation is retained if an aqueous solution is changed to a less polar alcoholic solution. They showed that the native structure of cardiotoxin was not affected by interactions with ethylene glycol or *n*-propyl alcohol, and only a rise in temperature to 88 °C or 6.5 M guanidinium chloride caused its denaturation.

In this paper, we show that some fluoroalcohols were capable of altering substantially the conformation of cardiotoxin, while other alcohols did not affect its native structure. Sodium dodecyl sulfate (SDS)[1] did not cause a large conformational change of cardiotoxin; this finding is considerably different than what has been observed with other proteins (Jirgensons, 1976; Jirgensons & Capetillo, 1970; Visser & Blout, 1971; Yonath et al., 1977). These effects are discussed in terms of a cardiotoxin unfolding–refolding phenomenon [for recent reviews of protein unfolding–refolding processes, see Ghelis & Yon (1982), Kim & Baldwin (1982), and Scheraga (1983)].

Experimental Procedures

Materials. Cardiotoxin analogue III was prepared and purified from the venom of the Taiwan cobra (*Naja naja atra*) (Yang et al., 1981). Prior to each measurement, the aqueous solution of cardiotoxin was filtered through a Millipore HATF 01300 filter. The protein concentration was determined spectrophotometrically using $E^{1\%}_{1cm}$ = 6.82 at 280 nm (Hung & Chen, 1977).

Hexafluoroisopropyl alcohol was purchased from Eastman; TFP was purchased from Aldrich; TFE and SDS were supplied by Fisher Scientific Co.

Methods. Circular dichroism was determined by using 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 mm. The molar absorbance of samples was adjusted to be within the range 0.5–1.5 absorbance unit. CD spectra were measured in silica cuvettes with path lengths varying from 0.1 mm to 2 cm. The spectra were measured at least 2 times on different days on newly prepared samples and the results averaged. The base line was measured each time for each spectrum. The CD 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 CD reading in degrees per full scale, l is the path length of the cuvette, and c is the mean residual concentration in moles per liter. The mean residue ellipticity is expressed in units of degrees centimeter squared per decimole with a mean residue weight of 112.3.

Results and Discussion

Conformation of Cardiotoxin. There is not much known about the tertiary structure of cardiotoxin; however, it should have many features in common with other snake toxins whose x-ray analyses have been performed (Kimball et al., 1979; Low, 1979; Tsernoglou & Petsko, 1976, 1977). For example, it has been shown in a number of papers dealing with NMR (Lauterwein et al., 1978; Steinmetz et al., 1981), conformation predictions (Dufton & Hider, 1977), and CD (Drake et al.,

† This work was supported in part by U.S. Public Health Service Grant AMO7300.

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

§ Harvard Medical School.

‖ National Tsing Hua University.

[1] Abbreviations: CD, circular dichroism; HFIP, hexafluoroisopropyl alcohol; NMR, nuclear magnetic resonance; TFE, 2,2,2-trifluoroethanol; TFP, 2,2,3,3-tetrafluoropropan-1-ol; SDS, sodium dodecyl sulfate; UV, ultraviolet; cmc, critical micelle concentration.

0006-2960/85/0424-5678$01.50/0

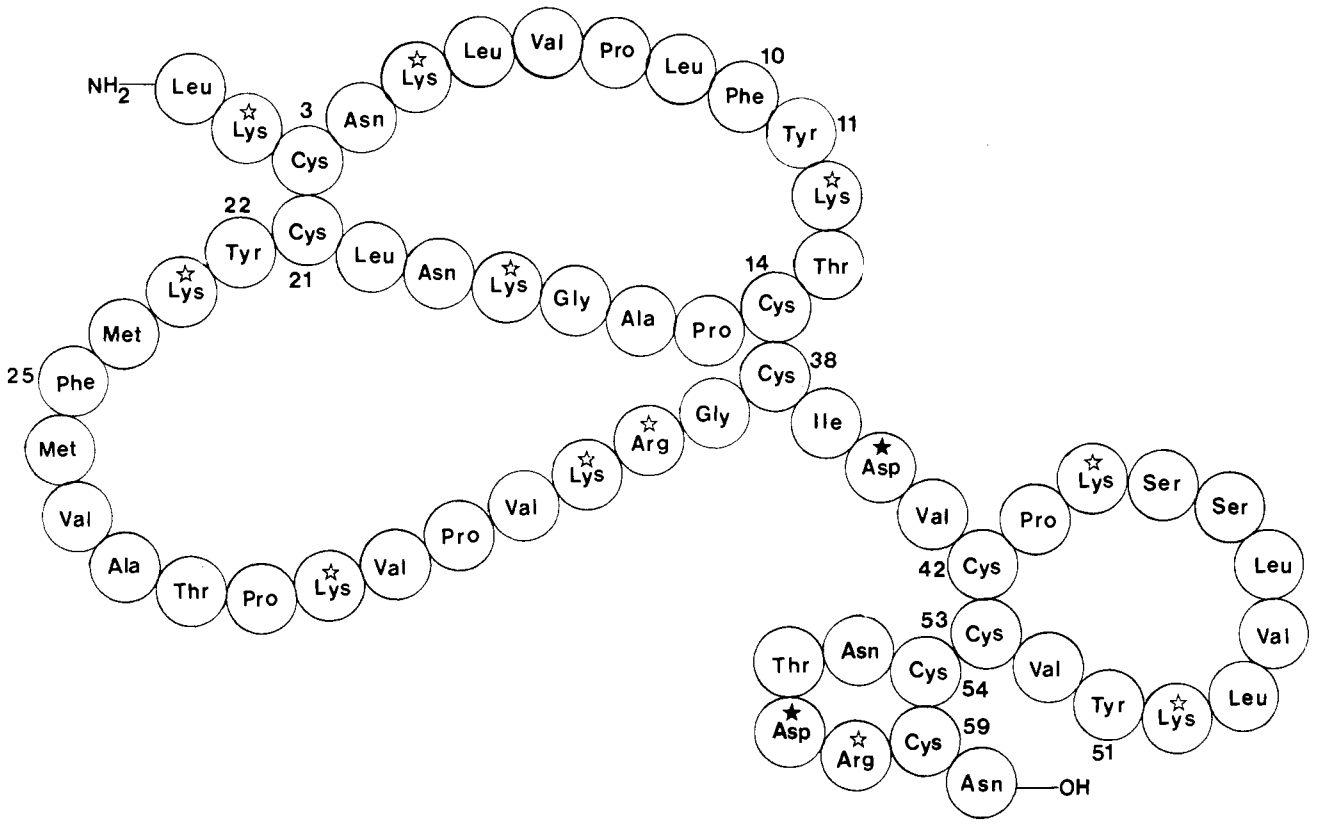

FIGURE 1: Sequence of cardiotoxin from Taiwan cobra (*Naja naja atra*) venom; the white stars indicate basic amino acids while the black stars indicate acidic amino acid residues.

1980; Dufton & Hider, 1982) that cardiotoxins obtained from different snakes exhibited similar spectral patterns which may suggest homology in their secondary and tertiary structures.

Using laser Raman spectroscopy and a conformation predictive method of Chou & Fasman (1974), it has been suggested by Hseu et al. (1977) that the cardiotoxin molecule consists mainly of antiparallel β sheets with only two β turns. Also, due to the fact that the cardiotoxin molecule lacks any α helix (Hseu et al., 1977), which is known to exhibit moderate or strong CD bands (Greenfield & Fasman, 1969), the contributions of aromatic chromophores to the far-UV region of the CD spectra are much more pronounced than for other globular proteins. For example, in a thorough study by Visser & Louw (1978), it has been shown that cardiotoxins obtained from various snakes exhibit somewhat different CD spectra especially in the near-UV region. These differences can be attributed to variable amino acid compositions of cardiotoxins isolated from different snakes (Yang, 1978) and to various spatial orientations of aromatic chromophores present in the proteins. These two factors are known to be responsible for distortion of the CD spectra below 250 nm of low molecular weight globular proteins (Strickland, 1974; Gałat et al., 1981).

The CD spectrum of cardiotoxin in water is the same as that reported by Hung & Chen (1977). The CD bands in the near-UV region are related to three tyrosines (Tyr-11, Tyr-22, and Tyr-51) and to two phenylalanines (Phe-10 and Phe-25). Also in this spectral region, some contributions of disulfide electronic transitions might be expected. The small band at about $\lambda = 234$ nm is due to a combination of the strong ^1L_A transition of Tyr residues, the $\pi \rightarrow \pi^*$ transition of Phe residues expected in this spectral region, and the $n \rightarrow \pi^*$ transition of the polypeptide chain (Bush & Gibbs, 1972; Strickland, 1974; Woody, 1978; Ziegler & Bush, 1971).

The ^1L_A transition usually shows a positive Cotton effect while the $n \rightarrow \pi^*$ transitions of different secondary structures

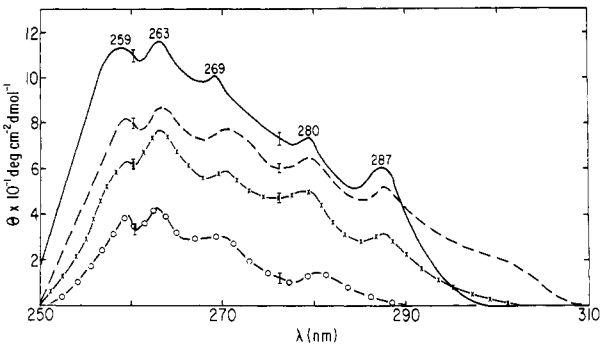

FIGURE 2: Near-UV circular dichroism spectra of cardiotoxin (mean residue ellipticity) in HFIP/H_2O mixtures: (—) 100% H_2O; (- - -) 1/8 ratio; (-×-) 1/6 ratio; (-○-) 1/5 ratio.

show negative Cotton effects. Hence, their mutual overlap leads to an unusual shaped CD curve in the far-UV region; a curve resolution process showed that a strong positive CD band may exist at about $\lambda = 218$ nm, $R = 0.168$ DBM and the other CD band at about $\lambda = 216$ nm, $R = -0.439$ DBM. The two short-wavelength CD bands are certainly related to the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions of amide chromophores of the different secondary structures present in the cardiotoxin molecule. Further discussion will be focused on the relation between changes of solvent with changes of the CD spectra and their implications for the cardiotoxin unfolding–refolding process.

Conformation of Cardiotoxin in HFIP/H_2O Mixtures. When HFIP was added to an aqueous solution of cardiotoxin, some slight changes were observed in the near-UV region, but only when the HFIP/H_2O ratio was lower than 1/10 was there observed a marked decrease of the intensity of the CD bands in the near-UV region (see Figure 2 and Table I). Additional amounts of HFIP further decreased the CD bands in the near-UV region, and at an HFIP/H_2O ratio of 1/4, all aro-

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

	I		II		III		IV		V	
HFIP/H_2O (v/v) ratio	λ	θ	λ	θ	λ	θ	λ	θ	λ	θ
0/100	287	60	280	75	269	100	263	115	259	110
1/10	287	50	280	65	269	80	263	95	259	80
1/8	287	50	280	60	269	80	263	85	259	80
1/6	287	30	280	50	269	55	263	75	259	60
1/5	287	15	280	15	269	30	263	40	259	30
1/4[b]										
reversed dilution										
1/10	287	45	280	75	269	100	263	120	259	115

[a] The positions of the Cotton effects (λ) are expressed in nanometers, the molecular ellipticity values (θ) are expressed in degrees centimeter squared per decimole, and the standard deviation of these values in the near-UV region is ±5 deg·cm²·dmol⁻¹. [b] No absorption at this ratio.

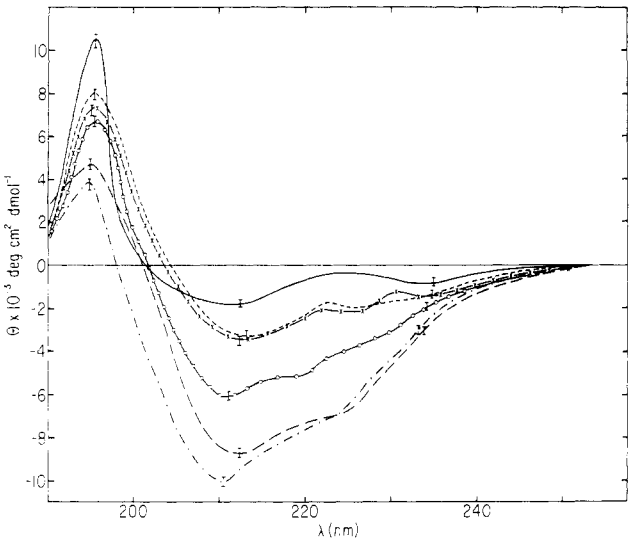

FIGURE 3: Far-UV circular dichroism spectra of cardiotoxin (mean residue ellipticity) in HFIP/H_2O mixtures: (—) 100% H_2O; (---) 1/8 ratio; (-×-) 1/6 ratio; (-○-) 1/5 ratio; (-·-) 1/2 ratio; (--) 2/1 ratio.

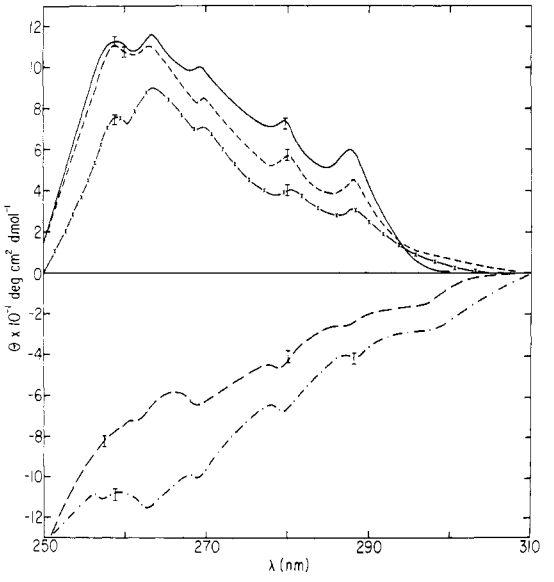

FIGURE 4: Circular dichroism spectra of cardiotoxin (mean residue ellipticity) in TFE/H_2O mixtures: (—) 0/10 ratio; (---) 1/1 ratio; (-×-) 2/1 ratio; (--) 5/1 ratio; (-·-) 10/0 ratio.

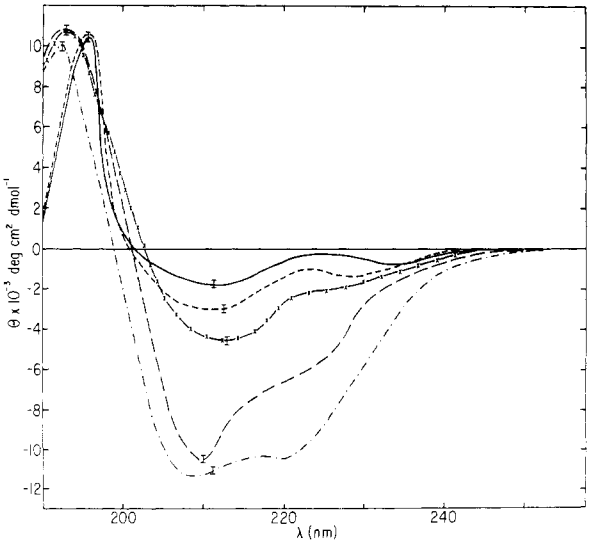

FIGURE 5: Circular dichroism spectra of cardiotoxin (mean residue ellipticity) in TFE/H_2O mixtures. Same representation used as in Figure 4.

matic contributions to the near-UV CD spectrum disappeared.

In the far-UV region, addition of HFIP to aqueous solutions of cardiotoxin resulted in a concurrent increase of the intensity of the bands originally positioned at λ = 234 and 214 nm, which finally led to a broad CD band with the maximum at λ = 210 nm and a substantial decrease in the Cotton effect at about λ = 195 nm (Figure 3, Table II).

Lack of Cotton effects in the near-UV region below a HFIP/H_2O ratio of 1/4 allows one to conclude that the tertiary structure of the protein has been disrupted by HFIP. The CD spectra in the far-UV region exhibited a characteristic profile for an α helix, although the magnitudes of the Cotton effects were low. This result suggests that some residual amounts of secondary structures were still retained due to the constraint of the short cardiotoxin loops. When the HFIP/H_2O ratio reached 2/1, the CD spectrum resembled that of an α helix. However, the intensities of the bands at about λ = 225 and 210 nm were both higher than the intensity of the band at about λ = 192 nm. This is not characteristic for an HFIP-induced α helix in synthetic polypeptides and globular proteins (Parrish & Blout, 1972; Timasheff, 1970). Addition of buffer to such a solution caused refolding of cardiotoxin to its original conformation.

Conformation of Cardiotoxin in TFE/H_2O Mixtures. Addition of TFE to an aqueous solution of cardiotoxin influenced its conformation only at relatively high concentrations of TFE. Similar behavior has been noted for cobrotoxin (Gałat et al., 1981) for which 50% TFE did not change the secondary structure of the neurotoxin significantly. Cardiotoxin, even at a TFE/H_2O ratio of 1/1, still maintained its tertiary structure almost intact, since the intensity of the Cotton effects in the near-UV region was only slightly decreased (Table III). Further increase of the TFE concentration to 2/1 caused about a 20–30% decrease in the intensity of the near-UV CD bands (see Figure 4), though in the far-UV

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

	I		II		III	
HFIP/H_2O (v/v) ratio	λ	θ	λ	θ	λ	θ
0/100	234	−800	214	−1600	195	10500
1/8	226	−2000	212	−3300	195	7900
1/6	226	−2300	212	−3400	195	7300
1/4			210	−6100	195	6600
1/2			210	−10600	195	3800
1/1			210	−9400	192	4700
2/1	220	−7600	210	−8800	192	4700
reversed dilution						
1/10	228	−1500	212	−3400	195	10300

[a] The positions of the Cotton effects (λ) are expressed in nanometers, the molecular ellipticity values (θ) are expressed in degrees centimeter squared per decimole, and the standard deviation of these values is ±200 deg·cm²·dmol⁻¹ for the bands positioned within 250–210 nm and ±500 deg·cm²·dmol⁻¹ for the bands below λ = 210 nm.

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

	I		II		III		IV		V	
TFE/H_2O (v/v) ratio	λ	θ	λ	θ	λ	θ	λ	θ	λ	θ
0/10	287	60	280	75	269	100	263	115	259	110
1/5	287	55	280	65	269	80	263	115	259	110
1/2	287	45	280	65	269	80	263	115	259	110
1/1	287	40	280	55	269	80	263	110	259	110
2/1	287	30	280	40	269	70	263	90	259	75
10/0	287	−40	280	−65	269	−100	263	−115	259	−110
reversed dilution with H_2O										
1/2	287	50	280	65	269	75	263	115	259	110
1/5	287	60	280	70	269	80	115	259	259	110

[a] The positions of the Cotton effects (λ) are expressed in nanometers, the molecular ellipticity values (θ) are expressed in degrees centimeter squared per decimole, and the standard deviation of these values in the near-UV region is ±5 deg·cm²·dmol⁻¹.

region more pronounced effects were noted (Figure 5, Table IV).

The decrease in absorption of circularly polarized light by aromatic chromophores at this TFE/H_2O ratio also caused some alteration in the far-UV Cotton effects. Although the intensity of the Cotton effects in the near-UV region was rather small due to the forbidden character of the magnetic dipole moment for the tyrosine and phenylalanine ^1L_b electronic transitions (Strickland, 1974), the contribution of the ^1B_b and ^1B_a transitions of both tyrosines and phenylalanines to the far-UV spectra might be of considerable magnitude, especially in the case of a small protein such as cardiotoxin. This is the reason why some small, or even seemingly insignificant, changes in the intensity of the Cotton effects in the near-UV region might have an influence on the intensity of the amide Cotton effects in the far-UV region.

In 100% TFE, the native structure of cardiotoxin was disrupted and, from the far-UV CD data, the unfolded state of cardiotoxin appeared to be related to an α helix. However, it was curious that pronounced near-UV Cotton effects were noted whose signs were opposite to those observed in aqueous solution. Also, the molecular ellipticity of the band at about λ = 192 nm was smaller than the molecular ellipticity values of the bands at about λ = 208 and 225 nm, which suggests that, due to geometrical constraints within the short loops, some parts of the polypeptide chain were converted into an unusually deformed α helix.

Interaction between Cardiotoxin and SDS. Stepwise addition of SDS to an aqueous solution of cardiotoxin changed its CD spectra in a different manner than with fluoroalcohols (Figures 6 and 7). With SDS, there were some small changes in the molecular ellipticity values of the Cotton effects in the far-UV region, but significant broadening of the band at λ = 195 nm occurred (see Table V and Figure 6). Beyond the 0.6/1 cardiotoxin/SDS molar ratio, no significant changes of

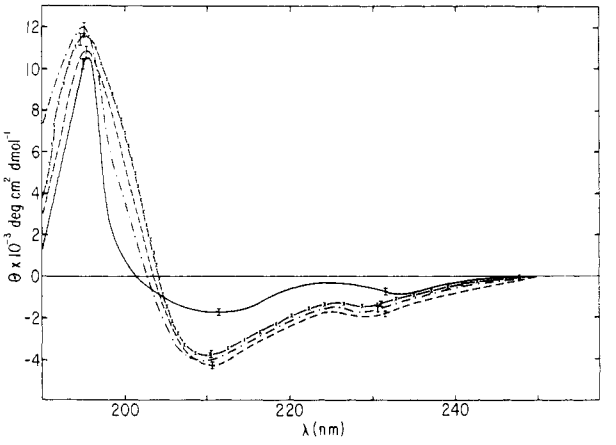

FIGURE 6: Circular dichroism spectra of cardiotoxin (mean residue ellipticity) in aqueous solution at different cardiotoxin/SDS ratios (mol/mol): (—) H_2O; (- - -) from 6/1 to 2/1 ratios; (-×-) 1.25/1 ratio; (-·-) 0.6/1 ratio.

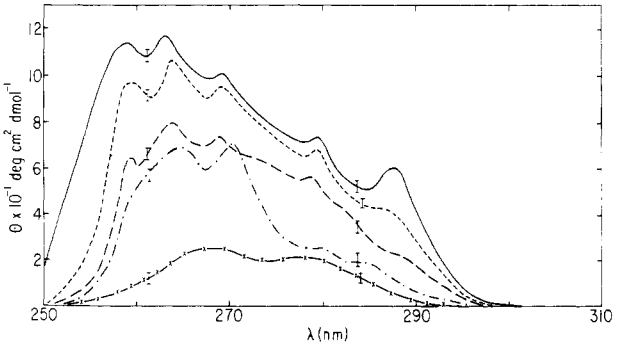

FIGURE 7: Circular dichroism spectra of cardiotoxin (mean residue ellipticity) in aqueous solution in different cardiotoxin/SDS ratios (mol/mol): (—) 100% H_2O; (- - -) 6/1 ratio; (– –) 2/1 ratio; (-×-) 1.25/1 ratio; (-·-) 0.6/1 ratio.

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

TFE/H_2O (v/v) ratio	I		II		III	
	λ	θ	λ	θ	λ	θ
0/10	234	−740	214	−1400	195	10900
1/5	234	−780	214	−1700	195	10800
1/2	229	−1100	212	−2800	195	9800
1/1	229	−1300	212	−2900	195	10800
2/1	228	−3000	210	−4300	195	11000
10/0	220	−10500	208	−11200	192	10000
reversed dilution with H_2O						
1/2	228	−1100	212	−2900	195	10000
1/5	234	−800	214	−1600	195	10800

[a] The positions of the Cotton effects (λ) are expressed in nanometers, the molecular ellipticity values (θ) are expressed in degrees centimeter squared per decimole, and the standard deviation of these values is ±200 deg·cm²·dmol⁻¹ for the bands positioned within 250–210 nm and ±500 deg·cm²·dmol⁻¹ for the bands below λ = 210 nm.

Table V: Positions and Ellipticity Values of Cobra Cardiotoxin in the Changes of SDS/Protein Ratios[a]

cardiotoxin/SDS (mol/mol) ratio	I		II		III	
	λ	θ	λ	θ	λ	θ
10/1	234	−800	214	−1600	195	10500
4/1	227.5	−1200	210	−3200	195	10000
2/1	227.5	−1650	210	−4050	195	10600
1.5/1	227.5	−1300	210	−3800	195	11500
0.8/1	227.5	−1700	210	−4000	195	12000
0.6/1	227.5	−1900	210	−4200	195	12100

[a] The positions of the Cotton effects (λ) are expressed in nanometers, the molecular ellipticity values (θ) are expressed in degrees centimeter squared per decimole, and the standard deviation of these values is ±200 deg·cm²·dmol⁻¹ for the bands positioned within 250–210 nm and ±500 deg·cm²·dmol⁻¹ for the bands below λ = 210 nm.

Table VI: Positions and Ellipticity Values of Cobra Cardiotoxin in the Changes of SDS/Protein Ratios[a]

cardiotoxin/SDS (mol/mol) ratio	I		II		III		IV		V	
	λ	θ	λ	θ	λ	θ	λ	θ	λ	θ
6/1	287	40	280	65	269	90	263	105	259	95
3/1	287	30	280	55	269	80	263	100	259	80
2/1	287	20	280	50	269	70	263	75	259	60
1.5/1	287	25	280	50	269	80	263	75	259	60
0.8/1	287	30	280	60	269	80	263	95	259	65
0.6/1	287	20	280	30	269	70	263	65	259	50
HFIP/H_2O addition										
1/25	287	20	280	30	269	50	263	65	259	50

[a] The positions of the Cotton effects (λ) are expressed in nanometers, the molecular ellipticity values (θ) are expressed in degrees centimeter squared per decimole, and the standard deviation of these values in the near-UV region is ±5 deg·cm²·dmol⁻¹.

the far-UV region were observed upon further increase of SDS concentration. However, in the near-UV region, some other effects were noted. When this ratio reached 1.5/1, the intensity of the Cotton effects in the near-UV region decreased substantially, and at the ratio of 1.25/1, the intensity of the Cotton effects reached its lowest value (see Table VI). Further increase of SDS concentration caused a reverse increase of the Cotton effects, and even at a very low cardiotoxin/SDS ratio, e.g., 0.6/1, the CD spectrum was similar to that at a 2/1 ratio. Addition of HFIP to such a solution in a 1/10 (v/v) HFIP/H_2O ratio did not change the conformation of the protein as has been noted in an analogous experiment with cobrotoxin where quasi-refolding of the protein occurred (Gałat et al., 1981). These results suggest that, in the beginning of the SDS interaction with the toxin (when the SDS concentration is low), a limited interaction took place between negatively charged SDS molecules and the positively charged Lys and Arg side chains present on the cardiotoxin surface. At these SDS ratios, the secondary structures remained almost intact which suggests that SDS did not enter into the interior of the toxin. This is in contrast to the behavior of cobrotoxin in an analogous experiment (Gałat et al., 1981) and to a number of other globular proteins where large changes of their conformations have been noted (Jirgensons, 1976; Jirgensons & Capetillo, 1970; Visser & Blout, 1971; Yonath et al., 1977). However, the tertiary structure of cardiotoxin was, in fact, affected. The electronegatively charged SDS molecules must be attracted by positively charged side chains of Lys and Arg residues [see, for comparison, Jones et al. (1984)], which caused an acceleration of their rotational movements. Because each Tyr residue is next to a Lys residue and Phe-10 is next to Tyr-11, fluctuations of Lys residues should decrease the energetic barrier of rotation of aromatic side chains. The interaction between SDS and the toxin must cause also some change in the dihedral angles of the S–S bridges. We postulate that these effects were the main reasons which caused distortions of the Cotton effects in the near-UV region at certain SDS concentrations. Under these conditions, it appeared that there was a partial unfolding of the peptide segments on the surface of cardiotoxin. However, even at a 1.5/1 (mol/mol) the cardiotoxin/SDS ratio, Cotton effects in the near-UV region were still detectable. Thus, they can be attributed to one of the three Tyr residues which is buried in the interior of the protein (presumably Tyr-22), which is strongly bond to a proton acceptor, and to the disulfide bonds which have a rigid conformation.

At high concentrations of SDS, the detergent molecules aggregate and form micelles which did not interact effectively with cardiotoxin. From our experiments, it is apparent that, at a molar concentration of about 0.3×10^{-3} M SDS, some recovery of the CD bands in the near-UV region was noted (see Figure 7). This SDS concentration value is very close to the cmc of SDS (Mysels & Mysels, 1965) which usually varies within the range 0.5×10^{-3} to 0.9×10^{-3} M depending on temperature, buffer, and protein (Burkhard & Stolzenberg, 1972).

Addition of HFIP to SDS-treated cardiotoxin did not change its conformation as long as the HFIP/H_2O ratio was maintained above 1/10. An increase in HFIP concentration in such solutions caused gradual collapse of the structure of cardiotoxin in a manner analogous to that described under Conformation of Cardiotoxin in HFIP/H_2O Mixtures.

Conformation of Cardiotoxin in EtOH/H_2O plus TFP Mixtures. It is known that the effect of alcohols on a protein depends on the length of the hydrophobic (methylene) chain of the alcohol (Bull & Breese, 1978; Franks & Eagland, 1975). However, this process presumably is not linear because the solubility of different alcohols in water is strongly dependent on the structure of the alcohol. For example, TFP is not miscible with water in room temperature, but addition of ethyl alcohol causes homogeneity of such mixtures. The CD spectrum of cardiotoxin in the mixture of H_2O/EtOH (1/1 v/v) (not shown) was identical with that in water which suggested that with this amount of EtOH there was no change in the conformation of cardiotoxin. Increasing the ethyl alcohol concentration in water did not affect the native conformation of cardiotoxin. Similar behavior was observed by Hung & Chen (1977), who noticed that 1,2-ethanediol and 1-propanol in the ratio 1/1 (v/v) H_2O/alcohol did not influence the native conformation of cardiotoxin. Furthermore, addition of TFP to the mixture H_2O/EtOH (1/1) in any proportion (v/v) did not change the conformation of cardiotoxin.

Comparison between the TFE- and HFIP-Induced Unfolding of Cardiotoxin and Neurotoxin from the Venom of Naja naja atra. It is well-known that cardiotoxins and neurotoxins from snake venoms exhibit considerably different physiological functions (Chang, 1979; Lee & Lee, 1979). Cardiotoxins are cytolytics and destabilizers of cell membrane structures, i.e., those of heart cells, whereas neurotoxins are effective blocking factors for the transmission of nerve impulses due to a specific interaction or distortion of the acetylcholine receptors at the neuromuscular junctions (Lee & Lee, 1979; Tu, 1977; Yang, 1974). It may be assumed provisionally that these different physiologial behaviors are related to the different ratios, basic amino acid residues/acidic amino acid residues, which is a property that distinguishes these two classes of proteins. For example, in cardiotoxin isolated from the venom of Taiwan cobra (*Naja naja atra*), there are nine Lys residues and two Arg residues whose positive charges are balanced partially by two negatively charged Asp residues, while in neurotoxin from the same venom there are three Lys and six Arg residues which are balanced by four Glu and two Asp residues. Also, every aromatic amino acid residue has Lys or Arg as a neighbor. In cardiotoxin, for example, Tyr-22 is linked by a peptide bond to Lys-23, Tyr-51 is next to Lys-50, and Tyr-11 is next to Lys-12. In neurotoxin, Tyr-25 is next to Lys-26, Trp-29 is surrounded by Arg-28 and Arg-30, and Tyr-35 is next to Arg-36 (Yang & Chang, 1976). However, comparison of the CD data of TFE- or HFIP-induced unfolding of these proteins (Gałat et al., 1981; Gałat, 1983)

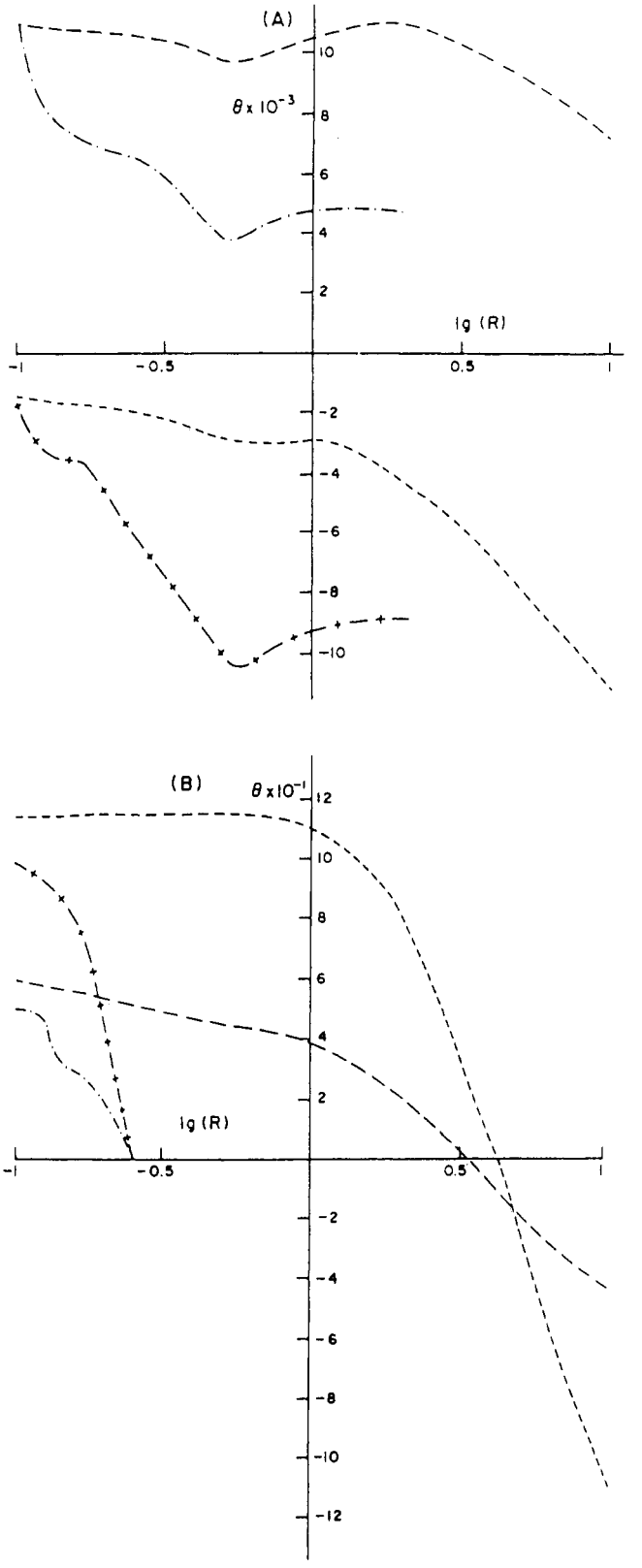

FIGURE 8: Unfolding curves of cardiotoxin. R = alcohol/water (v/v) ratio. HFIP: (–·–) λ = 195 nm (A, top) and λ = 287 nm (B, bottom); (×) λ = 212 nm (A, top) and λ = 263 nm (B, bottom). TFE: (– –) λ = 195 nm (A, top) and λ = 287 nm (B, bottom); (- - -) λ = 212–219 nm (A, top) and λ = 263 nm (B, bottom).

shows that their conformations are influenced differently by the fluoroalcohols. There must be several factors responsible for this difference, but the following two of them are presumably the most important. First, it may be due to the physical properties of the surface of both proteins, e.g., the

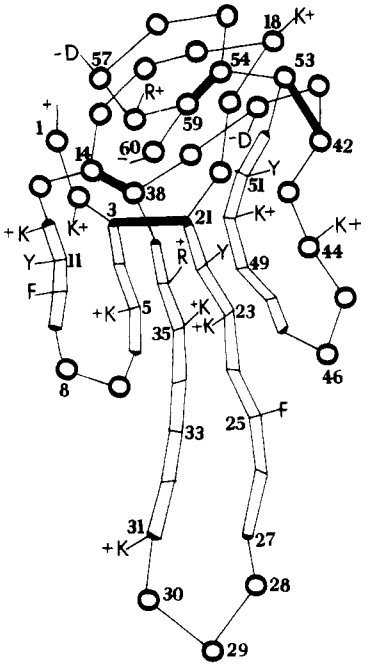

FIGURE 9: Scheme of the backbone of a cardiotoxin from Taiwan cobra (*Naja naja atra*) venom. Abbreviations: D, Asp residue; F, Phe residue; K, Lys residue; R, Arg residue; Y, Tyr residue.

number of charges on it. Second, this difference may be due to the chemical composition of their surfaces. In neurotoxin, there is a higher content of Arg residues over Lys residues, while in cardiotoxin this content is reversed. It has been shown that the charged side chains of Arg residues in proteins are less flexible and less solvated than Lys residues (Watenpaugh et al., 1980; Richardson, 1981). Therefore, the approach of HFIP molecules, which have a large dipole moment, to the surface of cardiotoxin may primarily disturb the solvation shells and cause an acceleration of fluctuations of lysine side chains.

These factors may explain why during the TFE- or HFIP-induced unfolding of cardiotoxin the intensities of the Cotton effects of the aromatic residues and disulfide bridges disappeared more rapidly (see Figure 8) than in the case of the TFE- or HFIP-induced unfolding of neurotoxin (Gałat et al., 1981; Gałat, 1983). The TFE- and HFIP-induced unfolding of cardiotoxin does not seem to be cooperative since the transition curves show only a modest descent and a number of inflection points (see Figure 8).

Why an Increase in Hydrophobicity of the Solution and SDS Did Not Change the Conformation of Cardiotoxin. It has been shown that the increase in hydrophobicity of the solvent does not influence the unique tertiary structure of cardiotoxin (Hung & Chen, 1977; this work). Under these conditions, the observed lack of conformational change may be due to the fact that the surface of cardiotoxin is a positively charged polyion (see Figure 9). Therefore, an increase of hydrophobicity around such a protein would involve an increase of the energy of interaction between its positively charged surface and the hydrophobic groups of the alcohol, e.g., CH_3CH_2 of ethyl alcohol. Thus, the whole system is much better optimized when, during addition of ethyl alcohol, the solvation sphere around the protein remains aqueous. However, this hypothesis would not be valid for larger proteins since their surface has about 60% amino acid residues which are essentially hydrophobic (Chothia, 1976), and these might be the sites where hydrophobic unfolding factors are primarily attracted. Therefore, methanol and its homologues influence both the tertiary and secondary structures of larger globular proteins (Tamura & Jirgensons, 1980).

Also, interactions with electronegatively charged SDS molecules were considerably different for both proteins; i.e., neurotoxin was very easily denatured by SDS (Gałat et al., 1981), while cardiotoxin was only partially unfolded by SDS, and after the cmc value of SDS was achieved, a quasi-refolding of tertiary structure has been observed. These findings indicate that the molecular interaction between the amphiphilic molecule SDS and the toxins is substantially different and this difference can be attributed to different numbers of charges on the surface of these proteins.

Finally, it may be concluded that the main difference between neurotoxin and cardiotoxin, during unfolding induced by different reagents, is due to their amino acid compositions—in particular, to the very high content of Lys residues in the cardiotoxin.

Registry No. HFIP, 920-66-1; TFE, 75-89-8; SDS, 151-21-3.

REFERENCES

Bull, H. B., & Breese, K. (1978) *Biopolymers 17*, 2121–2131.

Burkhard, R. K., & Stolzenberg, G. E. (1972) *Biochemistry 11*, 1672–1677.

Bush, C. A., & Gibbs, D. E. (1972) *Biochemistry 11*, 2421–2427.

Chang, C. C. (1979) *Handb. Exp. Pharmacol. 52*, 309–376.

Chothia, C. (1976) *J. Mol. Biol. 105*, 1–14.

Chou, P. Y., & Fasman, G. D. (1974) *Biochemistry 13*, 222–245.

Drake, A. F., Dufton, M. J., & Hider, R. C. (1980) *Eur. J. Biochem. 105*, 623–630.

Dufton, M. J., & Hider, R. C. (1977) *J. Mol. Biol. 115*, 177–193.

Dufton, M. J., & Hider, R. C. (1982) *CRC Crit. Rev. Biochem. 14*, 113–171.

Franks, F. (1978) in *Characterization of Protein Conformation and Function* (Franks, F., Ed.) pp 37–53, Symposium Press, London.

Franks, F., & Eagland, D. (1975) *CRC Crit. Rev. Biochem. 3*, 165–219.

Gałat, A. (1983) *Int. J. Biochem. 15*, 715–719.

Gałat, A., Degelaen, J. P., Yang, C. C., & Blout, E. R. (1981) *Biochemistry 20*, 7415–7423.

Ghelis, C., & Yon, J. (1982) *Protein Folding*, Academic Press, New York and London.

Greenfield, H., & Fasman, G. D. (1969) *Biochemistry 8*, 4108–4116.

Hseu, T. H., Liu, Y. C., Wang, C., Chang, H., Hwang, D. M., & Yang, C. C. (1977) *Biochemistry 16*, 2999–3006.

Hung, M. C., & Chen, Y. H. (1977) *Int. J. Pept. Protein Res. 10*, 277–285.

Jirgensons, B. (1976) *Biochim. Biophys. Acta 434*, 50–68.

Jirgensons, B., & Capetillo, S. (1970) *Biochim. Biophys. Acta 214*, 1–5.

Jones, M. N., Manley, P., & Holt, A. (1984) *Int. J. Biol. Macromol. 6*, 65–68.

Keung, W. M., Leung, W. W., & Kong, Y. C. (1975) *Biochem. Biophys. Res. Commun. 66*, 383–392.

Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459–489.

Kimball, M. R., Sato, A., Richardson, J. S., Rosen, L. S., & Low, B. W. (1979) *Biochem. Biophys. Res. Commun. 88*, 950–959.

Lauterwien, J., Lazdunski, M., & Wüthrich, K. (1978) *Eur. J. Biochem. 92*, 361–371.

Lee, C. Y., & Lee, S. Y. (1979) *Handb. Exp. Pharmacol. 52*, 547–590.

Low, B. W. (1979) *Handb. Exp. Pharmacol. 52*, 212–257.
Mysels, E. K., & Mysels, K. J. (1965) *J. Colloid Sci. 20*, 315–321.
Narita, K., & Lee, C. Y. (1970) *Biochem. Biophys. Res. Commun. 41*, 339–352.
Parrish, J., & Blout, E. R. (1972) *Biopolymers 11*, 1001–1020.
Richardson, J. S. (1981) *Adv. Protein Chem. 34*, 168–339.
Scheraga, H. A. (1983) *Carlsberg Res. Commun. 49*, 1–55.
Steinmetz, W. E., Moonen, C., Kumar, A., Lazdunski, M., Visser, L, Carlsson, F., & Wüthrich, K. (1981) *Eur. J. Biochem. 120*, 467–475.
Strickland, E. H. (1974) *CRC Crit. Rev. Biochem. 2*, 113–175.
Tamura, Y., & Jirgensons, B. (1980) *Arch. Biochem. Biophys. 199*, 413–419.
Timasheff, S. N. (1970) *Acc. Chem. Res. 3*, 62–68.
Tsernoglou, D., & Petsko, G. A. (1976) *FEBS Lett. 68*, 1–4.
Tsernoglou, D., Petsko, G. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 971–974.
Tu, A. T. (1977) in *Venoms, Chemistry and Molecular Biology*, Chapter 19, Wiley, New York.
Visser, L., & Blout, E. R. (1971) *Biochemistry 10*, 743–752.
Visser, L., & Louw, A. I. (1978) *Biochim. Biophys. Acta 533*, 80–89.
Watenpaugh, K. D., Sieker, L. C., & Jensen, L. H. (1980) *J. Mol. Biol. 138*, 615–633.
Woody, R. W. (1978) *Biopolymers 17*, 1451–1467.
Yang, C. C. (1974) *Toxicon 12*, 1–43.
Yang, C. C. (1978) in *Toxins: Animal, Plant and Microbial* (Rosenberg, A., Ed.) pp 261–292, Pergamon Press, Oxford.
Yang, C. C., & Chang, C. C. (1976) in *Animal, Plant and Microbial Toxins* (Oshaka, A., Hayashi, K., & Sawai, Y., Eds.) Vol. 1, pp 45–65, Plenum Press, New York.
Yang, C. C., King, K., & Sun, T. P. (1981) *Toxicon 19*, 645–659.
Yonath, A., Podjarny, A., Honig, B., Sielecki, A., & Traub, W. (1977) *Biochemistry 16*, 1418–1424.
Ziegler, S. M., & Bush, C. A. (1971) *Biochemistry 10*, 1330–1335.

cGMP- and Phosphodiesterase-Dependent Light-Scattering Changes in Rod Disk Membrane Vesicles: Relationship to Disk Vesicle–Disk Vesicle Aggregation†

Antonio Caretta and Peter J. Stein*
Department of Ophthalmology and Visual Science, Yale University School of Medicine, New Haven, Connecticut 06510
Received October 22, 1984

ABSTRACT: Visible light activates a large guanosine cyclic 3′,5′-phosphate (cGMP)- and phosphodiesterase (PDE)-dependent infrared light-scattering change in suspensions of photoreceptor disk membranes. Reconstitution experiments show that this signal requires bleached rhodopsin, G protein (three polypeptide subunits of M_r 39 000, 37 000, and 6000 which comprise the GTPase), phosphodiesterase, cGMP, and GTP. The lowest light intensity which elicits the light-scattering signal bleaches 0.002% rhodopsin. cGMP and GTP hydrolysis occurs more slowly than the initial phase of the scattering signal, and the kinetics of nucleotide hydrolysis do not correlate with any phase of the signal. Hydrolysis-resistant analogues of cGMP and GTP support the initial decreasing phase of the signal. Thus, the signal apparently depends upon nucleotide binding rather than hydrolysis. Microscopic observations made under the same conditions as light-scattering experiments show that vesicle–vesicle aggregation and disaggregation occur. The data suggest that light and nucleotide activations of the cyclic nucleotide cascade enzymes are responsible for the vesicle aggregation process and nucleotide hydrolysis for vesicle disaggregation. The vesicle aggregation–disaggregation phenomenon appears likely to be the physical basis of the cGMP- and PDE-dependent changes in infrared transmission.

A variety of infrared light-scattering signals have been reported to occur in suspensions of broken rod outer segment (ROS)[1] membranes after a flash of visible light. Small changes in transmittance, which occur in the absence of extrinsic membrane proteins, have been correlated with rhodopsin bleaching (Hofmann et al., 1976; Uhl et al., 1978). Similarly, large changes in transmittance dependent on ATP hydrolysis have been correlated with ion movements through disk membranes (Uhl et al., 1979a,b; Borys et al., 1983). An increase in transmittance dependent on GTP and extrinsic membrane proteins (Bignetti et al., 1980), attributed to light activation of the enzymes, has been demonstrated to depend on rapid interaction of rhodopsin, G protein, and GTP (Kuhn et al., 1981; Emeis et al., 1982). A slower and larger light-scattering change dependent on GTP and extrinsic membrane proteins also has recently been reported (Lewis et al., 1983).

In this paper, we describe a large infrared light-scattering signal. In contrast to those reported previously, the infrared transmission changes observed depend upon both GTP and cGMP. In addition, the signal requires the presence of G protein, PDE, and bleached disk vesicles. Reconstitution experiments demonstrate that the scattering signal can be triggered either by light, in dark-adapted membranes, or by nu-

† This work was supported in part by an International Research Scholar Award (A.C.) from Research to Prevent Blindness Inc., by National Institutes of Health Grant EY 05074 (P.J.S.), by AFOSR Grant 84-NL-024 (P.J.S.), and by the Connecticut Lions.

[1] Abbreviations: ROS, rod outer segment(s); G protein, three polypeptide subunits of M_r 39 000, 37 000, and 6000 which comprise the GTPase; PDE, phosphodiesterase; cGMP, guanosine cyclic 3′,5′-phosphate; 8Br-cGMP, 8-bromoguanosine cyclic 3′,5′-phosphate; cAMP, adenosine cyclic 3′,5′-phosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); DTT, dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/85/0424-5685$01.50/0