Conformation of Human Erythrocyte Glycophorin A and Its Constituent Peptides†

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ABSTRACT: We have examined the conformation of glycophorin A and its constituent tryptic peptides using circular dichroism. The conformation of native glycophorin A in aqueous solution is about 27% helix, 10% β form, and 63% unordered. In the presence of the anionic detergent sodium lauryl sulfate or the zwitterionic detergent Ammonyx-LO, the conformation of the glycoprotein changes only slightly, showing a small increase in helix. Despite the extensive glycosylation of glycophorin A, carbohydrate residues contribute little to the circular dichroism spectrum. The conformations of the four major tryptic peptides differ from each other greatly. The glycopeptides T1A and T3A possess some β form but are predominantly unordered. The hydrophobic peptide T6A, which represents the intramembranous segment of the molecule, can be examined only in the presence of detergents and is completely helical. The COOH-terminal peptide, T4A, has some helix but is predominantly unordered. Interestingly, the composite circular dichroism spectrum resulting from the addition of each of the individual peptide spectra is indistinguishable from that of the native glycoprotein. This indicates that the constituent peptides do not interact with each other in the whole glycoprotein in any way which affects their conformation. Thus the conformation of this glycoprotein appears to be determined solely by short-range interactions.

The conformation of glycophorin A and its constituent peptides is of considerable interest for a number of reasons. Glycophorin A, the major sialoglycoprotein of the human erythrocyte membrane, is a transmembrane protein (Bretscher, 1971; Cotmore et al., 1977) having three distinct chemical domains. The amino-terminal portion of the protein is located extracellularly and is heavily glycosylated, having 16 oligosaccharide chains per peptide chain (Tomita & Marchesi, 1975). A central portion of the protein has a sequence of 23 successive nonpolar amino acids which are thought to span the lipid bilayer and which may be involved in protein–protein interactions in the membrane (Furthmayr & Marchesi, 1976; Silverberg et al., 1976). The carboxyl-terminal portion of the protein consists of a relatively charged, proline-rich segment which extends into the cytoplasm (Cotmore et al., 1977). The combination of known amino acid sequence, known orientation with respect to the membrane, and such highly distinct peptide regions makes this protein an interesting and advantageous model for the study of certain questions common to transmembrane glycoproteins. Specifically, we wanted to determine whether peptides derived from the three distinct regions of the glycoprotein possessed distinct conformations and whether the conformations of these individual peptides could reasonably be expected to reflect their conformations when constituents of the native glycoprotein.

Since glycophorin A is 60% carbohydrate by weight, we anticipated that this degree of glycosylation might cause problems in the analysis of circular dichroism spectra. We have tried to assess the extent of these problems in three ways. The CD[1] spectra of glycophorin A following sialic acid removal and of the glycopeptides produced by pronase digestion of glycophorin A have been examined, and we have assessed the effects of analyzing our CD spectra over limited wavelength regions removed from those of greatest carbohydrate ellipticity.

Previous circular dichroism studies of human glycophorin have been done on the unfractionated sialoglycoproteins of the human red blood cell membrane (Decker & Carraway, 1975; Segrest & Kohn, 1973). The studies described in this paper have been done on a single sialoglycopeptide, glycophorin A, and the peptides derived from it.

Experimental Procedures

Specially pure sodium lauryl sulfate (SDS) was from BDH Chemicals. Ammonyx-LO (*N,N*-dimethyllaurylamine oxide) was from Onyx Chemical Co., Jersey City, NJ. Schwarz-Mann was the source of the ultrapure Tris base. Eastman Chemicals was the source of *d*-10-camphorsulfonic acid. Type VI pronase was obtained from Sigma. Neuraminidase from *Clostridium perfringens* was purified by affinity chromatography (Tomita & Marchesi, 1975). Trypsin-N^{α}-tosylphenylalanine chloromethyl ketone was from Worthington Biochemicals. All other chemicals were analytical reagent grade.

Isolation of Glycophorin A. Glycophorin A was isolated according to the procedure of Marchesi & Andrews (1971) as modified by Furthmayr et al. (1975). Following gel filtration in 0.1% Ammonyx-LO, the protein was exhaustively dialyzed and then lyophilized and extracted twice with absolute ethanol at 0 °C.

Neuraminidase Treatment of Glycophorin A. Glycophorin A was dissolved at a concentration of 5 mg/mL in 50 mM sodium acetate, 2 mM calcium chloride, 0.2 mM ethylenediaminetetraacetic acid (pH 5.6). Neuraminidase (0.05 unit) was added and the reaction mixture was incubated at 37 °C for 6 h. The reaction mixture was then applied to a gel filtration column equilibrated with 0.1% Ammonyx-LO as in the normal glycophorin A purification procedure, and the peak corresponding to desialylated glycophorin A was dialyzed, lyophilized, and ethanol-extracted as for glycophorin A. Desialylated glycophorin A, produced in this manner, was reduced in sialic acid content by more than 90%.

Pronase Digestion of Glycophorin A. Glycophorin A was dissolved at 0.5 mg/mL in 20 mM Tris-acetate, 2 mM calcium chloride, 0.01% sodium azide (pH 7.8), plus 7.5 μg/mL of pronase (4 units of activity/mg). This reaction mixture was

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[1] Abbreviations used: CD, circular dichroism; SDS, sodium lauryl sulfate.

0006-2960/79/0418-0275$01.00/0

incubated at 37 °C for 24 h and readjusted to pH 7.8 with Tris base, and an additional 7.5 μg/mL of pronase was added. After 24 h more of incubation, the mixture was chilled and centrifuged to remove insoluble material, and the supernate was lyophilized. The lyophilized material was chromatographed on a Bio-Gel P-4 column in 0.1 M ammonium acetate, and the void volume peak containing glycopeptides was lyophilized. Based upon quantitative amino acid analysis and galactosamine analysis, these glycopeptides contained fewer than three amino acids per oligosaccharide chain.

Preparation and Isolation of Tryptic Peptides. Glycophorin A, which had first been lipid extracted according to Silverberg et al. (1976), was treated with trypsin according to Furthmayr et al. (1975) but with an incubation time of 24 h. After acidification of the reaction mixture, the insoluble peptide T6A was isolated by centrifugation, washed several times, and then further purified by countercurrent distribution (Furthmayr et al., 1978). The soluble peptides were chromatographed on Ultra-gel AcA54 in 1.0 M ammonium acetate (pH 6.8) plus 0.2% sodium azide. Peptide T1A was isolated as an individual peak on this column. The peak containing peptides T3A and T4A was lyophilized, chromatographed on Cellex D, and eluted with a 0–500 mM sodium chloride gradient in 50 mM sodium formate. Peptides T3A and T4A were isolated as separate peaks on this column and were dialyzed and lyophilized. All peptides were checked for purity by amino acid analysis.

Circular Dichroism and Its Analysis. Circular dichroism spectra were obtained with a Cary Model 61 recording circular dichrometer at ambient temperature. The instrument was standardized using twice recrystallized *d*-10-camphorsulfonic acid. Spectra were recorded at a speed of 4 nm/min and at a full-scale range of 0.05 or 0.1°. The path length of the cells was 1 mm unless otherwise noted. All solutions were optically clear and were centrifuged before recording spectra. Glycophorin A in detergent solution was incubated at 37 °C for 30 min before the CD spectrum was recorded. Peptides in SDS solution were incubated at 80 °C for 30 min before CD analysis. Concentrations of peptides or proteins are given in the figure legends.

CD spectra were analyzed by the least-squares curve-fitting method of Chen et al. (1974) at 1-nm intervals. Coefficients for helix, β form, and unordered form were those of Chen et al. (1974). A BMDP-3R computer program was used for the analysis (UCLA Health Sciences Computing Facility).

Analytical Methods. Protein content was determined by quantitative amino acid analysis on a Durrum D500 analyzer, with L-norleucine used as an internal standard. Sialic acid was determined essentially by the method of Warren (1959). Galactosamine was measured after mild acid hydrolysis on the Durrum D500 amino acid analyzer (Fukuda & Osawa, 1973).

Results

Figure 1 shows an initial attempt to determine the contribution of carbohydrate residues to the CD spectrum of glycophorin A. Both glycophorin A and neuraminidase-treated glycophorin A, termed desialylglycophorin A, show very similar CD spectra. This is consistent with previous findings, although we succeeded in removing more than 90% of the sialic acid from the glycoprotein rather than the 60% previously reported (Decker & Carraway, 1975). Some differences in the spectra appear in the wavelength region below 220 nm, and these differences cause a slight change in the estimates of protein conformation. The third curve in Figure 1 shows the CD spectrum of glycopeptides produced by extensive pronase digestion of glycophorin A. These glycopeptides average less

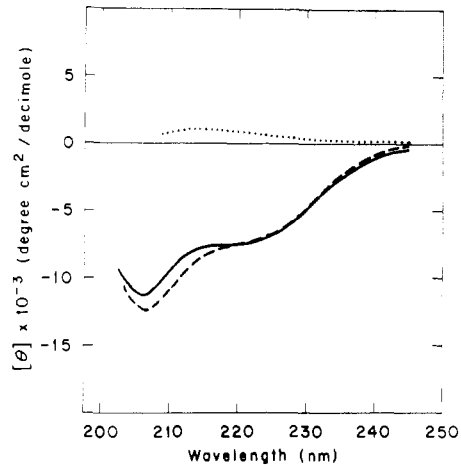

FIGURE 1: CD spectra of glycophorin A, desialylated glycophorin A, and glycopeptides derived from glycophorin A by pronase treatment. All spectra were recorded in 10 mM Tris-acetate, pH 7.0. The glycopeptide spectrum was done at a full-scale range of 0.02°. (—) Glycophorin A at 0.5 mg/mL; (--) desialylated glycophorin A at 0.5 mg/mL; (···) glycopeptides at 1 mg/mL.

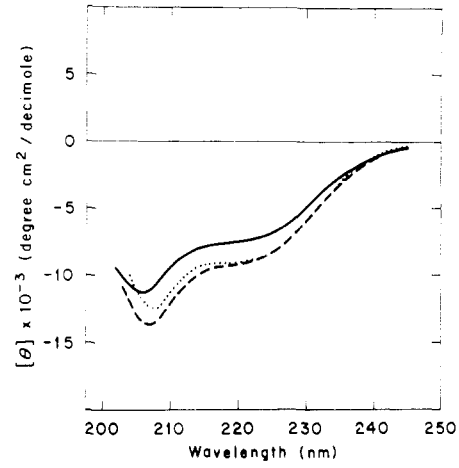

FIGURE 2: CD spectra of glycophorin A in two different detergents. All spectra were recorded at a glycophorin concentration of about 0.6 mg/mL in 10 mM Tris-acetate, pH 7.0. (—) No additions; (--) +1% Ammonyx-LO; (···) +1% SDS.

than three amino acids per oligosaccharide chain, and thus the CD spectrum should primarily reflect the ellipticity of the carbohydrate residues. The ellipticity of the glycopeptides has been normalized to the number of amino acids per galactosamine in native glycophorin A in order to give a direct comparison of the contribution of oligosaccharide chains to the CD spectrum of the native glycoprotein. As will be seen with the tryptic glycopeptides of glycophorin A, the glycopeptide solution in Figure 1 absorbs so strongly below 210 nm that CD measurements become impossible.

Based upon the data of Figure 1, the contributions of sialic acid residues or oligosaccharide chains to the CD spectrum of glycophorin A appear to be quite small.

Figure 2 shows the spectra of glycophorin A in buffer and in buffer plus 1% SDS or 1% Ammonyx-LO. Both detergents, one anionic and one zwitterionic, produce a change in the CD spectra. Analysis of these curves indicates that both detergents cause an increase in helix content. Unlike the conformation of glycophorin A in buffer, the conformation induced by the presence of these two detergents is stable even after heating at 80 °C for 30 min (data not shown). In view of this stability and similarity of structure in two very different detergent environments, it is conceivable that glycophorin A may have

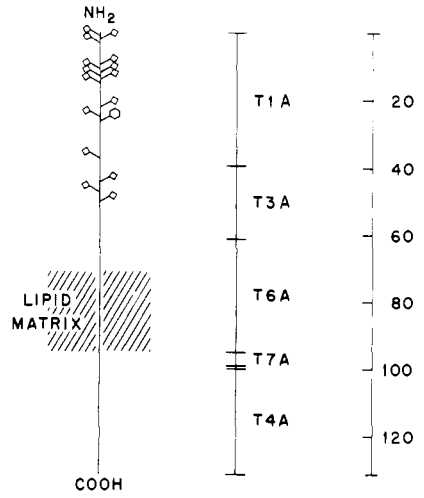

FIGURE 3: Schematic drawings of the arrangement of glycophorin A with respect to the cell membrane and of the arrangement of the major tryptic peptides within the protein. The drawing on the left illustrates the sites of glycosylation of the protein. The center drawing shows the arrangement of the tryptic peptides and corresponds to equivalent positions on the left with respect to the lipid matrix. The drawing on the right gives the amino acid residue numbers for equivalent positions to the left.

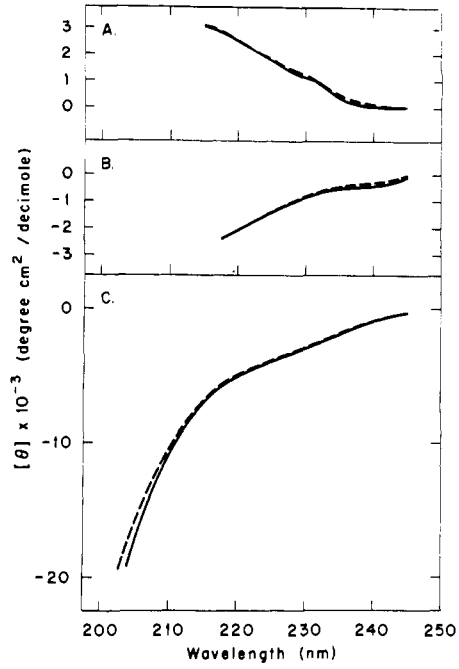

FIGURE 4: CD spectra of the major soluble tryptic peptides of glycophorin A: (A) peptide T1A at a concentration of 5 mg/mL; (B) peptide T3A at a concentration of 4 mg/mL; (C) peptide T4A at a concentration of 0.6 mg/mL. (—) In 10 mM Tris-acetate, pH 7.0; (--) in 10 mM Tris-acetate, pH 7.0, + 1% SDS.

this conformation when in the amphipathic milieu of the membrane.

To study the conformations of discrete regions of glycophorin A, we examined each of the constituent tryptic peptides by circular dichroism. Figure 3 is a schematic drawing indicating the order of these peptides in the protein and their orientation with respect to the lipid bilayer (Cotmore et al., 1977; Marchesi et al., 1976). It is apparent that the membrane bilayer must prevent associations between distant peptides in vivo.

Figure 4 shows the CD spectra of the three major soluble tryptic peptides of glycophorin A. The spectra of all three of these indicate little ordered structure and show virtually no

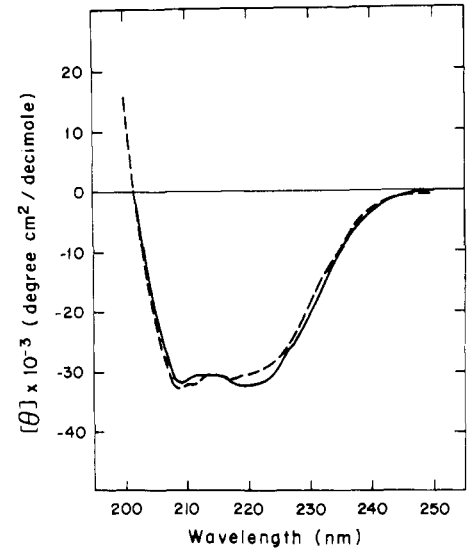

FIGURE 5: CD spectra of the hydrophobic tryptic peptide T6A at a concentration of 0.2 mg/mL. (—) In 10 mM Tris-acetate, pH 7.0, + 1% Ammonyx-LO; (--) in 10 mM Tris-acetate, pH 7.0, + 1% SDS.

change when 1% SDS is present in the medium. The glycopeptides T1A and T3A can only be analyzed over a relatively narrow range of the spectrum, probably because of the high absorbance of their carbohydrate residues at lower wavelengths.

The glycopeptide T1A is approximately 80% carbohydrate by weight, and its CD spectrum is shown in Figure 4A. This spectrum is not fit well by the coefficients of Chen et al. (1974) but does show positive ellipticity in the wavelength region around 220 nm, which is characteristic of a totally random structure. In this heavily glycosylated peptide the ellipticity contributions of sugar residues may not be negligible and so estimation of conformation is less certain.

Figure 4B shows the spectrum of the glycopeptide T3A. Analysis of this spectrum gives an estimate of conformation of 21% β form and little helix. However, this glycopeptide is approximately 60% carbohydrate by weight and here, too, ellipticity of the sugar residues could affect the estimate of conformation.

In an attempt to minimize possible contributions of carbohydrate to the CD spectrum, curve-fitting analysis for the T3A and T1A peptides was restricted to the wavelength region of 225 to 245 nm. Because the major absorbance peak of sugar *N*-acetyl groups is at a lower wavelength, this region might be expected to be less influenced by sugar group ellipticity. Estimates of glycopeptide conformation based on this restricted region (data not shown) were identical with those estimates made over the entire available spectrum; thus a contribution of sugar residue ellipticity is not obvious.

The CD spectrum of peptide T4A is shown in Figure 4C. Estimates of T4A conformation show some helix, but predominantly an unordered structure. Note that the CD spectrum of each of these peptides is unchanged in the presence of 1% SDS.

The other major tryptic peptide of glycophorin A, T6A, is insoluble in aqueous buffer. Figure 5 shows the spectrum of this peptide in solution containing either 1% SDS or 1% Ammonyx-LO. The CD spectrum of this peptide, in either SDS or Ammonyx-LO, indicates a completely helical conformation. A previous study had shown that the insoluble material resulting from the tryptic digestion of total red cell sialoglycoproteins produces a CD spectrum in trifluoroethanol also characteristic of a highly helical peptide (Segrest & Kohn,

Table I: Conformation of Glycophorin A and Peptides

compd[a]	wavelength range (nm) for curve-fitting	conformation		
		helix	β form	unordered form
glycophorin A	245–202	0.27	0.10	0.63
glycophorin A + 1% Ammonyx-LO	245–204	0.32	0.12	0.55
glycophorin A + 1% SDS	245–203	0.34	0.07	0.59
desialylglycophorin A	245–203	0.29	0.11	0.61
T1A[b]	245–215	−0.06	0.10	0.96
T3A	245–218	0.07	0.21	0.72
T6A + 1% SDS or Ammonyx-LO	245–203	1.0	0	0
T4A	245–203	0.23	0	0.77

[a] The standard solvent for all compounds is 0.01 M Tris-acetate, pH 7.0. [b] Computer analysis of this spectrum did not remain within the constraint of positive fractions and gave a small negative fractional percent of helix.

1973). The helical conformation of this peptide exists then in solvents of such diversity as SDS, Ammonyx-LO, and trifluoroethanol, and this conformation is stable in SDS to heating at 80 °C for 30 min (data not shown).

Table I shows the calculated conformation of all the spectra shown thus far. As mentioned earlier, both SDS and Ammonyx-LO produce small increases in the helical content of the whole protein, but the overall picture of glycophorin A is still one of only modest ordered structure. Table I also shows the great diversity of conformations of the constituent tryptic peptides of glycophorin A. As noted earlier, the spectrum of T1A is not fitted well by any peptide conformation and, in fact, is fitted best by a computer estimate using a small negative value for helix. This is due to the unusually high positive ellipticity of this CD spectrum, which probably is the result of a highly unordered state combined with some contribution of the carbohydrate side chains. Sialic acid derivatives as well as sialic acid polymers can show positive ellipticity in this range (Kabat et al., 1969; Jennings & Williams, 1976), and the total glycopeptides of glycophorin A display positive ellipticity as shown in Figure 1.

It is interesting that the extracellularly oriented glycopeptides T1A and T3A account for all the β form found in the peptides and virtually none of the helical content, while the transmembrane peptide T6A appears to be completely helical and accounts for much of the helical content of the whole protein. The intracellularly oriented peptide T4A also contains some helix, in spite of six proline residues interspersed among its 31 amino acids. As was noted earlier, the soluble peptides T1A, T3A, and T4A have CD spectra unaffected by the presence of 1% SDS, and thus the analyses presented in Table I apply to the peptides when in the presence of SDS also.

To determine if these peptide conformations are truly representative of their conformations when part of the whole protein, we reconstructed a whole-protein spectrum by the addition of all of the individual peptide spectra, weighted for each peptide's size relative to the whole protein. Figure 6 gives a comparison of this composite spectrum and the spectrum of the whole glycoprotein (from Figure 2). Once again, this comparison can only be made with all components in the presence of SDS because of the insolubility of the hydrophobic peptide T6A. As is clear from the figure, the composite peptide spectrum and the whole glycoprotein spectrum are virtually identical. It is likely, then, that the constituent peptides of glycophorin A retain their in vivo conformations even in the absence of long-range intramolecular interactions.

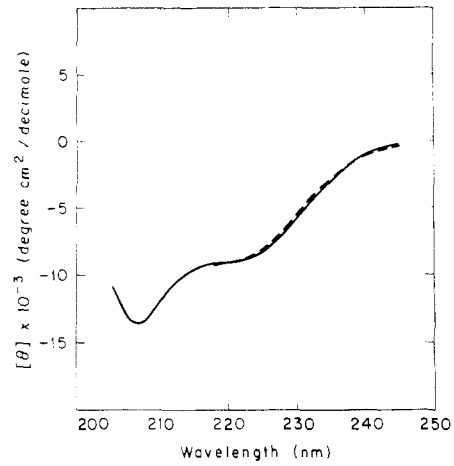

FIGURE 6: The CD spectrum of glycophorin A and the composite spectrum resulting from the addition of each of the individual peptide spectra. The solvent for each is 10 mM Tris-acetate, pH 7.0, plus 1% SDS. (—) Glycophorin A; (--) composite peptide spectrum.

Table II: Comparison of Glycophorin A Conformation with Additive Conformation of Constituent Peptides[a]

compd[b]	amino acid residues in		
	helix	β form	unordered form
T1A[c]	0	4	35
T3A	2	5	16
T6A	34	0	0
T4A	7	0	24
total of peptides[d]	43	9	75
glycophorin A	45	9	77

[a] These results are based upon the data of Table I and the known number of amino acid residues per peptide. [b] Since the peptide T6A can be analyzed only in detergent solution, the standard solvent for comparison of all of the above compounds is 0.01 M Tris-acetate + 1% SDS, pH 7.0. [c] Since the computer analysis of Table I gave a negative value for α helix, the actual conformation is assumed to be 0% helix, 10% β form, 90% unordered form for this peptide. [d] Note that the tetraptide, T7A, has not been analyzed.

Table II illustrates this striking additivity in another way. Using the data of Table I and the known number of amino acids of each peptide (Tomita & Marchesi, 1975), the number of amino acid residues in each conformation can be determined for each peptide. These totals can be compared to those for the intact glycoprotein, and the agreement is very good. The individual peptides of glycophorin A apparently comprise quite independent domains capable of maintaining their own conformations whether part of the parent protein or not.

Discussion

The use of circular dichroism as a tool to examine the conformations of proteins and their constituent peptides is not new. Generally, however, the conformations of protein components, whether on the level of constituent peptides or subunits, do not add up to the conformation of the whole protein. Usually, conformations of protein components show much less ordered structure than exists in the parent protein. This decrease in ordered structure of individual constituents can be attributed to the absence of long-range stabilizing interactions from other peptide regions of the native protein (Epand & Scheraga, 1968; Hermans & Puett, 1971; Bucci & Bucci, 1975). In this respect, we expected that the constituent peptides of glycophorin A might have conformations unaffected by such interactions since the transmembrane

orientation of the whole protein prevents the interactions of distant segments of the peptide chain. Furthermore, interactions between the neighboring glycopeptides T1A and T3A may be discouraged by the charge density of their component sialic acid groups. In fact, the data presented here indicate that the constituent peptides of glycophorin A have conformations in solution very similar to their conformations when part of the whole protein. Apparently, then, intrapeptide interactions do not influence their conformations to any significant extent.

The conformation of glycophorin A in aqueous solution is 27% helix, 10% β form, and 63% unordered form. We have tried to assess the contribution of carbohydrate to the CD spectrum and have found it to be essentially negligible for the whole glycoprotein. These findings are similar to those of Decker & Carraway (1975) although our estimates of conformation vary, primarily due to different methods of CD spectrum analysis and perhaps due to the relative hetergeneity of their glycoprotein preparation. The presence of the detergents SDS or Ammonyx-LO causes a change in the conformation of the whole protein, producing a small increase in helical content. The presence of detergents protects the protein conformation against thermal denaturation very effectively, however. After incubation at 100 °C for 3 min, glycophorin A in aqueous buffer shows a CD spectrum indicative of almost complete denaturation (data not shown). Glycophorin A in buffer plus SDS shows no change in CD spectrum after this type of treatment (Schulte & Marchesi, 1978). The underlying cause for such a drastic change in stability with only a small change in conformation is not known.

The constituent peptides of glycophorin A are very diverse both with respect to their chemical nature and to the environment with which they interact. We have found that the conformations of these peptides reflect this great diversity. The extracellularly oriented glycopeptides T1A and T3A show virtually no helix and some β form and are predominantly in an unordered form. The predominance of "unordered form", however, should not be misconstrued. The conformations of these peptides may be very constrained and specific, but just not in any regular conformation interpretable by circular dichroism. The cytoplasmically oriented T4A peptide shows a modest amount of helix but no β form. It would be this peptide which would likely be the site of interactions between glycophorin A and cytoplasmic or cytoskeletal components if, indeed, these interactions do exist (for a review, see Marchesi et al., 1976). T6A, the hydrophobic transmembrane peptide, has an entirely helical conformation in detergents such as SDS and Ammonyx-LO and is largely helical in the solvent trifluoroethanol (Segrest & Kohn, 1973). In addition, a peptide preparation composed largely of T6A appears 75% helical when incorporated into lecithin liposomes (Segrest, 1977). It is interesting that different theoretical methods for predicting conformation based on primary sequence predict a considerable amount of β structure for this peptide (Green & Flanagan, 1976) and varying amounts of helix, up to 55%. This peptide is of special interest because of indications that it may mediate glycophorin A self-association (Furthmayr & Marchesi, 1976; Silverberg et al., 1976). This peptide appears to be dimeric even in the presence of SDS (Furthmayr et al., 1977) and, when inserted into model phospholipid vesicles, may cause the appearance of intramembranous particles as seen by freeze–fracture electron microscopy (Segrest et al., 1974). Presumably this latter phenomenon, too, involves self-association. Our data now make it likely that these interactions are limited to interactions which can occur between neighboring helices. A model for glycophorin complexes in the erythrocyte membrane based upon interactions between hydrophobic peptide helices has been proposed (Segrest, 1977).

The remarkable similarity of the composite peptide CD spectrum to the spectrum of the parent protein is also striking. This similarity must indicate that the conformations of the isolated peptides are very close to the conformations in which they exist when part of the parent protein. Consequently, the conformations of these peptides are determined by relatively short-range interactions.

Based upon our data, glycophorin A has a tripartite structure not only with regard to its chemical nature and the environments it faces but also with respect to its conformation. The extracellular, glycosylated portion possesses some β form but is largely unordered. Similarly the intracellular portion is largely unordered except for some helix. Connecting these two domains is a totally helical segment spanning the bilayer. The conformations of these regions appear to be independent of each other.

Acknowledgments

The authors thank Dr. Joseph Coleman for the use of the Cary Model 61 circular dichrometer, Dr. Heinz Furthmayr for the countercurrent distribution purification of the T6A peptide as well as for many helpful discussions, and Dr. Jennifer Chen for invaluable assistance with the computer analyses.

References

Bretscher, M. S. (1971) *Nature (London), New Biol. 231*, 229–232.

Bucci, C. F., & Bucci, E. (1975) *Biochemistry 14*, 4451–4458.

Chen, Y. H., Yang, J. T. & Chau, K. H. (1974) *Biochemistry 13*, 3350–3359.

Cotmore, S. F., Furthmayr, H. & Marchesi, V. T. (1977) *J. Mol. Biol. 113*, 539–553.

Decker, R. V. & Carraway, K. L. (1975) *Biochim. Biophys. Acta 386*, 52–61.

Epand, R. M., & Scheraga, H. A. (1968) *Biochemistry 7*, 2864–2872.

Fukuda, M., & Osawa, T. (1973) *J. Biol. Chem. 248*, 5100–5105.

Furthmayr, H. F., Galardy, R. E., Tomita, M., & Marchesi, V. T. (1978) *Arch. Biochem. Biophys. 185*, 21–29.

Furthmayr, H., & Marchesi, V. T. (1976) *Biochemistry 15*, 1137–1144.

Furthmayr, H., Tomita, M., & Marchesi, V. T. (1975) *Biochem. Biophys. Res. Commun. 65*, 117–121.

Green, N. M., & Flanagan, M. T. (1976) *Biochem. J. 153*, 729–732.

Hermans, J., Jr., & Puett, D. (1971) *Biopolymers 10*, 895–913.

Jennings, H. J., & Williams, R. E. (1976) *Carbohydr. Res. 50*, 257–265.

Kabat, E. A., Lloyd, K. O., & Beychok, S. (1969) *Biochemistry 8*, 747–756.

Marchesi, V. T., & Andrews, E. P. (1971) *Science 174*, 1247–1248.

Marchesi, V. T., Furthmayr, H., & Tomita, M. (1976) *Annu. Rev. Biochem. 45*, 667–698.

Schulte, T. H., & Marchesi, V. T. (1978) *Biochim. Biophys. Acta 508*, 425–430.

Segrest, J. P. (1977) in *Surface Membranes of Specific Cell Types*, (Jamieson, A., & Robinson, D., Eds.) Vol. 3, pp 1–26, Butterworths, New York.

Segrest, J. P., Gulik-Kryzwicki, T., & Sardet, C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3294–3298.
Segrest, J. P., & Kohn, L. D. (1973) in *Protides of the Biological Fluids–21st Colloquium* (Peeters, H., Ed.) pp 183–189, Pergamon Press, New York.
Silverberg, M., Furthmayr, H., & Marchesi, V. T. (1976) *Biochemistry 15*, 1448–1454.
Tomita, M., & Marchesi, V. T. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2964–2968.
Warren, L. (1959) *J. Biol. Chem. 234*, 1971–1975.

Transient Electric Dichroism Studies of the Structure of the DNA Complex with Intercalated Drugs†

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ABSTRACT: We describe electric dichroism, fluorescence electric dichroism, and equilibrium binding studies whose purpose is to examine the structure of the complex between DNA and intercalating drugs (ethidium bromide, actinomycin D, 9-aminoacridine, and proflavin). We show that the DNA length increase which results from intercalation is not always 3.4 Å per bound drug as often idealized but varies from drug to drug, the range being 2–3.7 Å. From the limiting dichroism at high fields we calculate that the drugs are not perpendicular to the helix axis when intercalated but are tilted from perpendicularity by 21 ± 7° along their long axis. We propose that tilting occurs because base pairs neighboring the intercalated drug are flattened by the heterocycle and are therefore incompatible with adjacent propeller-twisted base pairs. We show that DNA is not bent or kinked by intercalators; therefore a conformational change must occur which compensates for the tilt of the complex. Others have suggested that 5′ pyrimidine 3′–5′ purine is the preferred sequence for intercalation. We propose that the conformational change which compensates for the tilt of the complex reflects the twofold rotational symmetry of the binding site, thereby maintaining the linearity of the helix. The magnitude of the conformational change required suggests that, as a result of intercalation, stacking is reduced between the 5′ pyrimidine and its 5′ neighbor.

Although the concept of intercalation was first proposed by Lerman 17 years ago (Lerman et al., 1961) and has been widely used as a model for ligand binding, the detailed structure of the intercalated complex in DNA is not known. Crystal structures have been solved at 3-Å resolution for ethidium bromide (Jain et al., 1977; Tsai et al., 1977), actinomycin (Sobell & Jain, 1972), proflavin (Neidel et al., 1977; Seshadri et al., 1977), and 9-aminoacridine (Sakore et al., 1977) complexes with nucleotides and dinucleotide helices. Such structures provide valuable information concerning local drug–nucleic acid interactions, but problems may arise when extending the models to long segments of DNA helix (Alden & Arnott, 1977).

So far, the structure of the intercalated complex in large DNAs has been impossible to measure at atomic resolution. However, as we have shown in previous papers (Hogan et al., 1978; Dattagupta et al., 1978; Crothers et al., 1978; Klevan et al., 1978), electric dichroism techniques can be used to measure the orientation of chromophores and the DNA length and conformation changes which result from ligand and protein binding. In combination, this information can put rigorous limits on the range of possible intercalated structures.

Here we report dichroism studies on the binding of several intercalators to DNA. Based upon these measurements we propose that, when intercalated, drugs are tilted by at least 20° along the long base-pair axis. Overall, the helix remains linear, due we believe to extensive conformational changes in the "excluded site" adjacent to the intercalator.

† From the Department of Chemistry, Yale University, New Haven, Connecticut 06520. *Received September 1, 1978.* Supported by a grant, CA 15583, from the National Cancer Institute. M.H. was supported by National Institutes of Health Training Grant GM 07223.

Dichroism Theory

Fiber diffraction data indicate that the bases in DNA form an extremely regular lattice. Therefore, when DNA is oriented in an electric field, chromophores bound to DNA will, like the bases, display linear dichroism. At any wavelength where the ligand absorbs light the reduced dichroism ρ is defined by

$$\rho = (A_{\parallel} - A_{\perp})/A \quad (1)$$

in which $A_{\parallel}$ and $A_{\perp}$ are the absorbances measured with light polarized parallel and perpendicular, respectively, to the applied electric field, and A is the absorbance in absence of the field. The dichroism due to a particular transition moment can be expressed by (Okonski et al., 1959)

$$\rho_i = \frac{3}{2}(3\cos^2\alpha_i - 1)\Phi \quad (2)$$

where α_i is the angle between the ith transition moment and the axis of orientation and Φ is the fractional orientation (Okonski et al., 1959), equal to the ratio (ρ/ρ_∞) of the measured dichroism to the dichroism at perfect orientation, achieved in the limit of infinite field. Dichroism remains constant over those wavelengths where absorbance arises from a single transition (Ding et al., 1972). Many intercalators display multiple visible and near-UV transitions and will therefore have associated with each transition a different dichroism value. From measurements at different wavelengths it is possible to measure independently the angles α_i between each of the optical transition moments and the axis of orientation.

The intense visible absorbances displayed by intercalated heterocycles arise from π–π^* transitions and are therefore directed in the aromatic plane. Several intercalators display

0006-2960/79/0418-0280$01.00/0