Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 4113–4116, June 1987
Biophysics

Structural basis of human erythrocyte glucose transporter function in proteoliposome vesicles: Circular dichroism measurements

(protein secondary structure/scattered-light-collecting device/cytochalasin B)

JAMES J. CHIN, EDWARD K. Y. JUNG, V. CHEN, AND CHAN Y. JUNG

Department of Biophysical Sciences, State University of New York and Biophysics Laboratory, Veterans Administration Medical Center, Buffalo, NY 14215

Communicated by David Harker, March 9, 1987 (received for review December 3, 1985)

ABSTRACT **The secondary structural compositions of the human erythrocyte glucose transporter in proteoliposome vesicles were assessed on the basis of circular dichroism (CD) spectra measured in the absence and in the presence of D-glucose or an inhibitor, cytochalasin B. We designed and used a scattered-light-collecting device, which corrects CD spectra for optical artifacts originating from light scattering. Relative contents of eight types of secondary structure were estimated by using basis spectra generated by the eigenvector method based on CD spectra of 15 proteins of known structure. Results indicate that the glucose transporter is composed of approximately 82% α-helices, 10% β-turns, and 8% other random structure, with no β-strands. In the presence of an excess of D-glucose, the α-helical content is reduced by more than 10% and there is a significant increase in the random structure content. Cytochalasin B does not appear to affect the secondary structural composition of the transporter to any significant degree.**

The movement of glucose across the plasma membrane of human erythrocytes is a typical example of carrier-mediated transport, in which an intrinsic membrane protein (transporter) catalyzes the translocation of selected hexoses across an otherwise practically impermeable membrane diffusion barrier (1). The glucose transporter protein has been isolated (2) and used to form a functional proteoliposome preparation (3). Recently, the complete amino acid sequence of this protein has been deduced on the basis of its extensive homology to the glucose transporter of the human hepatoma cell line HepG2 (4). Understanding at the molecular level how this protein selects and translocates sugar molecules requires detailed information on the secondary and higher structure and conformational dynamics of the protein.

Analysis of the ultraviolet circular dichroism (CD) is the most direct method known for estimating the secondary structure of protein in solution (5). For the membrane-bound protein, however, the scattering properties and particulate nature of the membrane suspension cause significant distortions in the CD spectra (6). Two major optical artifacts are the effects of differential scattering and absorption flattening (7). Several experimental devices have been introduced to eliminate the scattering distortion by enlarging the solid angle of detection for light-scattering membrane samples (8, 9). In the present study we have estimated the secondary structural compositions of the purified human erythrocyte glucose transporter in reconstituted liposomes on the basis of the CD spectra measured in the absence and in the presence of D-glucose and a well-known inhibitor, cytochalasin B. We have obtained CD spectra corrected for optical artifacts by using a specially fabricated device for collecting scattered light and by sonicating vesicle samples. The secondary structure was estimated by mathematical calculation of orthogonal basis CD spectra, using the CD spectra of 15 globular proteins of known structure, as described by Hennessey and Johnson (10). Results indicate that the transporter contains more than 80% of α-helix, with a small amount of β-turns and random coil, but no β-strands. The results further indicate that D-glucose, but not cytochalasin B, affects the secondary structure significantly by reducing the α-helical content and mostly increasing random coils.

METHODS

Sample Preparation. Human erythrocyte glucose transporter proteoliposomes were prepared by DEAE-cellulose chromatography of octyl glucoside-solubilized membranes followed by detergent removal, following the method of Baldwin *et al.* (3) as we have modified it (11). The proteoliposome preparation was sonicated in a bath sonicator for 20 min at 2°C, which uniformly reduced the vesicle size to less than 100 nm without loss of any cytochalasin B binding activity. The protein was quantitated by Lowry assay (12), using bovine serum albumin as a standard. Phospholipids were quantitated by phosphate determination (13).

Spectroscopy. CD spectra were measured with a Jasco model 41-C spectropolarimeter at 21°C with constant nitrogen flushing. The spectrapolarimeter employed a specially fabricated device for collecting scattered light, which includes a CD cell with a path length of 0.254 mm. The essential features of the device are diagrammed in Fig. 1. It uses aluminized and magnesium fluoride-overcoated elements (14, 15), which reflect and channel scattered light covering approximately a 340° solid angle from the sample. Forward-scattered light is channeled via an internally and externally aluminized, ¾-inch (19-mm) Pyrex tube to the photomultiplier. Additional scattering from 45° to 90° is directed by a 45° reflecting cone adjacent to the rear of the sample cell. Backscatter from 90° to 135° is directed by a similar 45° cone in front of the sample, to a concave mirror with a diameter of

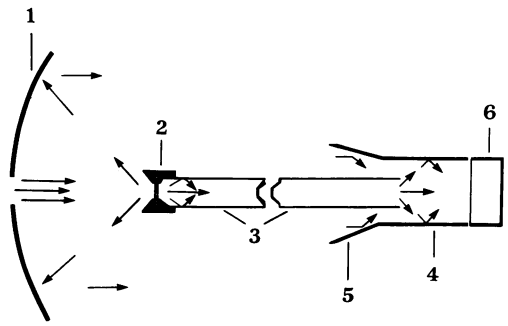

FIG. 1. Diagram of the light reflection device used in the present study. See text for details: *1*, concave mirror; *2*, sample holder; *3*, small light-channeling tube; *4*, large reflector tube; *5*, cone collector; *6*, spectrometer photomultiplier tube.

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138 mm and a focal length of 5 cm (Bausch & Lomb photospectrometer model 501). Backscattered light is then reflected directly or via reflection from the 30° reflecting cone, or bouncing off the surface of the smaller and the larger tubes to the photomultiplier. The scattered-light-collection device includes a sample cell as an integral part. The sample cell consisted of 19-mm-diameter and 0.15-mm-thick S1UV-A fused silica coverslips (Esco Products, Orkridge, NJ) and 0.254-mm polyethylene spacers. The internal diameters of the spacers were cut with a 45° slant and aluminized. The sample cell was placed in the special holder and positioned 6 cm in front of the concave mirror.

Spectra were obtained over wavelengths of 250–185 nm, with a scan rate of 1 nm/min and a time constant of 16 sec. The instrument was calibrated by using a standard solution of (+)*d*-10-camphorsulfonic acid at 290 nm (16).

For particulate samples, glucose transporter vesicles were suspended at a protein concentration of 150 μg/ml, in a buffer containing 12.5 mM Na^+, 0.5 mM K^+, 0.38 mM Ca^{2+}, and 0.25 mM Mg^{2+}, all as chlorides, and 1 mM Tris·HCl, at pH 7.4. For solubilized samples, 1% octyl glucoside was included in this buffer. Samples were also prepared with a final concentration of 10 μM cytochalasin B or 500 mM D-glucose when so specified. Baseline spectra were recorded with a blank and subtracted from the sample spectra. Cytochalasin B at 10 μM and glucose at 500 mM showed little or no effect on baseline spectra.

UV spectra were measured manually with a Gilford 2440 spectrophotometer with constant nitrogen flushing, over the range 600–185 nm, using the same scattered-light-collecting device and cell as used for the CD measurements.

Secondary Structure Estimation. The method introduced by Hennessey and Johnson (10) was used to estimate eight types of secondary structures. The method of Chang *et al.* (5) with helical length correction was also employed, where stated, for a comparison.

RESULTS AND DISCUSSION

The transporter vesicle preparations used in this study possessed glucose-sensitive cytochalasin B binding activity of at least 15 nmol/mg of protein. Gel filtration chromatography has indicated that more than 85% of the protein mass of each of these preparations represents the native glucose-sensitive cytochalasin B binding entity, P-2 (11). Sonication for 20 min at 2°C affected neither the cytochalasin B binding nor the glucose sensitivity of the inhibitor binding of the preparation (data not illustrated). The sonication, however, reduced vesicle size to an average diameter of approximately 80 nm, as estimated by negative stain electron microscopy, with at least 90% of the population less than 100 nm in diameter (not illustrated). The protein-to-lipid mass ratio of the proteoliposomes varied between 0.3 and 0.2.

Optical scattering of particulate samples is a major source of artifact in CD spectra of membrane proteins (6, 17). The use of our scattered-light-collecting device (Fig. 1) effectively overcame this problem by recovering most or all of the scattered light. This is illustrated in Fig. 2. When the scattered-light-collecting device was not used, the absorption spectra of the particulate and detergent-solubilized samples were quite different, even though the sample cell (1-mm path length Jasco CD cell) was placed directly next to the photomultiplier to make a total acceptance angle as large as possible. On the other hand, when the device was used, the difference in absorption spectra between the particulate sample and the detergent-solubilized sample was negligible, no more than 2% at all wavelengths. A calculation of the absorbance correction using $A_s(\lambda) = k\lambda^{-n}$ (18), in which A_s denotes apparent absorbance due to scattering, k and n are constants, and λ is the wavelength, revealed that the difference was no more than 0.002 *A* unit, well within the measuring error. This also demonstrates that there is negligible absorption flattening in this case. This may in part be effected by sonication, which reduced the particle diameter to less than 100 nm, together with a low protein content in our sample (7, 19, 20). In the present study, therefore, it was not necessary to make correction on CD either for differential scattering effects or for absorption flattening effects. The

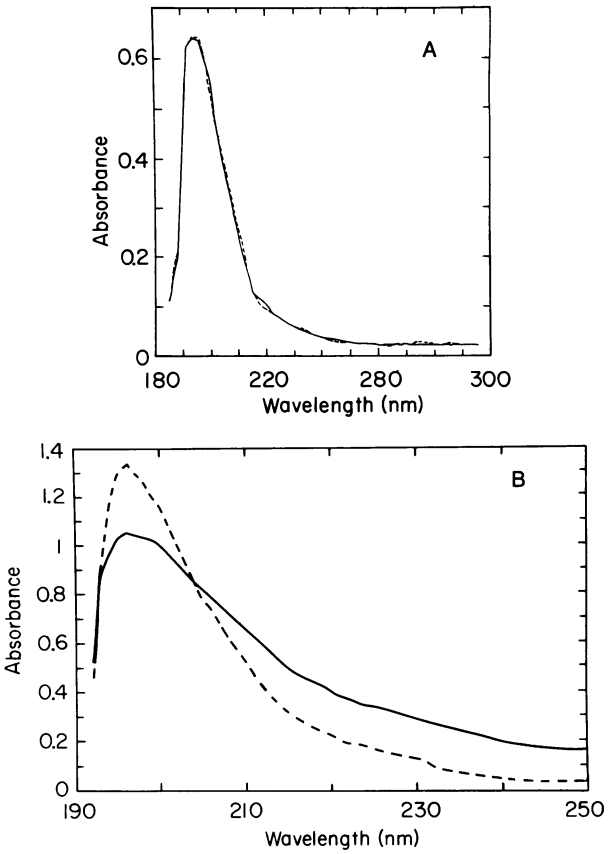

FIG. 2. Absorption spectra of the glucose transporter vesicles with (*A*) and without (*B*) the use of the scattered-light-collection device. A pair of samples containing no detergent (—) and 1% octyl glucoside (---) was used, and the spectra were measured under identical conditions to justify the comparison, except that a 1-mm path length Jasco CD cell was used in *B*.

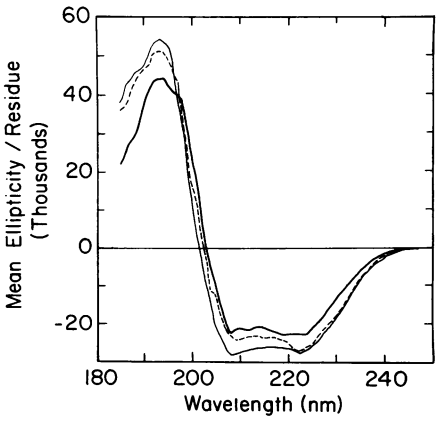

FIG. 3. CD spectra of the glucose transporter in vesicles. Spectra were measured with samples (150 μg of protein per ml) in the absence (—) and in the presence of 500 mM D-glucose (—), and 0.1 mM cytochalasin B (---). In each case the experimental conditions were otherwise identical.

Table 1. Fractional composition of secondary structure of human erythrocyte glucose transporter

Sample	H	A	P	I	II	III	T	O	Total	NRMSD*
Control	0.820	0.000	−0.017	0.033	0.013	0.046	0.025	0.081	0.991	0.068
+ cytochalasin B	0.790	0.000	−0.014	0.035	0.013	0.046	0.028	0.110	1.008	0.069
+ glucose	0.710	0.000	0.018	0.040	0.012	0.047	0.034	0.150	1.011	0.079

H, α-helix and 3_{10}-helix combined; A, antiparallel β-sheet; P, parallel β-sheet; I, type I β-turn; II, type II β-turn; III, type III β-turn; T, remaining β-turns combined; O, other random structures.

*Normalized root mean square deviation as given by the equation NRMSD = $[\Sigma_n (\theta_{exp} - \theta_{calc})^2/\Sigma_n \theta_{exp}^2]^{1/2}$, in which θ_{exp} and θ_{calc} are the experimental and calculated mean residue ellipticities and n is the number of data points used (20, 21).

scattered-light-collecting device recovered most of the scattered light, measuring light from the sample over a solid angle of approximately 340°. This would equal or better that of measurements from a fluorscat cuvette (8), and approaches the values obtained from fluorescence-detected CD (9). Our device is designed to capture all scattered light, as does the fluorescence-detected CD, but without the complications that may arise from interaction of an added fluorophore such as α-naphthylamine with the sample.

Using the scattered-light-collecting device, we obtained CD spectra of the vesicle sample at wavelengths as low as 185 nm. This would include enough information to analyze the positive peaks at 185 and 193 nm, which are the most prominent peaks within the 178- to 200-nm range. Hennessey and Johnson (10) have shown that having a truncated data set with a 184-nm cut-off made little difference in the analysis. More than 25 spectra were analyzed with data points at 2-nm wavelength intervals between 250 and 184 nm by a nonlinear least-squares curve-fitting procedure (19). Fig. 3 shows measured CD spectra of the glucose transporter in vesicles, in the absence and in the presence of 500 mM D-glucose or 0.1 mM cytochalasin B. It is clear that the circular dichrograph is modified in a specific manner by the presence of the substrate and the inhibitor.

Eight types of secondary structures, namely, α-helix, parallel and antiparallel β-strands, four types of β-turn, and "other" structures were estimated by using the basis spectra generated by the eigenvector method based on 15 water-soluble proteins according to Hennessey and Johnson (10). The computer program used assumed no constraint except that the total sum of the fraction of secondary structures be unity. No restriction is placed on negative coefficients. Results of analyses are summarized in Table 1. Fig. 4 shows spectra reconstructed by using the data in Table 1.

The transport protein is largely made of α-helix, which accounts for 82% of the total mass (Table 1, control). Approximately 10% β-turns and 8% random coil or "other" structures make up the rest of the mass. No β-strand was evident. The negative coefficients observed here are within the margin of error.

The present analysis does not account for the well-known chain-length effects on CD spectra of α-helices (5, 22). This may have caused an error in the calculations, particularly for the α-helical content. The α-helices of the water-soluble globular proteins whose CD spectra were used here are mostly made of 10–12 amino acids. This is considerably shorter than typical transmembrane α-helices, which would contain 20 or more residues.

A hydropathy analysis of the amino acid sequence of the HepG2 transporter (4) has predicted that approximately 50% of the peptide mass is in transmembrane disposition, probably in 12 α-helices, and approximately 30% is in nontransmembrane disposition, probably in 4 major hydrophilic segments. The α-helical content assessed in the present study is far in excess of that accounted for by the transmembrane mass alone in this prediction. This suggests that nontransmembrane hydrophilic segments also include a large amount of α-helix.

In the presence of an excess of D-glucose, the helical content is decreased significantly and there is a sizeable increase in random coil and β-turn contents (Table 1). This change in the helical content is considered to be significant,

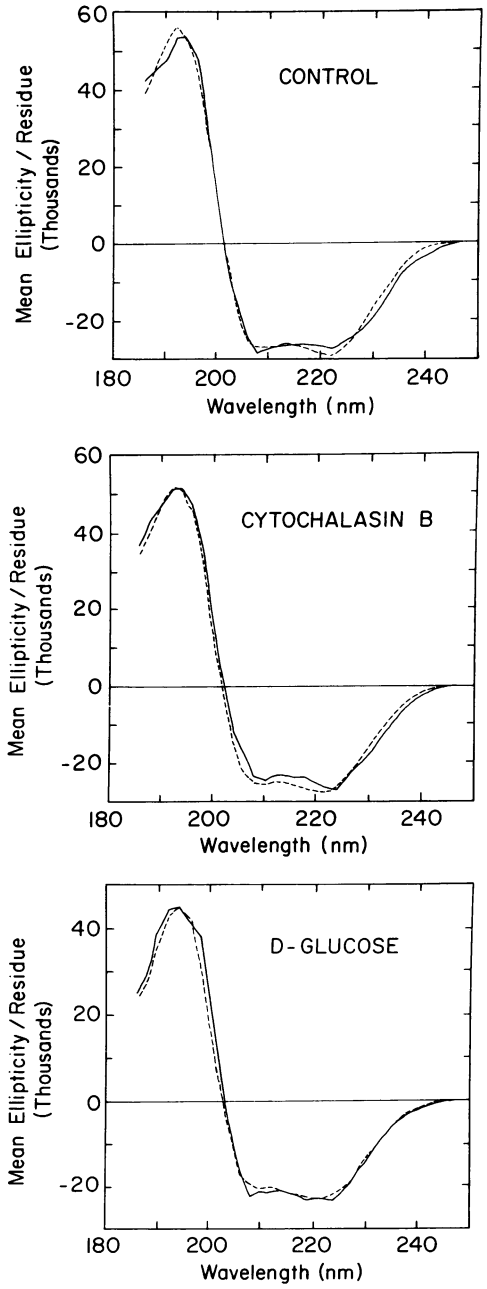

FIG. 4. Comparison of measured and reconstructed CD spectra of the glucose transporter in vesicles. Solid traces are measured CD spectra. Interrupted traces are spectra reconstructed by using the five basis spectra generated by Hennessey and Johnson (10). Degrees of fit are shown in Table 1.

as any error or artifact inherent in the data analysis is expected to be largely cancelled out in this comparison. The effect of cytochalasin B on the secondary structural composition of the transporter, on the other hand, is only minute, and may not be significant although, again, it tends to reduce α-helical content with a corresponding increase in random coil content (Table 1).

One salient feature revealed in these analyses is a consistent lack of β-form in this protein (Table 1). This is in agreement with our previous conclusion based on infrared spectroscopy of the transporter preparation (23). Virtual lack of β-form has been reported with rhodopsin (20), although this was refuted in other reports (19). It is interesting to note in this connection that a recently proposed hypothesis of peptide-chain insertion into membranes (24, 25) suggests that only helical conformations are likely to occur in the interior of membranes. A significant amount of β-structure was found in bacteriorhodopsin (5%) (26), Na^+,K^+-ATPase (31–38%) (27), a membrane-active plant toxin analog, crambin (22%) (28), and the major envelope protein of *Escherichia coli*, porin (>38%) (29). In each of these studies, however, if any of these β-structures are indeed in transmembrane disposition they are yet to be assessed.

We thank Dr. Grayson H. Snyder (Department of Biological Sciences, State University of New York, Buffalo) for the use of his spectropolarimeter and facilities. We thank Dr. Paul Binette (Electron Microscopy Laboratory, Veterans Administration Medical Center, Buffalo, NY) for his vesicle size measurements. We are grateful to Mr. Edward Hurely (Roswell Park Memorial Institute) for allowing us to use his equipment for metal overcoating during fabrication of the scattered-light-collecting device. We also gratefully acknowledge Charles Berenski for his preparation of human erythrocyte transporter vesicles. This work was supported by Grant AM-13376 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases and by the Veterans Administration Medical Center, Buffalo, NY.

1. Jung, C. Y. (1975) in *The Red Cell*, ed. Surgenor, D. M. (Academic, New York), Vol. 2, pp. 705–751.
2. Kasaraha, M. & Hinkle, P. C. (1977) *J. Biol. Chem.* **253,** 7384–7390.
3. Baldwin, S. A., Baldwin, J. M. & Lienhard, G. E. (1982) *Biochemistry* **21,** 3836–3842.
4. Mueckler, M., Caruso, C., Baldwin, S. A., Panico, M., Blench, I., Morris, H. R., Allard, W. J., Lienhard, G. E. & Lodish, H. F. (1985) *Science* **229,** 941–945.
5. Chang, C. T., Wu, C. S. C. & Yang, J. T. (1978) *Anal. Biochem.* **91,** 13–31.
6. Schneider, A. S. (1973) *Methods Enzymol.* **27,** 751–767.
7. Gordon, D. J. & Holzworth, G. (1971) *Arch. Biochem. Biophys.* **142,** 481–488.
8. Dorman, B. P., Hearst, J. E. & Maestre, M. F. (1973) *Methods Enzymol.* **27,** 767–796.
9. Reich, C., Maestre, M. F., Edmondson, S. & Gray, D. M. (1980) *Biochemistry* **19,** 5208–5213.
10. Hennessey, J. P., Jr., & Johnson, W. C., Jr. (1981) *Biochemistry* **20,** 1085–1094.
11. Rampal, A. L., Jung, E. K. Y., Chin, J. J., Deziel, M. R., Pinkofsky, H. B. & Jung, C. Y. (1986) *Biochim. Biophys. Acta* **859,** 135–142.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
13. Bartlett, G. R. (1959) *J. Biol. Chem.* **234,** 469–471.
14. Madden, R. P. (1963) in *Physics of Thin Films*, ed. Hass, G. (Academic, New York), Vol. 1, pp. 123–184.
15. Klingslake, R. (1965) in *Applied Optics and Optical Engineering*, ed. Kingslake, R. (Academic, New York), Vol. 3, pp. 309–327.
16. Cassin, J. Y. & Yang, J. T. (1969) *Biochemistry* **8,** 1947–1951.
17. Mao, D. E., Wachter, E. & Wallace, B. A. (1981) *Biochemistry* **21,** 4960–4968.
18. Leach, S. J. & Scheraga, H. A. (1960) *J. Am. Chem. Soc.* **82,** 4790–4792.
19. Glaeser, R. M. & Jap, B. K. (1985) *Biochemistry* **24,** 6398–6401.
20. Mao, D. & Wallace, B. A. (1984) *Biochemistry* **23,** 2667–2673.
21. Brahms, S. & Brahms, J. (1980) *J. Mol. Biol.* **138,** 149–178.
22. Chen, Y. J., Yang, J. T. & Chau, K. H. (1974) *Biochemistry* **13,** 3350–3359.
23. Chin, J. J., Jung, E. K. Y. & Jung, C. Y. (1986) *J. Biol. Chem.* **261,** 2561–2569.
24. Engelman, D. M. & Steitz, T. A. (1981) *Cell* **23,** 411–422.
25. Engelman, D. M. & Steitz, T. A. (1981) *Cell* **23,** 1406–1410.
26. Rothchild, K. J., Sanches, R., Hsao, T. C. & Clark, N. A. (1980) *Biophys. J.* **31,** 53–64.
27. Gresalfi, T. J. & Wallace, B. A. (1984) *J. Biol. Chem.* **259,** 2622–2628.
28. Wallace, B. A., Kohn, N. & Teeter, M. M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1406–1410.
29. Rosenbusch, J. P. (1984) *J. Biol. Chem.* **249,** 8019–8029.