Monosaccharide Transporter of the Human Erythrocyte. Characterization of an Improved Preparation†

Stephen A. Baldwin,‡ Jocelyn M. Baldwin,§ and Gustav E. Lienhard*

ABSTRACT: The human erythrocyte monosaccharide transporter has been purified through the use of the dialyzable detergent octyl glucoside. It was found that the transporter denatures in the detergent and that the rate of this process could be reduced by increasing the ratio of phospholipid to detergent. The transporter was obtained in higher yield and with a higher specific activity for cytochalasin B binding than has been previously reported. Scatchard plot analysis of cytochalasin B binding to the reconstituted preparations gave a dissociation constant of 1.5×10^{-7} M, and there were found to be 15.3 nmol of sites/mg of protein. On the basis of a value of 46 000 for the molecular weight of the polypeptide, this specific activity corresponds to 0.70 site/polypeptide chain; and there are reasons to believe that the value of the stoichiometry may be one site per functional transporter polypeptide. The complete amino acid composition and the N- and C-terminal residues of the transporter have been determined. Both the intact transporter and transporter that had been partially depleted of carbohydrate by treatment with endo-β-galactosidase were found to migrate anomalously upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis, relative to the behavior of standard proteins.

Using the reconstitution of D-glucose transport as an assay, Kasahara & Hinkle (1977) first purified the monosaccharide transporter from human erythrocytes. Subsequently, employing an assay based upon the binding of the high-affinity ligand cytochalasin B, we purified the same protein by a similar, but modified, procedure (Baldwin et al., 1979, 1980). Although Kasahara & Hinkle (1976) had originally found that the detergents octyl glucoside and Triton X-100 would solubilize the glucose transporter in a form allowing the reconstitution of transport activity, their purification was done only with Triton X-100. Previously we have used Triton X-100 (Baldwin et al., 1979) and octaethylene glycol dodecyl ether (Baldwin et al., 1980) in our isolation procedures.

Herein we describe the purification of the monosaccharide transporter through the use of the detergent octyl glucoside. The substitution of this detergent results in a substantial improvement both in the yield of transporter protein and in the specific activity for cytochalasin B binding. Because the number of cytochalasin B binding sites per milligram of protein of this new preparation is higher, the suggestion, which was based upon the specific activities of earlier preparations, that there may be only one cytochalasin B binding site per dimer of the polypeptide (Sogin & Hinkle, 1978; Baldwin et al., 1979) is no longer supported by experimental results. Initial chemical characterization of this new preparation of the transporter is also reported. A brief preliminary account of this work has been presented (Baldwin & Baldwin, 1981).

Experimental Procedures

Materials. *n*-Octyl β-D-glucopyranoside (octyl glucoside) was purchased from Calbiochem. Purified, protease-free endo-β-galactosidase (Fukuda & Matsumura, 1976) was a generous gift from Dr. M. Fukuda. Outdated blood, kindly provided by the blood bank of the Mary Hitchcock Memorial Hospital, was used for all preparations unless otherwise stated.

Purification of the Monosaccharide Transporter. The transporter was purified by a modification of previously reported procedures (Baldwin et al., 1979, 1980). All operations were carried out at 4 °C. Erythrocyte membranes (ghosts) were prepared from washed cells by the method of Steck & Kant (1974) and stored at −70 °C. The membranes were stripped of their peripheral proteins by treatment at pH 12 exactly as described by Gorga & Lienhard (1981). These protein-depleted membranes were also stored at −70 °C. Octyl glucoside was added to the protein-depleted membranes (2 mg of protein/mL) in 50 mM Tris-HCl[1]/2 mM dithiothreitol, pH 7.4 at 4 °C, to give a final concentration of 46 mM. After the mixture was shaked for 20 min, it was centrifuged at 130000*g* for 1 h. A sample (60 mL) of the supernatant (referred to as "the extract") was applied to a column (2.5 × 6.3 cm) of DEAE-cellulose equilibrated with 34 mM octyl glucoside in 50 mM Tris-HCl/2 mM dithiothreitol, pH 7.4, and the column was eluted with this buffer at a flow rate of 70 mL/h. Protein-containing fractions were detected by their absorbance at 280 nm and were pooled. This material (about 75 mL), which contained the transporter and erythrocyte phospholipids, was made 100 mM in NaCl and 1 mM in EDTA before reconstitution by dialysis against four changes, each of 2 L, of 50 mM Tris-HCl/1 mM EDTA/100 mM NaCl, pH 7.4, over a period of 48 h. When a sample containing ^{14}C-labeled octyl glucoside was reconstituted by this procedure, 99.966% of the radioactivity was found to have been removed after 48 h of dialysis, and the molar ratio of phospholipid to the remaining octyl glucoside was 80 to 1. The reconstituted material typically contained about 130 μg/mL protein and 350 μg/mL phospholipid, and unless otherwise stated, this material was used for further studies. Samples of the unfractionated extract were also reconstituted by this dialysis procedure.

In one experiment, the transporter was purified from a unit of freshly drawn blood, with protease inhibitors present at

† From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755. *Received January 29, 1982.* This research was supported by Grant GM 22996 from the National Institutes of Health.

‡ Present address: Departments of Biochemistry and Chemistry and of Chemical Pathology, Royal Free Hospital School of Medicine, University of London, London WC1N 1BP, England.

§ Present address: Department of Experimental Pathology, School of Medicine, University College London, London WC1E 6JJ, England.

[1] Abbreviations: dansyl or Dns, 5-(dimethylamino)naphthalene-1-sulfonyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; PTH, phenylthiohydantoin; Tris, tris(hydroxymethyl)aminomethane.

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various steps. The erythrocytes were lysed and the membranes washed in 5 mM sodium phosphate, pH 8, containing 1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, and 1.4 μM pepstatin A, compounds which are reported to be effective in minimizing proteolysis of erythrocyte membrane proteins (Bennett & Stenbuck, 1980). The same inhibitors were also included during the detergent solubilization of the protein-depleted membranes.

In some experiments, the final step in the isolation of the transporter was carried out in the presence of added lipid. For these, the ratio of phospholipid to detergent in the octyl glucoside solubilized material was increased by the addition, before centrifugation, of 0.033 volume of 31 mM dioleoylphosphatidylcholine dissolved in 171 mM octyl glucoside. The DEAE-cellulose column was equilibrated and eluted with 1.9 mM dioleoylphosphatidylcholine and 37 mM octyl glucoside in 50 mM Tris-HCl/2 mM dithiothreitol, pH 7.4.

The preparations of transporter were stored at −70 °C. In most instances, the measurements of cytochalasin B binding were made before freezing; however, the concentration of cytochalasin B binding sites was not altered by storage at −70 °C for periods up to 1 year.

Cytochalasin B Binding Measurements. Routine measurements of cytochalasin B binding to reconstituted extract and to the purified transporter, in 50 mM Tris-HCl/1 mM EDTA/100 mM NaCl, pH 7.4, were made by equilibrium dialysis using 5×10^{-8} M [^{3}H]cytochalasin B, according to our previously described method (Zoccoli et al., 1978). The ratio of bound to free cytochalasin B was determined, and a correction was made for nonspecific binding to lipids in the preparation by subtracting the ratio for a sample that had been held at 100 °C for 1 min. This value was less than 2% of the value for the undenatured mixture. As we have described in detail (Zoccoli et al., 1978), under these conditions the corrected value of the bound:free ratio is equal to the concentration of transporter-specific cytochalasin B binding sites divided by the dissociation constant for cytochalasin B. The ratio is referred to as the "cytochalasin B binding activity". Since octyl glucoside, like other detergents (Zoccoli et al., 1978), inhibits the binding of cytochalasin B to the transporter, it was necessary always to remove the octyl glucoside by dialysis before measuring cytochalasin B binding. The dissociation constant for this inhibition by octyl glucoside was about 5 mM (data not shown).

For determination of the dissociation constant as well as for more accurate determination of the concentration of sites, cytochalasin B binding was measured in duplicate over a range of concentrations. Stock solutions of [^{3}H]cytochalasin B were prepared by mixing a constant amount of the chemically synthesized [^{3}H]cytochalasin B (New England Nuclear) with varying amounts of the unlabeled natural product (Aldrich). The data were treated by the method of Scatchard, with correction for the small amount of nonspecific binding to the phospholipid in the preparations by our previously described method (Zoccoli et al., 1978).

Molecular Weight Estimation by Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed as previously described (Gorga et al., 1979). In some cases, pure sodium dodecyl sulfate was used, whereas in others the mixture of sodium C12, C14, and C16 alkyl sulfates sold by Pierce Chemical as "lauryl" sulfate was employed. We have used the terms "$NaDodSO_4$–polyacrylamide gel electrophoresis" and "alkyl sulfate–polyacrylamide gel electrophoresis" to designate electrophoresis with these two reagents. For estimation of the molecular weight of the protein moiety of the transporter, the glycoprotein was first depleted of carbohydrate by glycosidase treatment, according to a procedure similar to that given by Gorga et al. (1979).

S-Carboxamidomethylation and Delipidation of the Transporter. A sample (70 mL) of the reconstituted transporter containing about 10 mg of protein and 26 mg of phospholipid was centrifuged for 1 h at 150000g in order to pellet the material. The protein was reduced by resuspending the pellet to a final volume of 6 mL in 100 mM Tris-HCl/1 mM EDTA, pH 8.3, containing 2 mM dithiothreitol and 3% $NaDodSO_4$. After incubation of the mixture under N_2 for 30 min at 22 °C, sulfhydryl groups were alkylated by the addition of 12 mM iodoacetamide (radiolabeled if required) and further incubation for 1 h under N_2 in the dark at 22 °C. Excess reagent was then destroyed by the addition of 2-mercaptoethanol, and the mixture was applied to a column (91 × 2.5 cm) of Sephacryl S-300 in the dark. The column was eluted with 0.5% $NaDodSO_4$; and the protein-containing fractions, located by their absorbance at 280 nm, were pooled. One passage through the column resulted in the separation of the protein from all excess reagent and from more than 95% of the phospholipid. The protein-containing fractions were concentrated by ultrafiltration and rechromatographed on the same column in order to remove the remaining phospholipid. A yield of approximately 7 mg of protein was obtained, and the preparation contained less than 0.5 mol of phospholipid/mol of protein.

Determination of the Amino Acid Composition of the Transporter. Before amino acid analysis, $NaDodSO_4$ was removed from the delipidated, S-carboxamidomethylated glucose transporter by the procedure of Henderson et al. (1979). Samples were then hydrolyzed for periods of 24, 72, and 120 h at 110 °C under vacuum in constant-boiling HCl containing 2-mercaptoethanol (0.5 mL/L) and were analyzed with a Beckman 121 MB instrument. Cysteine was determined as *S*-(carboxymethyl)cysteine or as cysteic acid following performic acid oxidation of the delipidated transporter by the method of Hirs (1967). Tryptophan was determined spectroscopically with the S-carboxamidomethylated protein in $NaDodSO_4$ (Koziarz et al., 1978).

Amino acid analysis was also used to determine accurately the protein concentration in samples of the reconstituted glucose transporter. Samples were dialyzed against 4 mM NaCl; small changes in concentration due to dialysis were corrected for by measuring the phospholipid concentration before and after dialysis. Norleucine was added to the dialyzed samples as an internal standard; aliquots of each sample were lyophilized and hydrolyzed for 24 h. The protein concentration was calculated from the mean content of six stable amino acids in the hydrolysates and the complete amino acid composition.

N- and C-Terminal Analysis of the Transporter. N-Terminal analysis was performed on delipidated, S-carboxamidomethylated glucose transporter in $NaDodSO_4$ by the dansylation procedure of Gray (1972). A second method of N-terminal analysis was automatic Edman degradation. This was performed on a sample (4.3 mg) of $NaDodSO_4$-free, delipidated, S-carboxamidomethylated protein by using a protein QUADROL program (no. 122974) on the Beckman 890C sequencer. PTH-amino acids were identified both by thin-layer chromatography (Bridgen et al., 1975) and by high-performance liquid chromatography (HPLC) in system 4 of North & Mitchell (1981).

Carboxypeptidase A digestion of the delipidated, ^{14}C-labeled S-carboxamidomethylated glucose transporter in 0.4% $NaDodSO_4$ was performed by the method of Ambler (1972), with

Table I: Purification of the Monosaccharide Transporter[a]

fraction	protein (mg)	sites (nmol)	sp act. (nmol/mg)	$10^7 K_D$ (M)
protein-depleted[b] membranes	100	220	2.2	1.4
octyl glucoside extract	56	190	3.4	1.44
purified prepn[c]	10.2	123	12.1	1.43
	10.3	154	14.8	1.64
prepn purified with added lipid[d]	8.8	125	14.3	1.35
	12.2	198	16.2	1.73

[a] The values for the number of cytochalasin B binding sites and for the dissociation constant (K_D) were determined from Scatchard plots done on the reconstituted material. The protein contents of the purified preparations were obtained from amino acid analysis, whereas those of the other fractions were measured by the Lowry procedure. [b] Data from Baldwin et al. (1979). [c] Results for two separate preparations, the first of which is derived from the extract described in the line above. [d] Results for two separate preparations.

norleucine as an internal standard. Released amino acids were quantified with the amino acid analyzer. A time course of digestion was carried out, and a control incubation lacking the transporter was used to correct for the presence of any amino acids derived from the carboxypeptidase. The protein content of the samples was accurately determined from their radioactivity.

Other Procedures. The sulfhydryl content of the purified transporter was determined by reaction with DTNB (Ellman, 1959; Riddles et al., 1979) in the presence of 2% $NaDodSO_4$. The $NaDodSO_4$ was added to solubilize the membrane after addition of DTNB in an effort to avoid oxidation of newly exposed thiols before they could react with DTNB. Colorimetric tests were also used to measure protein (Peterson, 1977), $NaDodSO_4$ (Hayashi, 1975), octyl glucoside (Spiro, 1966), and phospholipid (Bartlett, 1959).

Results

Purification of the Transporter. Erythrocyte membranes that have been stripped of peripheral proteins are an optimal preparation for further purification of the monosaccharide transporter. It has been shown that these protein-depleted membranes retain all of the transporter-specific cytochalasin B binding sites present in the erythrocyte membranes but have lost the other types of high-affinity sites for cytochalasin B (Baldwin et al., 1979). Previously, we found that the detergents Triton X-100 (Baldwin et al., 1979) and octaethylene glycol dodecyl ether (Baldwin et al., 1980) led to the solubilization and reconstitution of 24 and 38%, respectively, of the transporter in the protein-depleted membranes, as determined by means of the cytochalasin B binding assay. In the current study, we have found that by use of the detergent octyl glucoside about 90% of the cytochalasin B binding sites can be solubilized and reconstituted (Table I). In addition, unlike the other detergents, octyl glucoside effected a somewhat preferential extraction of the transporter, such that a 1.5-fold increase in the specific activity of cytochalasin B binding sites was achieved in the solubilization step (Table I). The optimal detergent concentration for solubilization and reconstitution of the transporter was found to be between 42 and 51 mM, at a protein concentration of 2 mg/mL. Above 51 mM detergent, there was a reduction in the recovery of cytochalasin B sites, presumably as the result of denaturation of the transporter (see below). Table I summarizes the results of two purifications. The overall yield of purified transporter from the protein-depleted membranes is about 60%.

Table II: Rates of Inactivation of the Monosaccharide Transporter in the Solubilized State[a]

expt	sample	[PL] (mM)	[OG] (mM)	$t_{1/2}$ (h)
1	extract	1.36	*b*	35
	transporter	0.49	*b*	3.8
2[c]	extract	0.74	34	18
	transporter	0.24	31	3.3
	transporter + PL	0.85	36	7.6
3	extract	1.54	44	31
	transporter	0.70	39	7.5
4[d]	extract + PL	2.13	44	40
	transporter + PL	1.20	40	17

[a] For determination of the rates of inactivation, portions of the octyl glucoside extract and of the transporter-containing fractions from the DEAE-cellulose chromatography of this extract (see Experimental Procedures) were made 100 mM in NaCl/1 mM in EDTA and held at 8 °C. At various times thereafter, dialysis of samples was initiated, and subsequently the cytochalasin B binding activity of the samples was measured. PL and OG designate phospholipid and octyl glucoside. [b] These values were not measured, but should be about the same as those found in experiment 3, since the conditions were the same for the two experiments. [c] In this experiment, a small volume of dioleoylphosphatidylcholine in octyl glucoside (31 mM in 171 mM) was added to a portion of the solubilized transporter after purification. The use of a lower concentration of octyl glucoside than the normal one (46 mM) for the extraction of the protein-depleted membranes led to a lower concentration of erythrocyte phospholipid in the extract and in the purified preparation. [d] In this experiment, a small volume of dioleoylphosphatidylcholine in octyl glucoside was added to the extract, and the transporter was purified therefrom with additional phospholipid in the column buffer, as described under Experimental Procedures.

It has been suggested that the purified transporter may be a proteolytic fragment of a larger polypeptide (Mullins & Langdon, 1980). Although the results of immunological studies (Baldwin & Lienhard, 1980; Sogin & Hinkle, 1980a) indicate that this hypothesis is incorrect, it seemed worthwhile to examine the effects of protease inhibitors on the preparation. In addition, since the transporter was routinely purified from outdated units of blook-bank blood (drawn over 21 days previously), it was of interest to determine whether the properties of the transporter changed during storage of the blood. For these reasons, one preparation was carried out in the presence of protease inhibitors (see Experimental Procedures), with a freshly drawn unit of blood. The yield of transporter (8.5 mg/100 mg of protein-depleted membrane), the specific activity of cytochalasin B binding sites (13.2 nmol/mg), and the K_D value (1.43×10^{-7} M) were not significantly different than those of the standard preparation (Table I), and the polypeptide profile upon alkyl sulfate gel electrophoresis was identical with that of the standard preparation (see below).

Denaturation of the Solubilized Transporter. Denaturation of the transporter in detergent could account for the partial loss of cytochalasin B binding sites upon DEAE chromatography. In order to estimate the extent of such inactivation, we determined the stability of the transporter in octyl glucoside by measuring the cytochalasin B binding activity of extracts and purified preparations that had been reconstituted after various periods in the solubilized state. It was found that solubilized transporter undergoes a time-dependent inactivation that follows first-order kinetics (data not shown). Table II summarizes the results of several experiments. The transporter was more stable in the extract ($t_{1/2}$ about 33 h, experiments 1 and 3) than in the purified state ($t_{1/2}$ 3.8–7.2 h, experiments 1 and 3). The ratio of phospholipid to detergent in the extract is considerably higher than that in the fractions containing the

purified transporter (Table II) because only about 40% of the erythrocyte phospholipid passes through the DEAE-cellulose along with the transporter. This difference in the ratio of phospholipid to detergent appears to account, at least in part, for the difference in the stability of the transporter. When the ratio of phospholipid to detergent was increased, either by adding dioleoylphosphatidylcholine to the purified transporter (experiment 2) or by carrying out the purification in the presence of added dioleoylphosphatidylcholine (experiment 4, compare with experiment 3), the half-life of the purified transporter increased. The loss in activity was probably due to denaturation rather than to proteolytic degradation, since the profile of the purified protein upon $NaDodSO_4$–polyacrylamide gel electrophoresis (see below) was the same for samples reconstituted at once and after complete loss of the cytochalasin B binding activity.

The results of two purifications in which dioleoylphosphatidylcholine was added to the extract before chromatography are included in Table I. This modification led to a slight improvement in the purification: the average specific activity for the preparations was 15.3 nmol of cytochalasin B sites/mg of protein, whereas that for the transporter obtained in the absence of added lipid was 13.5 nmol/mg (Table I). Even with the addition of lipid it is probable that a small fraction of the transporter underwent denaturation during purification. A rough estimate, based on the data in Table II, is that 10% of the transporter may have denatured.

Cytochalasin B Binding to the Transporter. Accurate determination of the number of cytochalasin B binding sites per milligram of purified transporter protein requires several considerations. First, accurate measurement of the protein concentration is essential. Consequently, in this investigation, we have employed quantitative amino acid analysis. Triplicate samples of each preparation (Table I) were analyzed, and the individual values of the protein concentrations determined in this way routinely differed from the mean by less than 3%.

A second important consideration in the binding studies is the purity of the [^{3}H]cytochalasin B used. While the unlabeled compound employed in this study was known to be a natural product with unique stereochemistry (McLaughlin et al., 1970, and Aldrich Chemical), the labeled compound was a synthetic product obtained by chemical reduction of the keto group of cytochalasin A to a secondary alcohol with borotritide (Lin et al., 1974, and personal communication from Dr. P. R. Srinivasan of New England Nuclear). There are two possible diastereoisomers of this reaction, which could bind to the transporter with different affinities. Dr. P. R. Srinivasan of New England Nuclear kindly obtained the proton-decoupled tritium NMR spectrum of the labeled compound. The compound exhibited only a single tritium resonance, and thus the reduction and subsequent purification yielded only one of the two possible isomers (Chambers et al., 1978). Moreover, since the chemical shift of the tritium was virtually identical with that of the corresponding hydrogen nucleus in the natural product, the labeled compound must be the isomer that is stereochemically identical with the natural product (Chambers et al., 1978).

Figure 1 presents typical Scatchard plots for the binding of cytochalasin B to the purified transporter. Each preparation exhibited a single class of high-affinity sites with a K_D of about 1.5×10^{-7} M. This value of the dissociation constant is similar to that for the transporter in ghosts (Lin & Snyder, 1977; Jung & Rampal, 1977).

Chemical Characterization of the Purified Transporter. The amino acid composition of the delipidated, S-carboxamidomethylated glucose transporter, based on a molecular weight of 46 000, is given in Table III. The composition resembles that reported by Sogin & Hinkle (1978) for transporter purified in Triton X-100, although these workers did not determine tryptophan. However, we found a higher methionine content, 12.4 as opposed to 8.8 residues/chain, possibly as the result of the inclusion of 2-mercaptoethanol in the hydrolysis acid. In addition, we find less cysteine, about 4.8 as opposed to 8.3 residues/chain. The value of 4.8, determined as *S*-(carboxymethyl)cysteine after hydrolysis of the S-carboxamidomethylated protein, was confirmed by determination of the cysteic acid content of performic acid oxidized protein, which gave 5.0 residues/chain. These values for the cysteine content are very close to the value of 4.7 sulfhydryl groups/chain, which was obtained by reaction of the reconstituted transporter with DTNB in $NaDodSO_4$ without prior reduction, other than the presence of dithiothreitol during the octyl glucoside solubilization and DEAE-cellulose chroma-

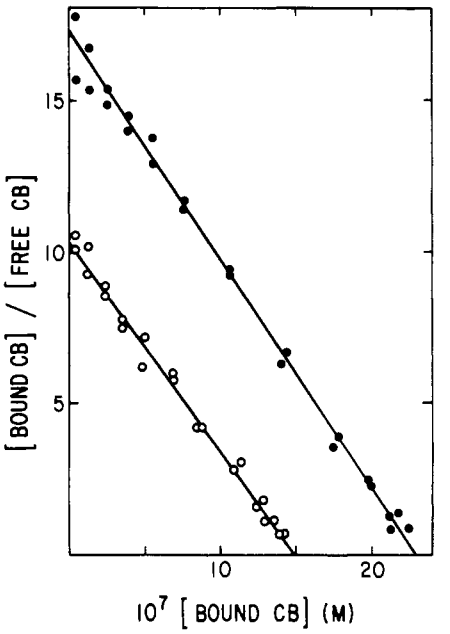

FIGURE 1: Scatchard plots for the binding of cytochalasin B (CB) to the reconstituted transporter purified without (○) and with (●) the addition of phospholipid. The data presented are those for the first preparations listed in Table I. The protein concentrations were 124 and 161 μg/mL, respectively. The data have been corrected for nonspecific binding to the phospholipid by the procedure of Zoccoli et al. (1978). The lines are the best fits according to linear least-squares analysis.

Table III: Amino Acid Composition of the Monosaccharide Transporter[a]

amino acid	residues/M_r 46 000	amino acid	residues/M_r 46 000
Cys	4.9 ± 0.1[b]	Met	12.4 ± 0.7
Asx	19.5 ± 0.2	Ile	27.5 ± 0.1[d]
Thr	21.3 ± 0.2[c]	Leu	51.3 ± 0.1[d]
Ser	31.0 ± 0.2[c]	Tyr	10.6 ± 0.1[d]
Glx	38.5 ± 0.3	Phe	31.1 ± 0.1[d]
Pro	21.4 ± 0.3	Lys	14.5 ± 0.1
Gly	38.4 ± 0.4	His	4.6 ± 0.1
Ala	26.8 ± 0.4	Arg	17.8 ± 0.1
Val	38.1 ± 0.1[d]	Trp	7.2[e]

[a] Unless noted, mean ± SEM for six determinations. [b] Average of duplicate determinations of *S*-(carboxymethyl)cysteine and of cysteic acid. [c] Extrapolated to 0-h hydrolysis, ± standard error of the estimate. [d] Mean of duplicate values from hydrolysis for 120 h or, for Tyr, 24 h. Tyr determined with lithium citrate buffer. [e] Single value determined spectrophotometrically.

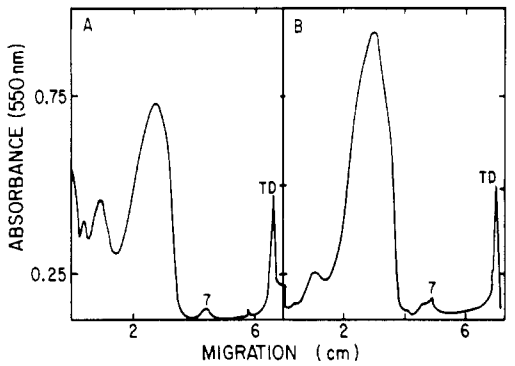

FIGURE 2: Polyacrylamide gel electrophoresis of the purified transporter (8.6 μg), with dodecyl sulfate (electrophoresis grade, from Bio-Rad) (A) or with the mixture of alkyl sulfates (sequanal grade "lauryl" sulfate from Pierce Chemical Co.) (B). Because the longer chain alkyl sulfates desorb from the protein less readily than does dodecyl sulfate and so cause a reduced intensity if the Coomassie Blue staining is done without prior washing, the gels were treated with 2-propanol/acetic acid/water (25:10:65, by volume) for 12 h before staining. TD and 7 indicate the positions of the tracking dye and band 7, respectively.

tography steps of the purification. Thus, it is unlikely that the purified undenatured transporter contains any disulfide bridges.

Amino terminal analysis of the purified transporter by the dansyl technique gave Dns-methionine, N^6-Dns-lysine, and *O*-Dns-tyrosine as the only dansylated amino acids. There was no trace of Dns-aspartic acid, despite the identification of the N-terminal residue of the transporter as aspartic acid by Hinkle & Sogin (1978). The identification of methionine as the only N-terminal amino acid was confirmed by the results of automatic Edman degradation. PTH-methionine was the only amino acid derivative obtained from the first cycle of degradation; the yield was about 38% based on a protein molecular weight of 46 000. The second cycle of degradation gave PTH-glutamic acid with a yield of about 20%. Although only traces of other amino acid derivatives were present at this stage, during succeeding cycles a background of several amino acids rapidly increased, and it was not possible to make further assignments with any confidence. The low yields from the first two cycles may be due to the hydrophobic nature of the polypeptide (Bailey et al., 1977).

The C-terminal residue of the transport protein was identified as valine by carboxpeptidase A digestion. In two experiments, the limit values for valine were 0.7 and 0.56 mol/mol of protein, based on a subunit molecular weight of 46 000. The only other amino acid released to any appreciable extent was alanine, which was obtained with a yield of 0.09 mol/mol of protein.

Polyacrylamide Gel Electrophoresis of the Purified Transporter. $NaDodSO_4$–polyacrylamide gel electrophoresis of the purified preparation on 10% gels showed one major, broad band of average apparent molecular weight of 55 000, along with species of higher molecular weight (Figure 2A). It was found that the amount of the higher molecular weight species was very much reduced when a mixture of alkyl sulfates was used in place of pure dodecyl sulfate (Figure 2B). Moreover, there was a corresponding increase in the amount of the 55 000-dalton species, such that the integrated intensities of the scans of the Coomassie Blue stained gels were the same for the two conditions. Presumably, the longer chain alkyl sulfates prevent the partial aggregation of the 55 000-dalton species that occurs in the presence of dodecyl sulfate alone. The only other polypeptide visible on the gels of the purified transporter was a small amount of erythrocyte band 7 [nomenclature according to Steck (1974)]. Staining of gels with the periodic acid–Schiff reagent (Steck & Yu, 1973) showed that the major sialoglycoproteins of the erythrocyte (Steck, 1974) were absent.

The transporter is a glycoprotein containing about 15% carbohydrate by weight (Sogin & Hinkle, 1978). Previously we have reported that removal of about 70% of this carbohydrate with the enzyme endo-β-galactosidase increases the mobility of the polypeptide upon $NaDodSO_4$–polyacrylamide gel electrophoresis and somewhat sharpens the profile of the band (Gorga et al., 1979). For estimation of the molecular weight of the polypeptide moiety, electrophoresis of the carbohydrate-depleted protein was performed in the presence of proteins of known molecular weight (Weber & Osborn, 1975). Moreover in order to ascertain the validity of this approach, we compared the results of electrophoresis on 7.5, 10, and 12.5% acrylamide gels. The results (data not shown) revealed that both the endo-β-galactosidase treated and the untreated transporter behaved anomalously when compared with the soluble, nonglycosylated standard proteins. The apparent molecular weight of the untreated transporter was found to be 53 000 on 7.5%, 56 500 on 10%, and 60 000 on 12.5% gels; that of the treated transporter was 42 700 on 7.5%, 45 800 on 10%, and 50 300 on 12.5% gels.

Discussion

The method described herein is a convenient one for the purification of substantial amounts of the monosaccharide transporter. We routinely obtain about 600 mg of erythrocyte membrane protein from a unit of blood (about 200 mL of cells) and recover approximately 36% of this protein in the protein-depleted erythrocyte membranes. Thus, the new procedure yields about 37 mg of purified transporter per g of erythrocyte ghost protein. This yield represents a substantial improvement over the previously described procedures that employed Triton X-100 or octaethylene glycol dodecyl ether; these gave about 7 (Kasahara & Hinkle, 1976), 14 (Baldwin et al., 1979), and 22 (Baldwin et al., 1980) mg of purified transporter per g of ghost protein. The specific activity of the current preparation (see Table I) is also higher than the values for preparations with other detergents, which were 7.4 (Baldwin et al., 1979), 11.2 (Baldwin et al., 1980), and 8–11 (Sogin & Hinkle, 1978, 1980b) nmol of cytochalasin B bound per mg of protein, based upon amino acid analysis.

A problem commonly encountered in the purification of an integral membrane protein is instability in the detergent-solubilized state. Such instability may be due to the displacement from the protein of lipid molecules that are needed for the maintenance of its native conformation. This view is supported by our observation that the rate of denaturation of the solubilized transporter is decreased when the ratio of phospholipid to detergent in solution is increased. Similar findings have been made for the agonist-regulated cation channel of the acetylcholine receptor (Anholt et al., 1981; Wu & Raftery, 1981), the tetrodotoxin binding component of the sodium channel (Agnew & Raftery, 1979; Barchi et al., 1980), and the lactose transporter from *Escherichia coli* (Newman & Wilson, 1980). Taken together, these results show that an important stratagem for the purification of an integral membrane protein in functional form is fractionation of the solubilized protein in the presence of mixed micelles of lipid and detergent, rather than of micelles of detergent alone.

Because the monosaccharide transporter is heterogeneously glycosylated, it runs as a broad band upon $NaDodSO_4$–polyacrylamide gel electrophoresis (Gorga et al., 1979). Endo-β-galactosidase treatment removes about two-thirds of the

carbohydrate and leads to some sharpening of the electrophoretic profile, but it is still not as sharp as that of a typical nonglycosylated protein (Gorga et al., 1979). Consequently, although it is clear from the results of polyacrylamide gel electrophoresis performed with the C12–C16 alkyl sulfates that the preparation does not contain significant amounts of contaminating polypeptides of higher or lower mobility, the presence of impurities of the same mobility is not excluded. However, two types of evidence suggest that the preparation contains less than about 20% of other polypeptide chains. First, the N- and C-terminal analyses gave only single amino acid residues in substantial yield. Second, the stoichiometry for binding of cytochalasin B to the most active preparations is 0.70 molecule/polypeptide of molecular weight 46 000 (see Table I and below).

It is worth noting that the preparation of the monosaccharide transporter almost certainly contains the erythrocyte nucleoside transporter as an impurity. Jarvis & Young (1981) have recently described a partial purification of the erythrocyte nucleoside transporter by a procedure that is virtually identical with the one for purification of the monosaccharide transporter in Triton X-100. Through the use of a binding assay with a high-affinity ligand, they found that the specific activity of the nucleoside transporter in the preparation was about 450 pmol/mg, a value that is about 3% of that for the glucose transporter. These relative amounts are in approximate agreement with the fact that the number of nucleoside transporters per erythrocyte (10 000) (Jarvis & Young, 1981) is about 4% of the number of glucose transporters (250 000) (Lin & Snyder, 1977). Thus, although the nucleoside transporter is probably present, it must be a minor component.

The anomalous behavior of the intact transporter and of the transporter with about two-thirds of the carbohydrate removed upon alkyl sulfate–polyacrylamide gel electrophoresis, relative to that of the water-soluble, nonglycosylated standards, is not suprising. $NaDodSO_4$–polyacrylamide gel electrophoresis has been found in several cases to underestimate the molecular weight of hydrophobic membrane proteins (Rizzolo et al., 1976; Bonitz et al., 1980) and often to overestimate the molecular weight of water-soluble glycoproteins (Leach et al., 1980). In this discussion, we have, for convenience, taken the molecular weight of the polypeptide to be 46 000, which is the middle value found for the endo-β-galactosidase-treated protein. However, it is evident that this value can only be considered to be an estimate and that accurate determination of the molecular weight will require a careful investigation by other methods.

In a recent review article, Klingenberg (1981) cited a stoichiometry of 0.5 cytochalasin B bound per polypeptide chain as evidence for the dimeric nature of the glucose transporter and, by implication, as support for the concept that the active site for transport of monosaccharide may be located at an interface between two subunits. Although the values of the stoichiometry for the earlier preparations of the transporter prompted these hypotheses, our current findings provide no support for them. Because of the uncertainly in the molecular weight, the stoichiometry can only be estimated. However, the average value of 0.70 per polypeptide of molecular weight 46 000 found for the preparations carried out in the presence of added lipid suggests that the true value may be one per functional polypeptide chain. In the absence of the estimated 10% denaturation in octyl glucoside, the observed value would have been about 0.8; and to the extent that there are protein impurities in the preparation, the value for the functional transporter would be even higher.

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Amino-Terminal Sequences of the L, M, and H Subunits of Reaction Centers from the Photosynthetic Bacterium *Rhodopseudomonas sphaeroides* R-26†

M. R. Sutton, D. Rosen, G. Feher, and L. A. Steiner*

ABSTRACT: We have determined the sequence of the 25–28 amino-terminal residues of the three subunits, L, M, and H, of the membrane-bound reaction center protein of the photosynthetic bacterium *Rhodopseudomonas sphaeroides* R-26. The sequences are as follows: L, H_2N-Ala-Leu-Leu-Ser-Phe-Glu-Arg-Lys-Tyr-Arg-Val-Pro-Gly-Gly-Thr-Leu-Val-Gly-Gly-Asn-Leu-Phe-Asp-Phe-(His)-Val-; M, H_2N-Ala-Glu-Tyr-Gln-Asn-Ile-Phe-Ser-Gln-Val-Gln-Val-Arg-Gly-Pro-Ala-Asp-Leu-Gly-Met-Thr-Glu-Asp-Val-Asn-Leu-Ala-Asn-; H, H_2N-Met-Val-Gly-Val-Thr-Ala-Phe-Gly-Asn-Phe-Asp-Leu-Ala-Ser-Leu-Ala-Ile-Tyr-Ser-Phe-Trp-Ile-Phe-Leu-Ala-X-Leu-Ile-. The H sequence, especially after the aspartyl residue at position 11, is rich in hydrophobic residues, consistent with the possibility that this section of the polypeptide chain is located within the membrane. The L sequence is hydrophilic near the amino terminus and then becomes moderately hydrophobic. The M sequence is of average polarity.

The primary electron transfer event in the purple photosynthetic bacterium *Rhodopseudomonas sphaeroides* R-26 is mediated by a membrane-bound reaction center composed of protein and a number of prosthetic groups including bacteriochlorophyll, bacteriopheophytin, ubiquinone, and iron [reviewed by Feher & Okamura (1978) and Okamura et al. (1982)]. The reaction center protein is isolated by treatment of chromatophore membranes with a nonionic detergent, *N,N*-dimethyllaurylamine oxide (LDAO).[1] It consists of three polypeptide chains, each of M_r ~30 000 and present in equimolar amounts. Two of these subunits, L and M, appear to be essential for photochemical activity; the function of the third, H, is not understood at present.

Initially, the subunits were isolated by elution from polyacrylamide gels after electrophoresis in sodium dodecyl sulfate ($NaDodSO_4$) (Okamura et al., 1974). More recently, a two-step procedure has been developed (Feher & Okamura, 1978; Rosen, 1979) in which the H subunit is dissociated from a complex of L and M by treatment with a chaotropic reagent, $LiClO_4$. The LM complex is then incubated in $NaDodSO_4$, and the L and M subunits are separated by affinity chromatography with an adsorbent containing organomercurial groups. The L subunit contains a free sulfhydryl group and binds to the adsorbent; the M subunit is devoid of cysteine and does not bind.

The amino acid compositions of the isolated subunits are notable for their high proportion of hydrophobic amino acid residues, consistent with their location in the bacterial membrane (Steiner et al., 1974a; Okamura et al., 1982). This is particularly so for the L and M subunits; the H subunit has a lower proportion of hydrophobic residues but still exceeds the range of such residues found in most water-soluble globular proteins.

To understand the primary photochemistry in detail, knowledge of the amino acid sequence and the three-dimensional structure of the reaction center protein, as well as of its arrangement with respect to the bacterial membrane, is required. We report here the amino-terminal sequences of the

† From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (M.R.S. and L.A.S.), and the Department of Physics, University of California, San Diego, La Jolla, California 92093 (D.R. and G.F.). *Received December 29, 1981.* This work was supported by grants from the National Science Foundation (PCM-78-13715 and PCM-78-13699), the Department of Agriculture, Competitive Research Grants Office (5901-0410-9-0378-0), the donors of the Petroleum Research Fund, administered by the American Chemical Society (PRF 11456-ACl,4), and the National Institutes of Health (GM-13191).

[1] Abbreviations: LDAO, *N,N*-dimethyllaurylamine oxide; $NaDodSO_4$, sodium dodecyl sulfate; PTH, phenylthiohydantoin; DEAE, diethylaminoethyl; GLC, gas–liquid chromatography; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography.

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