THE ACTIVITY OF THE ERYTHROCYTE MONOSACCHARIDE TRANSPORT SYSTEM IS NOT CORRELATED WITH CROSSLINKAGE OF BAND 3 PROTEIN

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Received March 28,1977

SUMMARY

We have prepared sealed vesicles from erythrocyte membranes in which 90% of band 3, the predominant intrinsic polypeptide, had been crosslinked by Cu^{2+}/o-phenanthroline treatment. The initial rates of L-sorbose uptake via the monosaccharide transport system in the vesicles varied from 72 to 176% of the rates for the corresponding control vesicles with uncrosslinked band 3. These results suggest that band 3 does not participate in monosaccharide transport.

Although a great deal is known about the kinetics, specificity, and inhibition of the human erythrocyte monosaccharide transport system (1-3) the identity of the polypeptide(s) that constitute this system has not as yet been unequivocally established. Kahlenberg recently reported evidence to suggest that band 3, the predominant transmembrane polypeptide of the erythrocyte membrane (4), may participate in transport. He found that when membranes are treated with Cu^{2+}/o-phenanthroline, the amount of D-glucose that binds specifically to the membranes in concentrated ammonium sulfate decreases by 70% (5). Cu^{2+}/o-phenanthroline treatment crosslinks band 3 polypeptides in the membrane through intermolecular disulfide formation (6,7). While the specific binding of D-glucose in concentrated ammonium sulfate is not an assay of transport activity *per se*, Kahlenberg *et al*. have shown that this interaction has the properties expected for a function of the transport system (8).

We have developed an assay for the transport system in membrane

ISSN 0006-291X

vesicles that is based upon the initial rates of L-sorbose uptake (9). Using this assay, we have examined the effect of crosslinking band 3 with Cu^{2+}/o-phenanthroline upon the activity of the system. We describe here that there is no correlation between the extent of crosslinking and the transport activity.

MATERIALS AND METHODS

Preparation of Vesicles. Freshly outdated units of blood in citrate-phosphate-dextrose were kindly provided by the Blood Bank of the Mary Hitchcock Memorial Hospital. Erythrocyte membranes in 5 mM sodium phosphate, pH 8, were isolated from the blood by the method of Steck and Kant (10). The preparation of vesicles with and without crosslinking was then carried out as follows. The freshly isolated membranes, at a concentration of 1.5 mg/ml protein, were divided into two 12 ml volumes. After 10 minutes at 25°, one received 0.4 ml 0.3 mM $CuSO_4$/1.5 mM o-phenanthroline. Both were maintained at 25° for an additional 20 minutes. Subsequent operations were performed at 3°. The membranes were separated by centrifugation at 48,000 xg (20,000 rpm in Sorval SS-34 rotor) for 20 minutes, and the supernate was removed by aspiration. In each case, the membranes were resuspended in 160 ml 0.5 mM sodium phosphate pH 8 (0.5P8). After 35 minutes, they were isolated by centrifugation at 48,000 xg for 20 minutes. The supernates were discarded, and the pellets left to stand for 12-18 hr. The pellets were then suspended to 4 ml with 0.5P8 and homogenized by four passages through a 27½ gauge needle. The membranes were diluted fourfold with 0.5P8 and 5.7 ml aliquots were layered over 6.2 ml Dextran T70 (density 1.03 g/ml) in 0.5P8. After centrifugation for 80 minutes at 37,000 rpm in a Beckman SW41 rotor, the sealed vesicles, which band at the interface, were collected in several ml and washed once with 40 ml 0.5P8 by centrifugation at 48,000 xg. Both control and treated membranes yielded about 7 mg vesicle protein. The above procedure, subsequent to the Cu^{2+}/o-phenanthroline treatment, is essentially that given by Steck for the preparation of sealed vesicles that are predominantly inside-out (11). We analyzed the sideness of our preparations by determining the accessibility of acetylcholinesterase (10). The control vesicles were 70-80% inside-out, and the ones with crosslinked band 3 were 60-70% inside-out.

In one experiment the crosslinking reaction was performed after preparation of the vesicles. Vesicles (1.5 mg protein/ml in 0.5P8) were incubated at 25° for 10 minutes, and then a small aliquot of 0.3 mM Cu^{2+}/1.5 mM o-phenanthroline was added to give 10 μM/50 μM. After 20 minutes, crosslinking was terminated by the introduction of 200 μM EDTA pH 8 from a concentrated stock solution (6,7). Control vesicles from the same preparation were treated in the same way except no additions were made.

Assay of Transport Activity. The activity of the monosaccharide transport system was assayed by measuring the initial rates of L-(^{14}C)sorbose (Amersham Searle) entry into vesicles at 12.5°. This procedure, which uses rapid filtration to separate the vesicles from the medium, is described in detail elsewhere (9).

Electrophoresis. A 100 μl sample (150 μg protein) for electrophoresis was removed from each assay mixture for L-sorbose uptake immediately after completion of the assay and treated with 10 μl 50 mM N-ethylmaleimide and then 10 μl 10% SDS/10 mM EDTA/100 mM Tris pH 8. The sample was divided in half. One half was reduced by holding it at 100° for 4 minutes after the addition of 5 μl 0.67 M dithiothreitol. Both received 5 μl 84% sucrose and 5 μl 12 mg/ml pyronin Y. SDS polyacrylamide gel electrophoresis and staining of 8-12 μl samples were performed as described by Steck and Yu (12). The

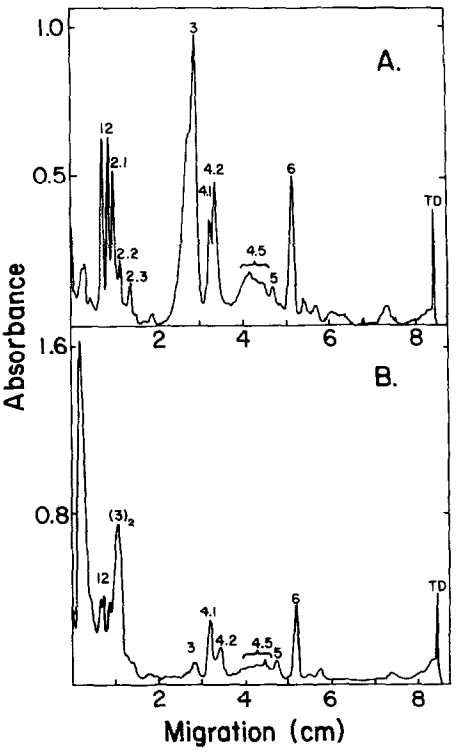

Figure 1. The polypeptide patterns obtained upon SDS polyacrylamide gel electrophoresis of unreduced control (A) and crosslinked (B) vesicles. The gels were stained with Coomassie blue and scanned at 550 nm. TD indicates the position of the tracking dye.

relative amount of band 3 was determined by scanning each gel at 550 nm and obtaining the band 3 and total areas from the scan.

Protein Concentration. The Lowry method was used (13), and then the values were corrected to give the concentration based upon the aminoacid content (see Reference 9).

RESULTS

Figure 1 presents the electrophoretic patterns of the unreduced polypeptides of vesicles that were prepared from Cu^{2+}/o-phenanthroline-treated and untreated membranes. The pattern for the control vesicles is virtually identical to that obtained after reduction with dithiothreitol. In agreement with earlier reports (6,7), band 3 polypeptides in the vesicles prepared from Cu^{2+}/o-phenanthroline-treated membranes are crosslinked,

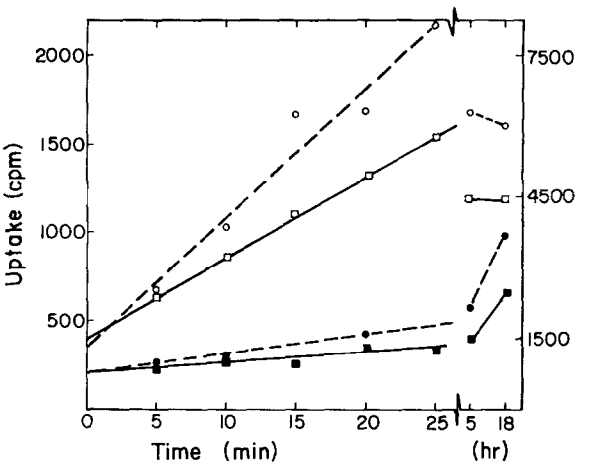

Figure 2. The initial rates of L-sorbose uptake at 12.5° by control vesicles (□), control vesicles plus 20 μM cytochalasin B (■), crosslinked vesicles (○), and crosslinked vesicles plus 20 μM cytochalasin B (●). The assay mixtures contained 0.50 mM L-(^{14}C) sorbose (4×10^6 cpm/ml) and 1.5 mg protein/ml in 0.5 mM sodium phosphate pH 8. The uptake values presented are those for 50 μl aliquots of the mixtures. The figure also shows the extent of uptake after 5 and 18 hr (right-hand ordinate).

largely in dimeric form. These vesicles also contain higher molecular weight aggregates, which have been shown to consist mainly of band 1, 2, and 2.1 polypeptides (6,7).

The rates of L-sorbose uptake were measured for control and crosslinked vesicles in the presence and absence of 20 μM cytochalasin B. Cytochalasin B is a potent inhibitor of the transport system and, at this concentration, should inhibit 97% of the entry that occurs by the system (14,15). Figure 2 shows that for both types of vesicles the initial rate of uptake is linear and inhibitable by cytochalasin B. The extent of cytochalasin B inhibition ranged from 65 to 90% for the different vesicle preparations. We assume that the uninhibitable fraction of the overall rate is due to entry by another process and have subtracted this fraction from the overall rate to obtain the rates for the transport system *per se*.

TABLE I

THE INITIAL RATES OF L-SORBOSE UPTAKE AT 12.5°

BY CROSSLINKED AND CONTROL VESICLES

In each case the initial rates in the presence and absence of 20 μM cytochalasin B were measured in duplicate under the conditions described in the legend to Figure 1. The duplicate determinations agreed within + 10% of the average. Preparations A-D are vesicles that were prepared from Cu^{2+}/o-phenanthroline treated membranes, while in the case of E vesicles were treated after their preparation. A and B were assayed for uptake immediately upon isolation of the vesicles, whereas C-E were assayed after 24 hr at 4°.

Preparation	Corrected V[a] (pmoles/min/mg)	% of Control V	% of Band 3 as Monomer[b]	% Crosslinked[c]
A. Control	59	100	90	10
Crosslinked	104	176	11	89
B. Control	110	100	91	9
Crosslinked	79	72	12	88
C. Control	95	100	83	17
Crosslinked	70	74	10	90
D. Control	56	100	92	8
Crosslinked	77	138	7	93
E. Control	77	100	105	0
Crosslinked	103	135	37	63

[a]At 0.5 mM L-sorbose, corrected for uptake in the presence of 20 μM cytochalasin B as described in the text. The values are averages from duplicate determinations.

[b]100 x band 3 monomer/band 3 total. The amounts of monomeric and total band 3 were obtained by electrophoresis of unreduced and reduced samples, respectively.

[c]100 - % band 3 as monomer.

The effects of crosslinking band 3 on the initial rates of L-sorbose uptake are summarized in Table I. In preparations A-D, although only 10% of the band 3 polypeptides remain uncrosslinked, the initial rates of sorbose uptake by the transport system vary from 72 to 176% of the corresponding control values. In the case of E, the vesicles were treated with Cu^{2+}/o-phenanthroline after their preparation. Since about 25% of the

vesicles were oriented right side-out and since the susceptible thiols are at the cytoplasmic surface and the reagent does not permeate the membrane (16), only 63% of band 3 is crosslinked. This limited crosslinking is associated with a small increase in transport activity.

DISCUSSION

Our vesicle preparations show variable activity for the monosaccharide transport system. The values of V for the control vesicles range from 56 to 110 pmoles/min/mg protein and those for crosslinked ones range from 77 to 104 pmoles/min/mg. We have not established the basis for this variation. However, in spite of its occurrence, the results indicate that extensive crosslinking of band 3 polypeptides has no consistent inhibitory or stimulatory effect upon transport. The values for crosslinked vesicles range from 72 to 176% of the corresponding control values, and the average is 120%. On the basis of the results of Kahlenberg, which are described in the Introduction, this outcome was unexpected.

There is substantial evidence to indicate that at least 90% of band 3 is a single species of polypeptide (17,18). We believe that our results suggest that this polypeptide is not a component of the monosaccharide transport system. Even if all the remaining band 3 were the monomeric form of this polypeptide, it is unlikely that the activity of the crosslinked vesicles is due to this uncrosslinked fraction. First, although the vesicles have one-ninth as much monomeric band 3 as the control vesicles, their activity is not consistently reduced. Second, although only 10% of the band 3 remains uncrosslinked, the uptake activity of the vesicles ranges from 26 to 39% of the activity for intact erythrocytes under the same conditions (265 pmoles/min/mg membrane protein, at 0.5 mM L-sorbose (9)). Consequently, if the major species in band 3 were involved, we must assume that crosslinking does not alter its function. It is worth noting that since the half-saturation constant (K_T) for L-sorbose is much higher than the concentration used here (18,19), the initial rate of entry is propor-

tional to the ratio of the maximal flux constant (J_{max}) to the half-saturation constant for transport (20). If crosslinking had altered either parameter, the initial rates would have changed. In this regard, there is good evidence that band 3 polypeptides are involved in anion transport (21,22), and Rice has reported that interchain disulfide formation changes the K_T, but not the J_{max}, for this process (23).

ACKNOWLEDGEMENTS

We thank the National Science Foundation (PCM73-06887), the National Institutes of Health (1R01GM22996), and the American Cancer Society (Faculty Research Award to G.E.L.) for support.

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