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Articles

Location of the Maltosyl Isothiocyanate Binding Site on the Human Erythrocyte Glucose Transporter†

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ABSTRACT: The covalent affinity probe maltosyl isothiocyanate (MITC) has been used previously to identify the glucose transporter of human erythrocytes as a component of band 3. By use of limited proteolysis, the site on the M_r 100 000 protein to which MITC attaches has been localized to a 17 000-dalton region near the center of the polypeptide chain which is intimately associated with the membrane. The erythrocyte anion transporter, which is probably homologous to the glucose carrier, has a corresponding segment which is known to bind the covalent affinity label 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid [Ramjeesingh, M., Gaarn, A., & Rothstein, A. (1980) *Biochim. Biophys. Acta 559*, 127–139]. These results suggest that, in addition to having structural features in common, the two carrier proteins may be quite similar with regard to functional organization.

The covalent affinity label maltosyl isothiocyanate (MITC)[1] has been used to identify the human erythrocyte glucose carrier (Mullens & Langdon, 1980a,b). The probe was bound almost exclusively to the M_r 90 000–100 000 component known as band 3 [nomenclature of Steck (1974) based on electrophoretic mobility)], glucose transport was inhibited in proportion to the amount of MITC bound, and labeling was decreased in the presence of glucose or sugars that compete with glucose for transport. We have recently demonstrated that band 3 can be purified and reconstituted into pure lipid vesicles with retention of glucose transport activity (Shelton & Langdon, 1983).

This broad electrophoretic band appears to represent a family of closely related transport proteins, containing in addition to the glucose carrier the anion transporter (Rothstein et al., 1976) and the (Na^+–K^+)-ATPase (Avruch & Fairbanks, 1972). Extensive peptide maps have been constructed by using limited proteolysis (Steck et al., 1976, 1978; Drickamer, 1976; Jenkins & Tanner, 1977), which gives fragments that appear homogeneous. This has led some investigators to propose that band 3 is homogeneous. Other studies, however, have clearly demonstrated that the transporters for glucose and for anions are distinct proteins (Mullins & Langdon, 1980a). We therefore believe that band 3 is comprised primarily of two proteins, the carriers for glucose and anions, and that the peptide maps generated for band 3 may be applied to both these transporters. In the present study, we have employed proteolytic dissection procedures described previously (Steck et al., 1978; Jenkins & Tanner, 1977) to localize the MITC binding site; it was found in a 17 000-dalton segment near the center of the polypeptide chain of the glucose transporter which is largely contained within the membrane, possibly forming a transmembrane channel through which substrates are transported.

Materials and Methods

α-Chymotrypsin and sodium dodecyl sulfate (lauryl sulfate, SDS) were purchased from Sigma; SDS was recrystallized from 95% ethanol. Dithiothreitol was obtained from Aldrich, trypsin from Worthington, [U-^{14}C]maltose from Amersham, and En^{3}Hance from New England Nuclear. Acrylamide, from Eastman, was recrystallized from acetone.

Fresh venous blood was anticoagulated by adding 1 mg of EDTA and 17 units of heparin/mL of blood. Leukocytes were

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[1] Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; EDTA, ethylenediaminetetraacetic acid; MITC, maltosyl isothiocyanate; SDS, sodium dodecyl sulfate.

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completely removed on a cellulose column as described by Beutler et al. (1976). Red cells were collected by centrifugation at 600*g* and washed 5 times in 5 volumes of 150 mM NaCl/5 mM sodium phosphate, pH 8; 25 mM [U-^{14}C]MITC (approximately 10 Ci/mol) was prepared by the method of Mullins & Langdon (1980a) and used immediately. One milliliter of cells was combined with 2.0 mL of MITC solution and incubated 10 min at 37 °C. The reaction was stopped by adding 40 mL of ice-cold 150 mM NaCl/5 mM sodium phosphate (pH 8)/100 mM glycine. Erythrocytes were pelleted, washed once in the stopping solution, and washed twice in 150 mM NaCl/5 mM sodium phosphate. The cells were hemolyzed by adding 40 mL of ice-cold 5 mM sodium phosphate, pH 8. Membrane ghosts were collected by centrifugation for 30 min at 15000*g* and washed twice, giving a final suspension of unsealed, packed ghosts containing ca. 2 mg of protein/mL by amino acid analysis (Shelton & Langdon, 1983).

Aliquots (100 μL) of membrane suspension were mixed with an equal volume of the following protease solutions: (1) 200 μg/mL α-chymotrypsin in 5 mM sodium phosphate, pH 8, incubated 1.0 h at 22 °C; (2) 1.0 μg/mL trypsin in 10 mM sodium phosphate (pH 7)/0.1 mM EDTA, incubated 1.0 h at 0 °C; (3) 100 μg/mL trypsin in 300 mM NaCl/5 mM sodium phosphate, pH 7, incubated 20 min at 37 °C. Reactions were stopped by adding 1.0 μL each of 0.1 M solutions of phenylmethanesulfonyl fluoride and diisopropyl fluorophosphate in 2-propanol. To the chymotrypsin reaction was added 1.0 mL of ice-cold 0.1 N NaOH. Ten microliters of 2 N NaOH was added to each trypsin reaction. Membranes were immediately pelleted by centrifugation at 100000*g* for 1 h. Each pellet, diluted to 200 μL, and aliquots of each supernatant were prepared for electrophoresis.

Samples for electrophoresis were made 2% in SDS and 5 mM in dithiothreitol and were heated 1.0 min at 100 °C. Slab gels (150 × 150 × 0.75 mm thick) containing 10% acrylamide were prepared by the method of Laemmli (1970). Two identical gels were run of labeled ghosts and proteolyzed membranes and their corresponding supernatants. Both gels were fixed by immersion with agitation in 200 mL of 50% methanol/10% acetic acid for 30 min, then in 5% methanol/7% acetic acid for 30 min, and finally in 10% glutaraldehyde for 30 min. One gel was washed 1 h in water, impregnated with En^{3}Hance for 1 h, washed 15 min in running water, and set for autoradiography for 21 days at −70 °C by using Kodak X-omat film. The other gel was silver stained by the method of Morrissey (1981).

Results

Figure 1 summarizes schematically the results of controlled band 3 proteolysis as described by Steck et al. (1976, 1978) and by Drickamer (1976). When some of these digestion conditions were repeated on ghosts prepared from erythrocytes which had been labeled with [^{14}C]MITC, the results shown on the left panel of Figure 2 were obtained. Unreacted ghosts are shown in lane A. Chymotryptic digestion of unsealed ghosts produces a 17000-dalton fragment from near the center of the band 3 polypeptide chain which lies predominantly within the membrane matrix (Steck et al., 1978) and is therefore retained in the membrane pellet (Figure 2, lane C). The soluble fragments released by this reaction appear in the supernatant (lane B). Trypsinolysis of unsealed ghosts at low ionic strength generates a soluble 41000-dalton peptide from the cytoplasmic N-terminal domain of band 3 which is further cleaved to 16000- and 22000-dalton fragments. Digestion must be carried out under mild conditions to avoid much more

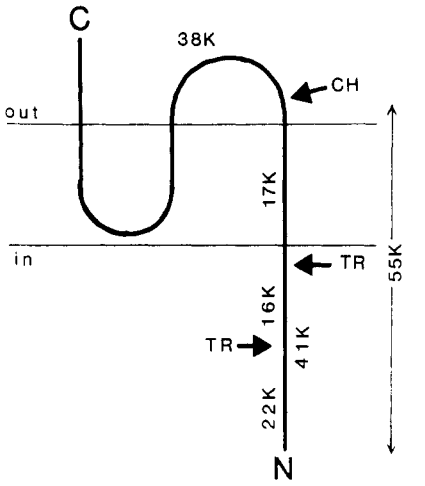

FIGURE 1: Topographic arrangement of major band 3 peptides. Treatment of intact erythrocytes with chymotrypsin gives complementary 38000- and 55000-dalton fragments, both of which remain with the membrane. Simultaneous digestion of the inner surface with chymotrypsin (by carrying out the reaction on open ghosts) removes a ca. 40000-dalton N-terminal segment, while a 17000-dalton intrinsic fragment remains with the membrane. Light trypsinolysis of open ghosts in hypotonic buffer produces a soluble 41000-dalton peptide from the N-terminus which is further cleaved into 22000- and 16000-dalton fragments. [Redrawn from Steck et al. (1978).]

extensive proteolysis; consequently, most of band 3 remains intact (Steck et al., 1978). These peptides, as well as several others, are evident in the supernatant from this digestion (Figure 2, lane D), while the membrane pellet has a large amount of undigested band 3 (lane E). Jenkins & Tanner (1977) found that when tryptic hydrolysis of unsealed ghosts is carried out in isotonic buffer rather than 5 mM sodium phosphate, band 3 is much less susceptible to degradation. They postulated that the proteins assume a more compact conformation as the ionic strength is raised, thereby sterically hiding many trypsin cleavage sites. One of the peptides that they obtained by treatment at high ionic strength migrates on electrophoresis as a diffusely staining broad band with an average apparent molecular weight of 55000. Although it is not possible to unambiguously locate this fragment on the map shown in Figure 1, it probably results from removal of a ca. 40000-dalton segment from the N-terminus. This would account for its broad migration on electrophoresis; loss of the cytoplasmic domain, which contains no carbohydrate, would leave a fragment with a higher relative degree of heterogeneous glycosylation than the parent protein and would therefore be expected to form a wider electrophoretic band. This reaction in our hands released many of the peptides produced by low ionic strength trypsinolysis (Figure 2; compare lane F to lane D); the intrinsic peptide of apparent average M_r 55000 is evident in lane G.

It has been demonstrated (Mullins & Langdon, 1980a,b) that MITC fully satisfies the stringent criteria proposed by Groman (1977) for an affinity label: it is a substrate analogue, inhibits glucose transport, and attaches to a single protein (see Figure 2, lane A) by a reaction which is first order with respect to transport activity, and labeling is inhibited by substrates and reversible inhibitors of transport. Furthermore, reaction is stoichiometric: the number of glucose transport sites suggested by experiments with MITC, 300000 per cell, agrees with that found by several other methods (Jones & Nickson, 1980). To determine which of the band 3 fragments described above contained the label, a gel identical with the one used for silver staining was run and subjected to autofluorography. Results are shown on the right panel of Figure 2. It is apparent

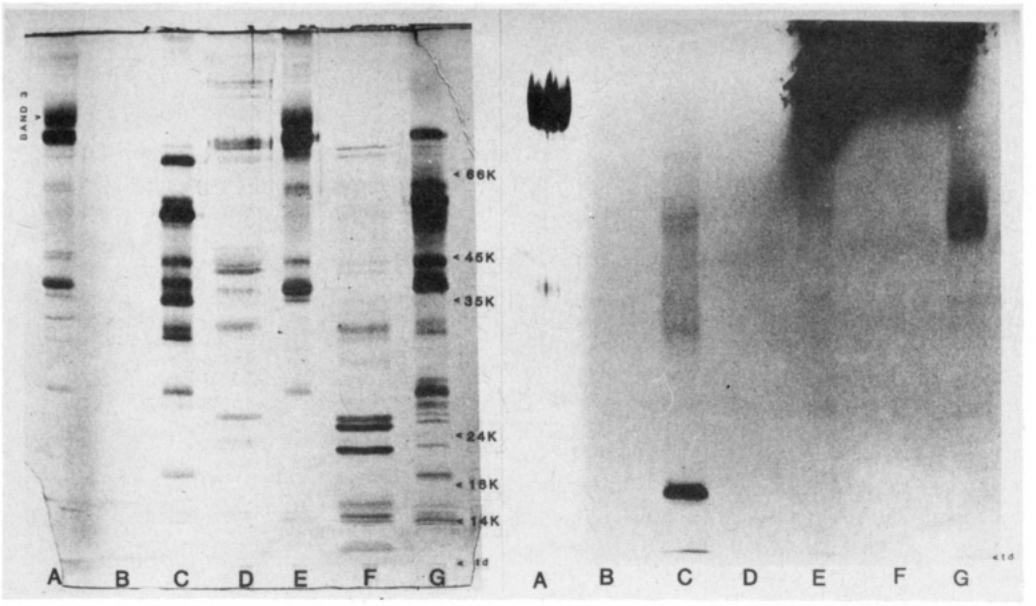

FIGURE 2: 10% polyacrylamide gels of [^{14}C]MITC-labeled ghosts before and after proteolysis. The gel on the left is silver stained; the one on the right is an autoradiogram. (A) Ghosts, with label in band 3. After proteolysis, membranes were collected by centrifugation. Chymotrypsin reaction: (B) supernatant; (C) pellet. MITC is found eclusively in a ca. 20000-dalton intrinsic peptide. Hypotonic trypsinolysis: (D) supernatant; (E) pellet. None of the soluble peptides released from the membrane contain detectable label. Isotonic trypsinolysis: (F) supernatant; (G) pellet. The 55000-dalton intrinsic peptide is labeled, while none of the soluble fragments contain MITC. See text for experimental details. Positions of molecular weight markers are indicated: bovine albumin (M_r 66000), egg albumin (M_r 45000), pepsin (M_r 35000), trypsinogen (M_r 24000), β-lactoglobulin (M_r 18000), and lysozyme (M_r 14000).

that band 3 is the only protein in erythrocyte membranes which is labeled significantly (lane A). None of the soluble peptides released by any of the three digestion conditions contains a detectable amount of radioactivity (lanes B, D, and F). The 17000-dalton intrinsic chymotryptic peptide contains the probe (lane C). This peptide is part of the 55000-dalton fragment which is obtained when chymotryptic cleavage is limited to the exoplasmic surface (Steck et al., 1978; see Figure 1) and which was noted by Mullins & Langdon (1980b) to contain the MITC binding site. In experiments similar to those described here, the probe was found exclusively on the larger 55000-dalton fragment. As is evident from Figure 2, lane C, no M_r 55000 peptide (labeled or cold) remains after cleavage from both faces, suggesting quantitative degradation of this fragment to smaller peptides, only one of which is labeled. The broadly migrating M_r 55000 peptide generated by tryptic hydrolysis in isotonic buffer is also labeled (lane G), which is consistent with the proposed relationship of this fragment to the map shown in Figure 1.

Discussion

Most of the currently available data strongly suggest that anions and glucose are transported across human erythrocyte membranes by distinct but similar proteins, both of which are components of band 3. Treatment of cells with the specific inhibitor 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) in concentrations sufficient to completely abolish the flux of anions has no effect upon that of glucose (Mullins & Langdon, 1980a), indicating that the transport sites for these two substrates are unique. Since Rothstein et al. (1976) have demonstrated that all of the membrane-spanning segments of the anion carrier are involved in forming a transmembrane channel for the passage of anions, it is unlikely that this protein also contains the transport site for glucose. Furthermore, Mullins & Langdon (1980b) obtained evidence using MITC-labeled membranes that the glucose transporter is more susceptible to degradation by endogenous proteases than the remainder of band 3.

Limited proteolysis of band 3 was reported by Steck et al. (1976, 1978) to give peptides which appeared homogeneous when analyzed by electrophoresis; it was suggested that band 3 consisted of a single polypeptide chain whose broad migration on electrophoresis was attributable to heterogeneous glycosylation. An alternative explanation is that there is more than one quantitatively significant component, but the several proteins are homologous. Of the 700000 copies of band 3 present per erythrocyte (Shelton & Langdon, 1984), approximately 300000 are anion transporters (Ho & Guidotti, 1975) and roughly 300000 are glucose transporters (Mullins & Langdon, 1980a,b; Jones & Nickson, 1980); combined, the two make up almost 90% of band 3. taken together, the results suggest that the glucose and anion carriers are homologous and are the predominant components of band 3. As a consequence of this, data obtained from band 3 proteolysis may provide information about the structure of both carriers.

Of particular interest, the affinity label DIDS, which completely inhibits anion flux (Rothstein et al., 1976) without affecting glucose transport (Mullins & Langdon, 1980a), interacts with a region of the anion carrier reminiscent of that bound by MITC on the glucose carrier. DIDS attaches exclusively to the ϵ-amino group of a lysine residue (Ramjeesingh et al., 1981) approximately 6000 daltons from the N-terminus of the 17000-dalton transmembrane segment of the anion transporter (Ramjeesingh et al., 1980). This suggests that, in addition to sharing gross structural features, the carriers for glucose and anions have a similar functional organization in the region of their substrate binding sites. The glucose carrier may therefore, by analogy to the anion carrier, form a channel through the membrane through which glucose passes, and within which a substrate-specific binding site exists that causes the glucose analogue MITC to be covalently bound nearby. Much more work is necessary to elucidate the structure of this region.

It should be mentioned that several groups have proposed that the native glucose carrier is a protein with an apparent average molecular weight of approximately 50000 which

migrates on electrophoresis as a broad band within zone 4.5. This hypothesis is based primarily on two observations. First, under certain conditions, such a protein can be isolated from erythrocytes and has glucose transport activity (Kasahara & Hinkle, 1977; Sogin & Hinkle, 1978; Wheeler & Hinkle, 1981; Nickson & Jones, 1982). Recent studies from our laboratory (Shelton & Langdon, 1983) and others (Phutrakul & Jones, 1979; Nickson & Jones, 1980; Acevedo et al., 1981) have shown that this material is in fact a partially active fragment of the 100 000-dalton band 3 glucose transporter produced by the action of endogenous proteases when necessary precautions to inhibit their activity are not taken during membrane preparation. Thus, the protein within band 4.5 is not present in substantial amounts when membranes are isolated by our method. Second, when erythrocytes are photoaffinity labeled with cytochalasin B, a very potent inhibitor of glucose transport (Taverna & Langdon, 1973), the probe is found exclusively on material in zone 4.5 (Carter-Su et al., 1982; Shanahan, 1982, 1983). However, cytochalasin B is not a substrate analogue, it interacts with a site remote from the one associated with transport (Krupka & Deves, 1980; Deves & Krupka, 1980), the kinetics of its covalent attachment have not been evaluated, and the efficiency of incorporation is so low that no effect on carrier activity can be demonstrated. Because it is not a true affinity label for the transporter, the significance of its behavior as a photoaffinity label is unclear.

In summary, the MITC binding site has been localized to a 17 000-dalton intrinsic segment of the glucose transport protein; this corresponds to the region of the anion carrier to which the affinity probe DIDS is attached (Ramjeesingh et al., 1980). On the basis of currently available data, it is probable that these two transporters have structures that are very similar but not identical, and it may be speculated that they are also quite similar with regard to the functional organization of at least some of their domains.

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