The Intramembrane Topography of the Mannitol-specific Enzyme II of the *Escherichia coli* Phosphotransferase System*

(Received for publication, September 23, 1982)

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The D-mannitol-specific Enzyme II of the phosphoenolpyruvate-dependent phosphotransferase system of *Escherichia coli* is an integral cytoplasmic membrane protein responsible for concomitant transport and phosphorylation of this hexitol. We have investigated the intramembrane topography of this enzyme/acceptor complex, membrane-impermeable reagents, and antibodies against the purified protein. The results of these experiments suggest that this protein spans the membrane in a single orientation with a sizeable proportion of its mass extending into the cytoplasm, but with little of the polypeptide exposed at the outside surface of the membrane. Such an orientation is consistent with the reception and transport roles of the mannitol Enzyme II in *E. coli*.

In recent years, it has become clear that most integral membrane transport proteins that have been studied in detail both span the lipid bilayer and are situated in a single orientation with respect to the inside and outside of the system bounded by the membrane. Furthermore, at least two classes of such proteins have been recognized (1, 2); those proteins that have most of their mass embedded in the hydrophobic domain of the membrane, and those that have a sizeable fraction of their polypeptide exposed to aqueous environments at either or both faces of the bilayer. An example of the former is the hydrophobic bacteriorhodopsin polypeptide of *Halo bacterium halobium* (3, 4) and examples of the latter include ion-translocating ATPases (5-7) and other transport proteins such as the anion channel of human erythrocytes (8).

An understanding of the intramembrane topography of transport proteins is obviously essential in determining the molecular mechanisms of transmembrane transport. To this end, proteolytic enzymes (6, 7, 9-14) and other membrane-impermeable reagents (e.g. Refs. 15, 16) have been especially useful in helping to elucidate the orientations of many membrane proteins.

In a previous communication, one of us reported the purification to apparent homogeneity of the protein responsible for the tightly coupled transport and phosphorylation of D-mannitol in *Escherichia coli* (17). This protein, the mannitol-specific Enzyme II of the PTS (for reviews see Refs. 18 and 19), comprises a single kind of polypeptide chain, *M* = 60,000, and is integrally associated with the cytoplasmic (inner) membrane of this organism. These conclusions have independently been confirmed recently in *E. coli* minicells harboring a hybrid plasmid containing the genes necessary for mannitol utilization (20).

We have sought to determine the orientation of Enzyme II in *E. coli* membranes using limited proteolysis and membrane-impermeable reagents. Our results lead to a working model for the disposition of this protein in the phospholipid bilayer. Since Enzyme II is a multifunctional protein, the functions of which can be dissected genetically (21), immunologically (22), and proteolytically (this report), these probes of intramembrane topography should also be useful in elucidating relationships of structure to function in this enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phosphoenolpyruvate (tricyclohexylammonium salt), D-mannitol, D-mannitol-1-phosphate, soybean trypsin inhibitor, bovine pancreatic trypsin inhibitor, PCMPS, and egg white lysozyme (used in spheroplast preparation) were all purchased from Sigma. *E. Chymotrypsin* (treated with *N*-*p*-tosyl-L-lysine chloromethylketone) and papain were also from Sigma, while trypsin (treated with *L*-tosylamide-2-phenylethylcholoromethylketone) was a product of Worthington. D-[14C]Mannitol was purchased from New England Nuclear. IgG fractions from preimmune and anti-Enzyme II rabbit sera were prepared as previously described (30). Other compounds were obtained from commercial sources and were the highest grade available.

**Cell Growth, Enzyme Purification, and Preparation of Spheroplasts**—Enzyme II was purified from mid-exponential phase *E. coli* K12 (strain KL141) by a modified procedure as previously described (22). As a source of the soluble phospho-transfer proteins of the PTS, Enzyme I and HPr, a cytoplasmic fraction from *Salmonella typhimurium* strain L414 (cpd-401, *cyaA150/F* 198) was prepared. This strain contains the pts operon on an *E. coli* episome and overproduces these proteins about 5-fold relative to wild type cells (22). Spheroplasts were formed from early exponential phase *E. coli* K12 strain KL141, which had been grown on medium 63 (24) supplemented with thiamin (10 µg/ml), arginine (50 µg/ml), uracil (20 µg/ml), and 0.5% mannitol to an OD600 of 0.3. The procedure involved lysozyme-EDTA treatment in the presence of sucrose exactly as described by Osborn et al. (25) for *S. typhimurium*. Spheroplasts were kept on ice until used and were freshly prepared for each experiment. Only prepara-

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* This research was supported by a grant from the Boston University Graduate School and supported by National Institutes of Health Grant 1 R01 GM28226 from the National Institute of General Medical Sciences. Portions of this report were presented in a preliminary form at the Annual Meeting of the American Society of Biological Chemists, April, 1982 in New Orleans, LA. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: Enzyme II, membrane-bound permease of the PTS specific for D-mannitol; PTS, phosphoenolpyruvate-sugar phosphotransferase system; Enzyme I and HPr, cytoplasmic-phosphate-transfer proteins of the PTS; PEP, phosphoenolpyruvate; PCMPS, para-chloromercuri phenyl sulfonate; mannitol-1-P, D-mannitol-1-phosphate.

2 Physiological functions ascribed to Enzyme II include: unidirectional and exchange group translocation, PEP- and mannitol-1-P-dependent phosphorylation of D-mannitol, primary chemotactic receptor for D-mannitol, autoinduction of PTS protein synthesis, control of non-PTS permease activity, and control of adenylate cyclase activity (19).
Enzyme I and HPr are soluble, general phospho-transfer proteins of the PTS and function in the Enzyme II-mediated transport and phosphorylation of PTS sugars. Both Reactions 1 and 2 operate in a vectorial fashion in whole bacterial cells (sugar-in --- sugar-out) and nonvectorially in membranes and in purified systems (18, 19). For the PEP-dependent reaction, incubation mixtures contained 0.1 M Tris-HCl, pH 7.0, containing 0.1 M dithiothreitol, 10 mM MgCl₂, 1 mM dithiothreitol, and saturating amounts of S. typhimurium cytoplasm containing HPr and Enzyme I in a total volume of 0.1 ml. The size of the sample to be assayed was chosen such that Enzyme II⁺⁺ was present in rate-limiting amounts. After 30 min at 37 °C, mixtures were rapidly cooled to 4 °C and the [¹⁴C]mannitol-1-P formed was determined by DAEAE-filter paper (Whatman DE52) as previously described (17). Transphosphorylation activity was determined in mixtures containing 0.1 M potassium phosphate, pH 7.0, containing 5 mM MgCl₂, 10 mM KF, 1 mM dithiothreitol, 1 mM PEP, 1 mM [¹⁴C]mannitol (5 µCi/µmol), and saturating amounts of S. typhimurium cytoplasm containing HPr and Enzyme I in a total volume of 0.1 ml. The samples were incubated at 30 °C for 30 min and then diluted with 1 ml of H₂O to 4 °C to stop the reaction. [¹⁴C]Mannitol-1-P formed was then determined by the Dowex 1-X2 resin column procedure (27).

The uptake of [¹⁴C]mannitol into spheroplasts mediated by Enzyme II⁺⁺ was measured after various treatments at room temperature. [¹⁴C]Mannitol (5 µCi/µmol) was added to untreated or untreated spheroplasts to a final concentration of 0.1 mM. At various times, 1-ml aliquots were removed and centrifuged for 1 min in an Eppendorf microcentrifuge. The supernatant was carefully and thoroughly removed, the pellet was resuspended in 1 ml of H₂O to lyse the spheroplasts, and this solution was then transferred to a vial containing 10 ml of standard toluene/Triton X-100-based scintillation fluid for counting.

RESULTS AND DISCUSSION

Proteolytic Modification of Enzyme II⁺⁺.—Purified Enzyme II⁺⁺ is rapidly inactivated by trypsin with the concomitant cleavage of its 60,000-dalton polypeptide into fragments approximately half this mass (17, 22). In these experiments, the ability of the enzyme to catalyze PEP-dependent phosphorylation of D-mannitol was measured. We have confirmed and extended these observations, and the results of these experiments are shown in Fig. 1. Both PEP- and mannitol-1P-dependent activities of purified Enzyme II⁺⁺ in Lubrol PX were inactivated at similar rates by trypsin (0.5 pg/ml). Similarly, chymotrypsin at 0.5 µg/ml inhibited both activities in a time-dependent manner, although in this case, PEP-dependent phosphorylation was inactivated much more quickly than transphosphorylation. In membranes derived from French press ruptured cells, chymotrypsin also partially dissected these activities (inset, Fig. 1).

In order to determine the localizations of the trypsin- and chymotrypsin-sensitive sites with respect to the cytoplasmic membrane, we performed the experiments presented in Fig. 2A. Spheroplasts of E. coli were prepared in which the outer membrane of the cell was disrupted to expose the outer surface of the inner membrane to the medium (25). These

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1 Certain inner membrane proteins of E. coli can be labeled when spheroplasts are treated with ³²P and lactoperoxidase (cf. Ref. 19). This strongly suggests that the surface of the inner membrane should be available to molecules of the size of these proteases in spheroplasts. Furthermore, prolonged incubation of E. coli spheroplasts with proteases can lead to lysis (G. Jacobson and D. Kelly, unpublished observations), further supporting this contention.
spheroplasts, and membranes derived from them by French pressure cell lysis, which yields mainly everted (“inside-out”) vesicles (26), were then subjected to proteolysis for varying times. Subsequently, these preparations were tested for their abilities to catalyze PEP-dependent phosphorylation of D-mannitol in the presence of the soluble PTS enzymes. The results of these experiments show (Fig. 2A) that spheroplasts treated with trypsin or chymotrypsin, then lysed in a French pressure cell and assayed, were largely or completely insensitive to inactivation by these proteases. In contrast, everted membrane vesicles treated with trypsin or chymotrypsin rapidly lost their ability to phosphorylate D-mannitol.

Next, we investigated the effects of increasing concentrations of the relatively nonspecific protease, papain, on Enzyme II\textsuperscript{mut} activities in spheroplasts and everted vesicles. Again, nearly complete inactivation of both PEP-dependent and transphosphorylation activities was observed in everted vesicles, while the enzyme in spheroplasts was essentially insensitive even to relatively high concentrations of papain (Fig. 2B). These results demonstrate, therefore, that the sites sensitive to these proteases are exposed to the aqueous environment on the inner, cytoplasmic surface of the inner membrane.

Finally, we tested the effects of these proteases at 5 \mu g/ml on the transport rate of \textsuperscript{14}C]mannitol into spheroplasts (see “Experimental Procedures”). No significant difference in uptake rate (>5%) was seen with any protease tested over a 30-min period at room temperature (not shown). Therefore, sites on Enzyme II\textsuperscript{mut} necessary for activity that are cleaved by these proteases do not become available on the outside surface, even during transport of mannitol by the enzyme.

Effects of Antibody on Membrane-bound Enzyme II\textsuperscript{mut}—The preparation of polyclonal antibodies against purified Enzyme II\textsuperscript{mut} has been described previously (20). These antibodies specifically inhibited the catalytic activities of both purified enzyme and of the protein in crude membrane fractions (22). In order to determine the localization of these antibody-combining sites with respect to the membrane, we carried out the experiments shown in Fig. 3. Spheroplast suspensions were treated with partially purified preparations of either control (preimmune) or anti-Enzyme II\textsuperscript{mut} IgG, and then measured for their abilities to take up \textsuperscript{14}C]mannitol. No significant difference in initial uptake rate was observed for the two preparations (Fig. 3). In contrast, when the same spheroplast preparation was lysed in the French press and treated with an identical amount of anti-Enzyme II\textsuperscript{mut} IgG, PEP-dependent phosphorylation of mannitol was inhibited more than 90% relative to a control sample (see legend to Fig. 3). Similar results were obtained when the same experiments were performed with “right-side-out” membrane vesicles prepared by osmotic lysis of spheroplasts (19).

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\section{Chemical Modification Experiments—Enzyme II\textsuperscript{mut}}
Enzyme II\textsuperscript{mut} is known to be inactivated by reagents that modify free sulphydryl groups in proteins (17, 19). Previously, it had been shown that vectorial transphosphorylation catalyzed by this protein in whole E. coli cells or in right-side-out membrane vesicles was insensitive to the reagent PCMPS when this compound was added to the outside of these suspensions, implying that the sulphydryl group(s)

\begin{thebibliography}{99}
\bibitem{} S. Dills and G. Jacobson, unpublished results.
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necessary for this reaction was localized on the inside surface of the membrane (28). We have confirmed and extended these results in our systems. Treatment of whole cells or spheroplasts with 1 mM PCPMS for 10 min at room temperature, followed by removal of excess reagent by centrifugation and washing, had no effect on transphosphorylation activity of Enzyme II\textsuperscript{pp} which had been extracted by Lubrol PX from membranes prepared from these samples (Table I). In contrast, if everted vesicles were similarly treated with PCPMS, washed, and extracted, over 90% of Enzyme II\textsuperscript{pp} transphosphorylation activity was lost relative to a control (Table I). Finally, we found that PCPMS had no significant effect on mannitol uptake into either whole E. coli cells or spheroplasts (not shown). These results demonstrate that over 90% of the required sulfhydryl groups must be oriented such that they are accessible to this reagent only from the inside surface of the membrane. They also show, therefore, that most, if not all, of the Enzyme II\textsuperscript{pp} molecules must have a single, asymmetric orientation in the cytoplasmic membrane.

In an effort to modify Enzyme II\textsuperscript{pp} from the outside surface of the cytoplasmic membrane, we investigated the effects of the powerful oxidant, KMnO\textsubscript{4}. This compound has the potential to oxidize nearly all amino acid side chains, and thus might be expected to inactivate many enzymes (29, 30). Because permanganate anion would not be expected to traverse intact membranes rapidly because of its hydrophilicity and charge, we tested the effects of KMnO\textsubscript{4} on Enzyme II\textsuperscript{pp} in intact spheroplasts and in everted membrane vesicles. The results of a representative experiment are shown in Fig. 2C. Rapid time-dependent inactivation of PEP-dependent phosphorylation of D-mannitol occurred with KMnO\textsubscript{4} when everted membranes were used as the source of Enzyme II\textsuperscript{pp}. In contrast, spheroplasts treated with KMnO\textsubscript{4} under the same conditions reproducibly lost only part of their PEP-dependent phosphorylating activity, and inactivation reached a plateau by about 30 min of KMnO\textsubscript{4} treatment (Fig. 2C). These results suggest that KMnO\textsubscript{4} is not highly permeable to the E. coli cytoplasmic membrane, and partially inactivates Enzyme II\textsuperscript{pp} from the outside surface of the bilayer. Thus, Enzyme II\textsuperscript{pp} appears to span the phospholipid bilayer.\textsuperscript{8}

**Topography of Enzyme II\textsuperscript{pp}**

The results presented in this report strongly support the schematic model shown in Fig. 4 for the topography of Enzyme II\textsuperscript{pp} in the cytoplasmic membrane of E. coli. They suggest that Enzyme II\textsuperscript{pp} spans the membrane, but that relatively little of the Enzyme II\textsuperscript{pp} polypeptide is exposed at the outside surface, while a sizeable proportion of the protein most likely protrudes into the cytoplasm. This conclusion is based on the fact that, with the sole exception of KMnO\textsubscript{4}, no reagent or protease tested was able to significantly inactivate Enzyme II\textsuperscript{pp} from the outside surface of intact spheroplasts. Our results also show that nearly all (>90%) active Enzyme II\textsuperscript{pp} molecules have a single, asymmetric orientation in the cytoplasmic membrane of E. coli because the enzyme extracted by Lubrol PX from spheroplasts treated with papain or PCPMS is ≥90% as active compared with a control treatment, while the same extraction of treated everted vesicles yields little active enzyme (cf. Table I and Fig. 2B).

The orientation of Enzyme II\textsuperscript{pp} in the membrane shown in Fig. 4 is also consistent with a number of other properties of the protein. Presumably, it must interact with phospho-HPr, and possibly also Enzyme I of the PTS,\textsuperscript{6} on the cytoplasmic surface of the membrane. This would require that part of the polypeptide be exposed to the cytoplasm. Also, evidence has

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**TABLE I**

**Activity of Enzyme II\textsuperscript{pp} in whole cells, spheroplasts, and everted vesicles after treatment with para-chloromercuri-benzene-sulfonate, a membrane-impermeable sulfhydryl reagent**

| Sample      | % Activity relative to untreated control sample |
|-------------|-----------------------------------------------|
| Whole cells | 99                                             |
| Spheroplasts| 105                                            |
| Everted vesicles | 9                                   |

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\textsuperscript{6} Because KMnO\textsubscript{4} oxidation reproducibly (3 experiments) inhibited Enzyme II\textsuperscript{pp} in spheroplasts by about 30%, and this inactivation plateaued after about 30 min, we conclude that the partial inactivation seen is not due to diffusion of KMnO\textsubscript{4} across the membrane and oxidation from the inside. Rather, because ≥95% of the spheroplasts remained intact throughout the experiment, we believe that KMnO\textsubscript{4} oxidizes the enzyme from the outside surface, leading to a species with impaired catalytic function relative to the unoxidized enzyme. An alternative explanation, that KMnO\textsubscript{4} oxidizes phospholipids important for Enzyme II\textsuperscript{pp} function, however, cannot be ruled out by these experiments.

\textsuperscript{8} Direct interaction of Enzyme I with membrane-bound Enzymes II of the PTS as shown in Fig. 4 has not yet been shown. However, a significant proportion of total cellular Enzyme I (~10%) is bound to membranes after lysis of E. coli, and this Enzyme I is active in the PEP-dependent phosphorylation of PTS substrates, including D-mannitol (G. Jacobson and D. Finlay, unpublished observations). This binding could also be due to indirect association of Enzyme I with an Enzyme II via an Enzyme I-HPr-Enzyme II complex.
transphosphorylation (Fig. 1). This result shows that there is at least one site on the enzyme that is more important for PEP-dependent phosphorylation than for transphosphorylation, the structure of which is altered by chymotryptic hydrolysis. A reasonable candidate for this site would be a residue on the protein whose structural integrity (or conformation) is necessary in the PEP-dependent reaction for interaction with phospho-HPetr in the cytoplasm. However, it remains to be determined whether chymotryptic inactivation of either reaction is due to effects on substrate K₁ values, Vmax of the reaction, or both. Partial dissection of these activities of Enzyme IImt also recently has been demonstrated immunologically in E. coli (22) and in S. typhimurium by isolation of certain mutants in the gene coding for this protein (21). It will be interesting to identify which domains of the protein are involved in these functions either by determining the sites hydrolyzed by chymotrypsin or by further genetic studies.

The experiments reported here constitute a first step in understanding how the structure of Enzyme IImt is related to its in vivo functions of mannitol transport, phosphorylation, and chemoreception. Since Enzyme IImt is quantitatively a relative minor protein of the E. coli inner membrane (17), it has not yet been possible to determine the sites of the membrane-bound and hydrophobic domains, for example by limited proteolysis. The availability of a plasmid carrying the mannitol operon which can be selectively expressed in E. coli minicells (20), however, should allow these questions to be explored in more detail.

Acknowledgments—We thank Dr. Milton Saier for helpful and stimulating discussions during the course of the work reported in this paper, and Drs. H. Hausman and S. Peterson for helpful criticisms of the manuscript.

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The intramembrane topography of the mannitol-specific enzyme II of the
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J. Biol. Chem. 1983, 258:2955-2959.

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