in our laboratory are directed at determining how these fundamental requirements are fulfilled in the hexokinase–outer mitochondrial membrane system.

RESULTS

A "hexokinase binding protein" (HBP) has been highly purified from outer mitochondrial membranes by a procedure involving dissociation of the membrane with octyl glucoside followed by reconstitution of lipid vesicles into which the HBP is selectively incorporated (5). Based on its inaccessibility to exogenous proteases, the HBP (subunit mol wt, 31,000) is thought to be an integral protein of the outer mitochondrial membrane. This protein appears to be the sole membrane component responsible for specific interaction with hexokinase. Indirect evidence suggested involvement of the N-terminal region of the enzyme in the binding. The N-terminal amino acid sequence has now been determined. The first three residues are a triplet of aromatic amino acids, Tyr-Tyr-Phe. This unusually hydrophobic sequence may provide a "wedge" by which the N-terminal portion of the enzyme secures access to the HBP buried in the membrane. A speculative model for the hexokinase-membrane interaction is shown in the accompanying figure.

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THE MEMBRANE ENZYME COMPLEX REQUIRED FOR L-PROLINE TRANSPORT AND UTILIZATION IN *ESCHERICHIA COLI* K12

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L-proline is actively accumulated by *Escherichia coli* K12 and can be oxidized to L-glutamate, serving as sole carbon and/or nitrogen source for bacterial growth. Two specific transport systems, denoted proline porters I and II (PP-I and PP-II), catalyze proline uptake (1).[1] Proline oxidation to Δ^1-pyrroline carboxylate is coupled via the respiratory chain with the reduction of O_2 (2). The membrane-associated enzyme proline dehydrogenase catalyzes that reaction and probably also the NAD^+-linked oxidation of Δ^1-pyrroline carboxylate to glutamate (2, 3). Proline dehydrogenase is encoded in the *putA* gene and mutations at the adjacent locus, *putP*, inactivate PP-I (1). Although *putP* and *putA* are transcribed separately, their expression is controlled coordinately by catabolite repression and specific induction (1, 3). In addition to its enzymatic

TABLE I
ESCHERICHIA COLI K12 STRAINS

Strain*	Genotype*	Gene dosage‡	
		putP	*putA*
CSH4	F⁻ *trp lacZ rpsL thi*	1	1
RM2	CSH4 Δ*putPA100*	0	0
JT31	CSH4 *putA1*::Tn*5*	1	0
JT34	CSH4 *putP3*::Tn*5*	0	1
WG2400	RM2 *recA srl*::Tn*10*	0	0
WG2404	WG2400/pLC35-38	>1§	0
WG2405	WG2400/pLC43-41	>1	>1
WG2406	WG2400/ColE1	0	0

*The derivation of these strains and genetic nomenclature have been described (1, 4).

‡Gene dosages are given as numbers of copies of the intact gene per chromosome.

§Although pLC35-38 complements *putP* defects, the plasmid insert has not been shown conclusively to include *putP* (4).

[1]Stalmach, M. and J. M. Wood. Unpublished results.

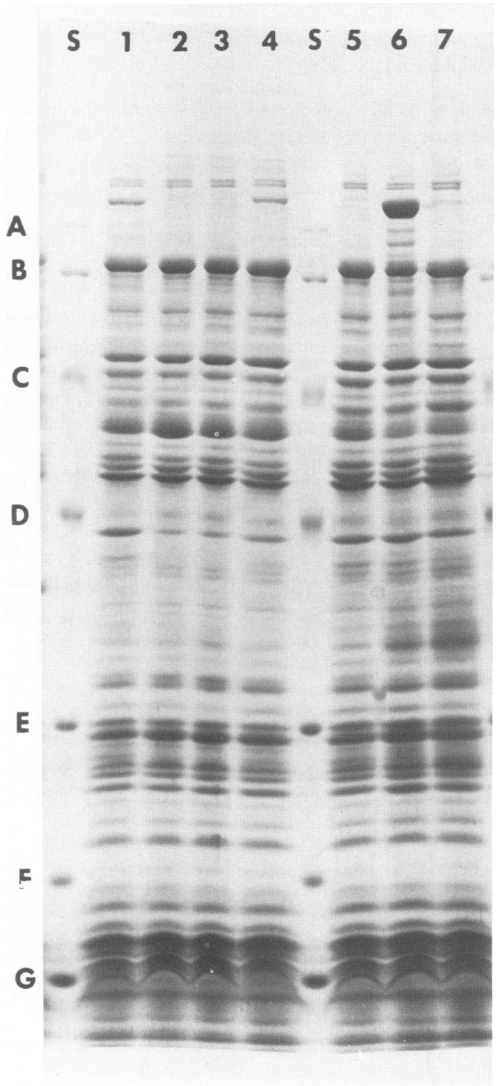

FIGURE 1 Polyacrylamide gel electrophoresis of bacterial membrane proteins. Bacterial cultures were prepared as described before (4) with sodium succinate (0.2%) as carbon source, NH_4Cl (95 mM) as nitrogen source, and L-proline (0.2%) or glycyl-L-proline (0.2%, strain JT34 only) as inducer. Membranes were prepared essentially as described by Hertzberg and Hinkle (5) using 0.5 M Na cacodylate, pH 6.8, as the preparation buffer (3). Electrophoresis was performed in sodium dodecyl sulphate as described before (4) except that a linear acrylamide concentration gradient from 10–14% was used. Samples were prepared by incubating at 25°C for 18 h and were clarified by centrifugation at 30,000 *g* for 60 min. Samples boiled for 5 min yielded similar patterns with the exception that a major outer membrane protein showed reduced electrophoretic mobility, as expected. The samples in the lanes labeled *S* were molecular weight standards identified as follows: *A*, β-galactosidase (112,000); *B*, phosphorylase *b* (94,000); *C*, bovine serum albumin (68,000); *D*, chicken ovalbumin (43,000); *E*, carbonic anhydrase (30,000); *F*, soybean trypsin inhibitor (21,000); and *G*, cytochrome *c* (12,4000). The other samples are identified in the text.

activities, the *putA* gene product is a negative genetic effector controlling *putP* and *putA* expression (1, 3). I have isolated mutant bacteria with specific defects in *putP* and *putA* (1) and have identified hybrid plasmids bearing *putP* and *putA* that complement those defects (4). Expression of the hybrid plasmids in bacterial minicells led to tentative identification of the *putP* and *putA* gene products among the plasmid-encoded proteins (4).

RESULTS AND DISCUSSION

The objective of the experiments described here was to visualize the *putP* and *putA* gene products as components of total membrane protein mixtures separated by polyacrylamide gel electrophoresis. The genotypes and *putA* gene dosages of the bacterial strains employed are listed in Table I. The effects of *putP* and *putA* mutations on the membrane protein composition were examined by comparing membranes from strains CSH4, RM2, JT31, and JT34 (Fig. 1, lanes 1, 2, 3, and 4, respectively). Proline dehydrogenase has a molecular weight of ~ 130,000 (1, 2). A protein of that size is present in strains CSH4 and JT34 but absent from strains RM2 and JT31, as expected from their genotypes. A protein of 25,000 mol wt was tentatively identified as the *putP* gene product and that size is consistent with the protein coding capacity of the chromosomal insert in plasmid pLC43-41 (4). No protein of that size is present in strains CSH4 and JT31 and absent in strains RM2 and JT34, as would be predicted from their genotypes, nor does any other protein follow that pattern. Membranes from strains containing multiple copies of the *put* genes have also been examined. Strain WG2406 contains the unmodified plasmid vector ColE1 (lane 5), strain WG2405 contains a recombinant derivative of ColE1 including genes *putPA* (lane 6), and strain WG2404 contains a recombinant derivative of Col1E1 that complements *putP* but not *putA* (lane 7) (4). As expected, the membranes from strain WG2405 contain a high concentration of the protein identified as *putA* gene product. No other polypeptide is present in strain WG2405 (or WG2404) that is absent in strains WG2406, RM2 and JT34. The *putA* gene product can therefore be clearly identified by polyacrylamide gel electrophoresis among the membrane proteins of *E. coli* K12. The *putP* gene product, however, is not visible. It may be present in too small a quantity to be detected by this technique or it may not be a cytoplasmic membrane component.

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MEMBRANE-IMPERMEANT, CLEAVABLE CROSS-LINKERS

NEW PROBES OF NEAREST NEIGHBOR RELATIONSHIPS AT ONE FACE OF A MEMBRANE

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We have developed a new type of chemical probe for the determination of nearest neighbor relationships of membrane components at one face of a membrane: membrane-impermeant, cleavable cross-linkers. These bidentate compounds form covalent adducts with primary amino groups and can therefore be used to probe protein-protein and, in principle, protein-aminolipid and aminolipid-aminolipid interactions. An internal disulfide bond in each of the cross-linkers allows the cleavage of the cross-linking bridge to facilitate the analysis of the components of the cross-linked complex. What distinguishes these reagents from other cleavable cross-linkers (reviewed in reference 1) is that these probes do not permeate biological membranes, so that one can determine not only what interactions occur in a membrane, but also at which face of a membrane they occur.

RESULTS AND DISCUSSION

We have synthesized prototypes of two chemical classes of membrane-impermeant, cleavable cross-linkers (Fig. 1). Diisethionyl-3,3'-dithiobispropionimidate (DIDIT) is a bis(alkyl imidate) which forms amidines from primary amino groups (2) (Fig. 1, Eq. I). 3,3'-Dithiobis(sulfosuccinimidylpropionate) (DTSSP) is a bifunctional active ester which forms amide linkages from primary amines[1] (Fig. 1, Eq. II).

The chemical properties of these compounds are complementary. Alkyl imidates, such as DIDIT, have the distinct advantage that the product amidines (Fig. 1, Eq. I) are charged at neutral pH like the primary amines from which they were derived. Even extensive amidination does not markedly change the physical properties of many proteins (3). However, the pH maximum for the reaction of alkyl imidates with primary amines is far above physiological, e.g., pH 9–10.5 for methyl acetimidate with α- or ϵ-amino groups (4). Even when the reaction is carried out at high pH, hydrolysis can effectively compete with aminolysis, reducing the yield of the cross-linking reaction. Niehaus and Wold (5) showed that when erythrocyte membranes are reacted with dimethyladipimidate at pH 9.6, only 20% of the modified lysines were involved in cross-links; the remainder were modified with reagent which had hydrolyzed at its second reactive functionality. Further, when the reaction is carried out below pH 8, a side reaction becomes significant in which *N,N'*-disubstituted amidines (which constitute non-cleavable cross-links) are formed (6).

The benefits and liabilities of active esters such as DTSSP are essentially the converse of those for alkyl imidates. The aminoacyl adduct formed (Fig. 1, Eq. II) is not charged at neutral pH, since on reaction, the primary amino group becomes an amide nitrogen. Therefore, one must be cognizant of potential structural changes on reaction due to a change in charge. However, the reaction can be carried out at neutral pH; by analogy with the closely related *N*-hydroxysuccinimide esters (7), the rates of hydrolysis are very slow, so the chemical yield of cross-linked products can be relatively high.

We have tested both DIDIT and DTSSP using a series of procedures designed to show that the probes are cross-linkers, that they are cleavable, and that they are membrane-impermeant (2). Rabbit muscle aldolase, a tetrameric protein having four identical 40,000 dalton subunits, was treated in solution with a series of concentrations of each reagent, and the products were subjected to electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. When gels were run under non-reducing conditions, bands were observed corresponding to dimers, trimers, tetramers and, at higher reagent concentrations, traces of

[1]The chemistry of *N*-hydroxysulfosuccinimide and of the active esters formed with this compound is discussed elsewhere (Staros, J. V., in preparation).