Coupling of Alanine Racemase and D-Alanine Dehydrogenase to Active Transport of Amino Acids in Escherichia coli B Membrane Vesicles*

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SUMMARY

Isolated membrane vesicles from Escherichia coli B grown on Dl-alanine-glycerol carry out amino acid active transport coupled to D-alanine oxidation by a membrane-bound dehydrogenase. Several other D-amino acids are substrates for this D-alanine dehydrogenase and also drive concentative uptake of solutes. Additionally, L-alanine and L-serine can energize solute transport by virtue of conversion to oxidizable D isomers by a membrane-bound alanine racemase. No other physiological L-amino acids were effective. Both membrane enzymes and consequent solute transport are markedly reduced in vesicles from glucose-grown cells.

Respiratory chain uncouplers abolish the racemase-dehydrogenase-supported transport activity. When aminooxyacetate at 10^{-4} M is added to the vesicles, the racemase activity and transport driven by L-alanine and L-serine is specifically and reversibly inhibited. D-Alanine-driven transport is unaffected. Similarly, N-chloro-L-alanine is an irreversible inactivator of the bound racemase but not the D-alanine dehydrogenase.

Both the D and L isomers of N-chloroalanine support oxygen uptake by the vesicles and initially stimulate L-L-proline active transport. However, oxidation of the N-chloro-D-alanine rapidly uncouples active transport from substrate oxidation. This transport inactivation can be protected partially by dithiothreitol, putatively scavenging a reactive product of chloroalanine oxidation. Authentic N-chloro-pyruvate produces the same transport uncoupling. When N-chloro-L-alanine is employed as a substrate, no such transport inactivation is observed. This difference may stem from the possibility that the alanine racemase eliminates HCl from N-chloro-L-alanine producing pyruvate, not the N-chloropyruvate that would arise from racemization and then dehydrogenation. We have shown that exogenous pyruvate is oxidized by the vesicles and will also stimulate active transport of amino acids.

The active transport of many metabolites is carried out by isolated bacterial cytoplasmic membrane vesicles in the presence of an appropriate energy source, usually an oxidizable substrate (1). Additionally, artificial electron donors such as reduced phenazine methosulfate have been used to probe membrane transport capacity in several bacterial species where physiological electron donors are unknown (2). In search of physiological oxidizable substrates for electron donors, Kaback and his colleagues have demonstrated that in Escherichia coli vesicles L-lactate oxidation is preferentially coupled to transport. In Staphylococcus aureus, however, either a L-lactate dehydrogenase or a-glycerol phosphate dehydrogenase may be the important coupling agent depending on growth conditions (3). In vesicles from other species, physiological substrates such as L-malate, succinate, NADH, glucose, and ethanol (in a marine pseudomonad) can undergo oxidation by membrane-bound dehydrogenases and are coupled to active transport (4).

Recently Jenkins and co-workers, in a study of the oxidation of alanine by an E. coli B membrane preparation, have demonstrated that L-alanine is first racemized to D-alanine and then the D isomer, specifically, is oxidized to pyruvate (5, 6). No evidence for an L-alanine dehydrogenase, such as found in Bacillus subtilis (7), was detected. Further, the alanine racemase and D-alanine dehydrogenase activities sedimented with the membrane fraction.

These observations prompted us to evaluate the possible role of these two membrane-associated enzymes in the active transport of solutes by isolated cytoplasmic membrane vesicles from E. coli B. The experiments presented here confirm that, when E. coli B cells are grown on DL-alanine, as inducer, membrane vesicles prepared from these cells can couple oxidation of both L-alanine and D-alanine to the active transport of several amino acids. Through the use of both reversible (aminooxyacetate) and irreversible (N-chloroalanine) inhibitors of alanine racemase, we have been able to evaluate the interaction of both the racemase and D-alanine dehydrogenase in this process. This represents the first clear instance of coupling of amino acid racemization and oxidation to the active transport of solutes in bacterial systems. Additionally, we have found that membrane-bound pyruvate oxidase also appears to couple oxidation to solute active transport.

EXPERIMENTAL PROCEDURES

Materials—D-Proline, N-chloro-D-alanine HCl, and N-chloro-L-alanine HCl were supplied by Cyle Chemical Co. Other unlabeled...
amino acids were obtained from Sigma Chemical Co. 2-Hydroxy-3-butynoic acid and 2-hydroxy-3-butenoic acid (vinylglycolate) were prepared as described previously (8). Pyruvic acid (Aldrich) was distilled in vacuo and neutralized as the potassium salt before use. Aminoxyacetic acid HCl was from K & K Laboratories. Radioactive compounds were purchased from New England Nuclear, ICN, Calitonic, and Amerham/Searle. Other chemicals used were commercially available and of reagent grade quality.

**Membrane Preparation**—Escherichia coli B (wild type) was used for preparing membrane vesicles. The bacteria were grown at 37° with aeration on a minimal medium supplemented with 0.5% D-alanine and 2% glycerol (5). Membrane vesicles were prepared by the method of Kaback (9) with the following alterations. The concentration of lysozyme used in the lysozyme-EDTA step was 50 μg/ml of 30 mM Tris-HCl pH 8.0, 50% sucrose buffer, as higher concentrations caused severe clumping of spheroplasts. Also three to four low speed spins (800 × g) were carried out for 10 min each on the homogenized lysed cells to maximize the yield of vesicles. The purified membrane vesicles were suspended in 0.1 M potassium phosphate buffer, pH 7.5, at a protein concentration of 10 mg/ml and stored in liquid nitrogen.

**Transport Assays**—Radioactive amino acid uptake in the presence of various energy sources was assayed by established procedures (9, 10). Samples were analyzed by liquid scintillation counting with approximately 85% efficiency. The specific activities and final concentration of 4C-labeled substrates employed at the following saturating concentrations: L-proline (230 μCi/mmol), 1.2X 10^{-5} M; L-tyrosine (185 μCi/mmol), 1.7X 10^{-5} M; L-phenylalanine (450 μCi/mmol), 0.6X 10^{-4} M; L-tyrosine (450 μCi/mmol) 5.7X 10^{-4} M; glycine (80 μCi/mmol), 2.3X 10^{-4} M; L-alanine (120 μCi/mmol), 2.2X 10^{-4} M; D-alanine (37 μCi/mmol), 6.6X 10^{-4} M; and lactate (14.0 μCi/mmol) 8X 10^{-4} M (11).

**Measurement of Membrane Dehydrogenase Activities**—Both pyruvate oxidase and D-alanine dehydrogenase activities were measured by monitoring substrates-dependent rates of oxygen uptake in suspensions of membrane vesicles with a Clark type electrode (YSI model 53 oxygen monitor) (12). An alternate method involved following the reduction of an acceptor dye, dichlorofluorescein, spectrophotometrically at 600 nm (13). Alanine racemase activity was similarly determined, starting with the L isomer of the amino acid to be tested.

**Recovery of Transported Amino Acids**—Vesicles were loaded with either L-[^14]C]proline, L-[^14]C]serine, or L-[^14]C]phenylalanine in the presence of 20 mM D-alanine as an energy source. The uptake was terminated after 10 min for proline or 15 min for other substrates by the addition of 18 volumes of 0.1 M LiCl and immediately filtered through a 0.45-μm membrane filter. The filter was washed four times with the same volume of LiCl and dried in an oven for 10 min at 100°. The radioactive amino acids were recovered from the filters by the extraction procedure of Konings (14). Identification of the recovered compounds was achieved by comparison with authentic standards using descending paper chromatography in an isobutyl alcohol-acetic acid-water (5:4:1, v/v) solvent system. Radioactivity on the chromatogram was determined by strip scanning.

**RESULTS**

**Coupling of Active Transport to L-Alanine and D-Alanine Oxidation**—When Escherichia coli B membrane vesicles prepared from D-alanine-glycerol-grown cells were assayed for their ability to support concentrative uptake of solutes, specifically the amino acid L-[^14]C]proline, the data of Fig. 1 were obtained. In the absence of an oxidizable energy source, only a low endogenous rate of accumulation prevailed. On addition of 20 mM D-alanine to the external medium, a 16-fold stimulation occurred in the initial rate of proline uptake, reaching a steady state at 12 min of around 1.2 nmol accumulated/mg of membrane protein. Other data to be presented below confirm that this is indeed active transport against a concentration gradient. We have measured by phase microscopy the average size of the E. coli vesicles to be 0.8 μm in diameter, which is quite similar to that seen by Kaback and Stadtman for E. coli ML vesicles where an internal volume of 2.2 μl/mg of protein has been calculated1 (15). Therefore, at steady state conditions the intravesicular concentration of radioactive proline is 522 μM. Since the exogenous concentration added at the start of the experiment is 13.3 μM, the uptake of proline represents a 39-fold concentration.

This result, then, establishes that the D-alanine dehydrogenase activity reported by Jenkins and colleagues (5, 6) can be coupled to drive active transport in isolated membrane vesicles.

The remaining curve of Fig. 1 shows that L-alanine also can energize active transport over the endogenous rate. The L isomer stimulates the rate (measured at 10 min) of L-[^14]C]proline uptake about 7 fold, approximately 15% as well as D-alanine. The slight initial lag may reflect the fact that very low levels of D isomer, from racemization of L-isomer, are available at early times to drive transport. The K_m for D-alanine-driven L-[^14]C]proline uptake was determined to be 5 mM for the D-amino acid with normal hyperbolic saturation kinetics (data not shown). A K_m for the L isomer has not been determined.

**The data of Fig. 1 represents the first instance of active transport of solutes coupled to oxidation of either enantiomers of aα-amino acid. To demonstrate that L- and D-alanine-coupled uptake is not specifically restricted to the concentration of the**

1 H. R. Kaback, personal communication.
positive. This is somewhat predictable because Lombardi employed and physiological energy sources were at a final concentration of 20 mm. Initial uptake rates are corrected for endogenous activity by subtracting the rate of amino acid transport in the presence of no energy source.

### Table I

<table>
<thead>
<tr>
<th>Substrate transported</th>
<th>Energy sources</th>
<th>Rate (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Proline</td>
<td>D-Alanine</td>
<td>0.106</td>
</tr>
<tr>
<td></td>
<td>T-Alanine</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td>n-Lactate</td>
<td>0.123</td>
</tr>
<tr>
<td></td>
<td>Ascorbate-PMS</td>
<td>0.183</td>
</tr>
<tr>
<td>L-Serine</td>
<td>D-Alanine</td>
<td>0.068</td>
</tr>
<tr>
<td></td>
<td>T-Alanine</td>
<td>0.033</td>
</tr>
<tr>
<td></td>
<td>n-Lactate</td>
<td>0.100</td>
</tr>
<tr>
<td></td>
<td>Ascorbate-PMS</td>
<td>0.249</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>D-Alanine</td>
<td>0.154</td>
</tr>
<tr>
<td></td>
<td>T-Alanine</td>
<td>0.046</td>
</tr>
<tr>
<td></td>
<td>n-Lactate</td>
<td>0.119</td>
</tr>
<tr>
<td></td>
<td>Ascorbate-PMS</td>
<td>0.184</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>D-Alanine</td>
<td>0.115</td>
</tr>
<tr>
<td></td>
<td>T-Alanine</td>
<td>0.045</td>
</tr>
<tr>
<td></td>
<td>n-Lactate</td>
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</tr>
<tr>
<td></td>
<td>Ascorbate-PMS</td>
<td>0.264</td>
</tr>
<tr>
<td>Glycine</td>
<td>D-Alanine</td>
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<td></td>
<td>T-Alanine</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>n-Lactate</td>
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</tr>
<tr>
<td></td>
<td>Ascorbate-PMS</td>
<td>0.110</td>
</tr>
</tbody>
</table>

* PMS, phenozine methosulfate.

In addition to L-proline, L-serine, L-phenylalanine, L-tyrosine, and glycine also are accumulated against a concentration gradient. Lombardi and Kaback have previously demonstrated in E. coli ML vesicles that these five amino acids represent four separate specific carrier systems (11). In the first four cases 20 mm D-alanine is to 4 fold more effective an electron donor than 20 mm L-alanine in the rate of solute accumulation. Further, when the stimulatory ability of D-alanine is contrasted to that of D-lactate, an acknowledged preferred oxidizable substrate for transport, the two are quite comparable. The last column indicates that the artificial electron donor system is again not significantly more effective than D-alanine as a coupling agent. However, neither isomer of alanine significantly stimulates the transport of glycine. This is somewhat predictable because Lombardi and Kaback have shown that alanine and glycine share a common transport system (11), at least in ML vesicles, and the very high concentrations of alanine employed probably competitively inhibit the binding of glycine to its carrier protein. Both D-lactate and ascorbate-phosphate methosulfate drive glycine uptake as expected.

At this point it is germane to point out again that one demonstrable virtue of the isolated membrane vesicle preparation over whole cells is the absence of subsequent metabolism of a solute once it has been accumulated in the intravesicular space. This is essential if radioactive uptake is to be equated with accumulation. To check this issue we recovered L-[14C]phenylalanine, L-[14C]serine, and L-[14C]proline after transport into vesicles and demonstrated that only the chemically unmodified amino acids were present.

Two other observations deserve mention at this point. First, while Kaback (9) has previously demonstrated that D-lactate-coupled active transport of proline shows a pH optimum at pH 6.6, we found higher pH values to be optimal in this system, consistent with the pH optimum reported by Jenkins (5) for the D-alanine dehydrogenase. Second, our investigations so far have been concerned only with amino acid uptake. We have examined the E. coli B vesicles for [14C]lactose uptake which was negligible (data not shown). This is explained because the lac operon was not induced in these bacterial cells.

### Table II

<table>
<thead>
<tr>
<th>Energy source</th>
<th>Rate (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Alanine</td>
<td>0.106</td>
</tr>
<tr>
<td>D-Methionine</td>
<td>0.080</td>
</tr>
<tr>
<td>D-Serine</td>
<td>0.073</td>
</tr>
<tr>
<td>D-Asparagine</td>
<td>0.053</td>
</tr>
<tr>
<td>D-Phosphate</td>
<td>0.014</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>0.026</td>
</tr>
<tr>
<td>L-Serine</td>
<td>0.023</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>0</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>0</td>
</tr>
<tr>
<td>L-Proline</td>
<td>0.083</td>
</tr>
<tr>
<td>L-α-Aminobutyrate</td>
<td>0</td>
</tr>
<tr>
<td>β-Chloro-D-alanine*</td>
<td>0.034</td>
</tr>
<tr>
<td>β-Chloro-L-alanine</td>
<td>0.045</td>
</tr>
</tbody>
</table>

* In the presence of 10 mm dithiothreitol (see text).

FIG. 2. Transport of L-proline driven by D- and L-amino acids. A, L-[14C]proline uptake was monitored as described in Fig. 1 except that 100-μl assay volumes were employed (0.5 mg of membrane protein). Incorporation of radioactive substrate by membrane vesicles in the presence of 20 mm D-serine ( ), 20 mm L-serine ( ), and no energy source ( ) are shown. B, transport of L-[14C]proline driven by D- and L-phenylalanine was also investigated. Radioactive substrate was added to a 50-μl incubation mixture containing 20 mm D-phenylalanine ( ), 20 mm L-phenylalanine ( ), and no energy source ( ).
isomers drive proline uptake; $r$-isomers do not. The only exceptions for physiological substrates are $L$-alanine and $L$-serine. As we shall see in a subsequent section, the ability of these $L$ isomers alone to energize solute uptake is related to their ability to be acted on by the racemase. In both these instances the $L$ isomers are less stimulatory than the corresponding $D$ isomers.

The data of Table II indicate that, while $D$-alanine is the most effective amino acid electron donor for transport, a considerable structural variation can be tolerated including the $S$-methyl group of $D$-methionine and the $\beta$-carboxamide of $D$-asparagine. Even the unphysiological $\beta$-halo-$\alpha$-amino acid, $\beta$-chloro-$D$-alanine, can be accommodated although special incubation conditions were required. The consequences of $\beta$-chloro-$D$-alanine functioning as a substrate to drive transport will be elaborated in a subsequent section of this paper.

Comparison of Vesicles from $D,L$-Alanine-glycerol-grown Cells with Those from Glucose-grown Cells—One might expect that the novel coupling of a variety of $D$-amino acids to solute active transport was intimately related to growth of the $E$. coli B cells on $D,L$-alanine. Ramsey and Jenkins indicated a 9-fold increase in $D$-alanine dehydrogenase activity in membrane fractions derived from cells grown on $D,L$-alanine-glycerol compared to those grown on glucose (5). This expectation is borne out by the data of Fig. 3 which illustrates $L$-$[14C]$proline uptake into isolated cytoplasmic membrane vesicles prepared from cells grown on 0.5% glucose. The stimulation of $L$-$[14C]$proline uptake by $D$-lactate is slightly higher than in the vesicles from the alanine-glycerol cells. However, 20 mm $D$-alanine is now only 50% as effective an electron donor in the glucose membranes. This is correlatable with a decline in $D$-alanine dehydrogenase activity. Even more striking is that there is no detectable stimulation of $L$-$[14C]$proline transport by $D$-alanine. This dramatic switch off can be related to the previous observation by Lambert and Neuhaus on $E$. coli whole cells where levels of alanine racemase were depressed 25-fold on replacing $D,L$-alanine with glucose in the growth media (16). This experiment is one index that it is the racemase which allows $D$-alanine to funnel electrons into the membrane respiratory chain to drive solute uptake.

To this point, we have not discussed two salient features about amino acid driven uptake of amino acids in $E$. coli B membrane vesicles. First, can an amino acid drive uptake of that same radioactively active amino acid? The second point relates to the demonstration of active transport of alanine itself.

The answer to the first question is negative and this is as one might expect. In the vesicles from alanine-glycerol grown cells, $D$-phenylalanine would not stimulate active transport of $L$-$[14C]$phenylalanine although Fig. 2B shows $D$-phenylalanine can drive proline uptake and Table I indicates $L$-$[14C]$phenylalanine is actively taken up in the vesicles. This result is not surprising if enantiomers of amino acids bind competitively to the same carrier protein system. A much higher concentration of amino acid (5 mm) is needed to saturate as an electron donor compared to the concentration of amino acid needed to saturate the transport carrier protein ($K_m = 0.42$ $\mu$m for phenylalanine) (11). The excess of unlabeled substrate dilutes out the radioactive molecules binding to the carrier so that the effective specific activity of molecules transported drops significantly.

What about the transport of alanine into vesicles? The data of Fig. 4 show the uptake of $L$-$[14C]$alanine into the induced $E$. coli B vesicles driven by ascorbate-phenazine methosulfate. The unusual observation is the high endogenous rate of alanine transport in the absence of an energy source, 0.085 nmol in 5 min. This high level is unique to alanine uptake; for instance, Fig. 1 shows a very low endogenous rate of $L$-$[14C]$proline uptake. This low rate was also seen for $L$-$[14C]$serine and $L$-$[14C]$phenylalanine (data not shown). When $n$-$[14C]$alanine was examined, the endogenous accumulation was even higher than $L$-$[14C]$alanine, 0.190 nmol in 5 min. The data of Fig. 4 show that it is possible to see some stimulation of $L$-$[14C]$alanine uptake and the same is true with $n$-$[14C]$alanine active transport (0.670 nmol/mg of protein in 5 min in the presence of ascorbate-phenazine methosulfate; data not shown).

Currently, we are at a loss to explain the singular ability of either isomer of $[14C]$alanine to be so efficiently scavenged for concentrative uptake by the induced vesicles. The process is clearly active transport and not some high capacity facilitated diffusion because 1 mm dinitrophenol (Fig. 4) will completely abolish all of the endogenous $[14C]$alanine uptake.

The data of Fig. 4 also point out that this effect is peculiar to the vesicles from alanine-glycerol grown cells. In vesicles from glucose-grown cells, ascorbate-phenazine methosulfate stimulates $L$-$[14C]$alanine uptake as expected while, in the absence of an exogenously added energy source, the background level of uptake is essentially zero. At present one might suppose that growth of cells on alanine may induce large amounts of the alanine carrier protein and so generate a high capacity for subsequent alanine transport in isolated membrane vesicles. Perhaps the residual endogenous energy supply is tightly coupled to this transport system but this is not understood and merits further study.
FIG. 4. Uptake of alanine in alanine-glycerol and glucose-grown Escherichia coli B membrane vesicles. Vesicles, 25 ~1 (0.25 mg of membrane protein), were diluted to a final volume of 50 ~1 (50 mM potassium phosphate, pH 7.5) including 10 mM magnesium sulfate. This mixture was preincubated at 25° for 5 min under an oxygen atmosphere. Sodium ascorbate, 10 mM, and 10 ~ phenazine methosulfate then were added to each reaction mixture in the dark, followed immediately by the addition of L-[14C]alanine. The assay medium was continually gassed with oxygen until the reaction was terminated with 0.1 M lithium chloride. The endogenous rate of n-alanine uptake was determined by adding L-[14C]alanine without prior addition of ascorbate and phenazine methosulfate or incubation under an oxygen atmosphere. In cases where the endogenous rate of transport was studied in the presence of 2,4-dinitrophenol, 1 mM final concentration, dinitrophenol was added 5 min before addition of the radioactive substrate. The uptake of L-alanine was studied in alanine-glycerol grown vesicles stimulated by ascorbate and phenazine methosulfate (O—O), no energy source (□—□), and no energy source plus 1 mM dinitrophenol (△—△). The transport of L-alanine in glucose grown vesicles in the presence of ascorbate and phenazine methosulfate (O—O) and no energy source (□—□) also was determined.

Effect of Respiratory Chain Uncouplers on Alanine Driven Transport—Because a 39-fold concentration gradient of L-[14C]proline can be sustained by the exogenous addition of n-alanine, one would expect that a continual supply of electrons from the n-alanine dehydrogenase would be necessary to avoid the collapse of the gradient. Kaback has mapped the pathway of electrons derived from n-lactate into the membrane respiratory chain and out to oxygen as terminal electron acceptor (1). If n-alanine should funnel electrons into the same chain, uncouplers of the respiratory chain should abolish the capacity to drive transport.

Thus, the curves in Fig. 5 demonstrate that 1 mM 2,4-dinitrophenol, a classic respiratory chain uncoupler in both mitochondrial and bacterial membranes, suffices to completely abolish active transport driven by both n-alanine and L-alanine. The endogenous background rate also is abolished by dinitrophenol, indicating that this process is indeed active transport. A similar inhibitory effect of carbonyl cyanide m-chlorophenylhydrazone is found on L-[14C]proline uptake driven by n-alanine (data not shown). Carbonyl cyanide m-chlorophenylhydrazone, 3 X 10^-3 M, gives 50% inhibition in a 5-min preincubation with vesicles. From these data, keeping in abeyance the controversy of whether carbonyl cyanide m-chlorophenylhydrazone uncouples by collapsing proton gradients or alkylating -SH groups, or both (17), the involvement of membrane respiratory chain components in D- and n-alanine-stimulated solute uptake is strongly inferred.

Activities of Alanine Racemase and n-Alanine Dehydrogenase in Vesicles—Raunio and Jenkins indicated that in membrane fractions from alanine-glycerol grown cells of E. coli B, both alanine racemase and n-alanine dehydrogenase activities were detectable (5). There was no detectable L-alanine dehydrogenase, either dependent on NAD as in Bacillus subtilis (7) or NAD-independent. The racemase was susceptible to pyridoxal phosphate enzyme inhibitors, specifically aminoxyacetate. The n-alanine dehydrogenase was NAD independent and, as Jenkins suggested (5), is most likely a flavoprotein, bolstered by the view that other bacterial membrane dehydrogenases such as those oxidizing lactate in E. coli (18), α-glucosyl phosphate in Staphylococcus aureus (19), and L-glutamate (20) and L-malate (21) in Azotobacter vinelandii appear to be flavoenzymes.
Substrate oxidized/nmol of solute transported. By this criterion, n-alanine is actually a more efficient coupler to active transport than n-lactate in the alanine-glycerol-grown membrane vesicles. But nonetheless reliable, index of oxidation by the membrane of amino acids to support oxygen uptake. This assay is, as Jenkins and colleagues (6) have pointed out, a somewhat circuitous, exceptions, as yet inexplicable.

The rates of n-alanine, L-alanine, n-serine, and L-serine oxidation were measured by oxygen electrode assay as described in Table III. Assays also were performed with a 5-min preincubation of 10⁻⁴ M aminooxyacetate before the addition of substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>No aminooxyacetate</th>
<th>10⁻⁴ M Aminooxyacetate</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>2.85</td>
<td>0.40</td>
<td>86</td>
</tr>
<tr>
<td>L-Serine</td>
<td>3.75</td>
<td>0.87</td>
<td>79</td>
</tr>
<tr>
<td>D-Alanine</td>
<td>19.5</td>
<td>22.0</td>
<td>0</td>
</tr>
<tr>
<td>D-Serine</td>
<td>5.70</td>
<td>4.89</td>
<td>14</td>
</tr>
</tbody>
</table>

Raunio and Jenkins (5) also indicated other d-amino acids could be oxidized by the vesicles and in general we agree qualitatively with their observations and have found additional substrates as indicated in Table III. d-Lactate is listed as a reference point to indicate that the d-lactate dehydrogenase in the membranes has about a 3-fold higher specific activity than d-alanine, the best substrate for the d-amino acid dehydrogenase.

As one would predict from the data of Table II, all the d-amino acids which drive transport are also oxidized by the d-amino acid dehydrogenase. The Kₘ for n-alanine in driving transport is 5 mm; Jenkins reports a Kₘ of 6.5 mm as a substrate for the dehydrogenase. In general the relative effectiveness in powered transport is correlated with substrate ability of the d isomer for the dehydrogenase. d-Phenylalanine and d-proline appear to be exceptions, as yet inexplicable.

The data of Table I and III together indicate that 20 mm d-lactate, oxidized at 66 nmol of oxygen/min/mg of membrane protein, drives transport at 0.12 nmol of L-[¹⁴C]proline/min/mg of protein, a ratio of 550 nmol of substrate oxidized/nmol of solute transported. d-Alanine, on the other hand, is oxidized at 18.8 nmol of oxygen/min/mg of protein, while stimulating 0.11 nmol of L-[¹⁴C]proline/min/mg of protein, a ratio of 171 nmol of substrate oxidized/nmol of solute transported. By this criterion, d-alanine is actually a more efficient coupler to active transport than d-lactate in the alanine-glycerol-grown membrane vesicles.

Also contained in Table III are the abilities of various L isomers of amino acids to support oxygen uptake. This assay is, as Jenkins and colleagues (6) have pointed out, a somewhat circuitous, but nonetheless reliable, index of oxidation by the membrane vesicles. As will be indicated below from aminooxyacetate inhibition data, L isomers must first be racemized to the d isomers prior to oxidation. Thus, in addition to L-alanine, L-serine appears to be a good substrate for the alanine racemase. To our knowledge, there is little known about alanine racemase specificity (22) and serine appears to be a novel substrate. This assignment correlates well with the fact (Figs. 1 and 2A) that L-alanine and L-serine are singular among L-amino acids in driving active transport in the vesicles (Table II).

The other L-amino acids assayed in Table III do not result in oxygen usage, by this criterion are not racemase substrates and, in harmony with this conclusion, are inactive as energy sources for active transport (Table II).

Finally, both the d isomer and the L isomer of β-chloroalanine generate oxygen uptake when added to the alanine-glycerol vesicles, albeit at low levels. The consequences of apparent susceptibility of appropriate isomers to racemase and dehydrogenase are explored in a subsequent section.

We have repeated all the oxygen electrode assays in Table III with an alternate assay, reduction of the blue dye dichlorophenolindophenol to its leuco form. In general, conclusions of Table III are borne out (data not shown). There is, however, a systematic underestimation of enzymatic activity with the better substrates (d-lactate, d-alanine, d-methionine) which we do not understand but may be related to a new rate-determining step introduced with dichlorophenolindophenol as the electron acceptor. This may be worth investigating.

Effect of Aminooxyacetate on Alanine Oxidation and Alanine-driven Solute Active Transport—In their incisive experiments demonstrating that L-alanine is obligatorily racemized prior to its membrane-catalyzed oxidation, Jenkins and colleagues made use of the pyridoxal phosphate inhibitor aminooxyacetate to specifically inhibit the racemase in the membranes (6). We have used this substituted hydroxylamine to demonstrate that inhibition of racemization of L-alanine and L-serine is coordinate with loss of the ability of the L isomers, but not of the d isomers, to drive transport of solutes.

Thus, in Table IV the first two lines indicate that 10⁻⁴ M aminooxyacetate causes 86% inhibition where L-alanine is substrate and 79% inhibition when L-serine is substrate. The last two lines indicate a much lower and probably nonspecific inhibition of oxygen uptake by the d isomers of alanine and serine. This confirms Jenkins’ data for L-alanine, reinforces the view that L-serine is an authentic substrate for the racemase, and indicates that aminooxyacetate is specifically inhibiting the racemase and not the d-specific dehydrogenase.

The effect of the same concentration of aminooxyacetate on alanine-driven L-[¹⁴C]proline uptake is depicted in Fig. 6. d-
product molecules should protect against this inactivation and will be examined.

When the vesicles were similarly pretreated with 0.1 mM vinylglycolate under the same conditions, which are sufficient to completely abolish glucose active transport, 79% of the D-alanine-driven L-[14C]proline uptake remains.

These data suggest the independence of D-alanine-energized transport from both a functional phosphotransferase system and an active lactate dehydrogenase and suggest some insensitivity of the D-amino acid dehydrogenase to Michael addition by keto-butyrate. We shall see in the next section a specific substrate for this amino acid dehydrogenase which can destroy the active transport capacity of the vesicles.

Effects of β-Chloro-D-alanine on Alanine-driven Transport—In a previous section aminoxyacetate, a reversible pyridoxal-phosphate inhibitor, was shown specifically to block active transport from L-alanine without affecting that driven by D-alanine. Manning and his colleagues have recently reported that the D-isomer of β-chloroalanine is bacteriocidal (26). Specifically, treatment of dialyzed crude cell extracts with 40 mM β-chloro-D-alanine for 3 hours at 37° resulted in loss of 96% of the alanine racemase activity, suggesting perhaps an irreversible inactivation. We have examined this question in the alanine-glycerol E. coli B vesicles.

The data of Table III indicate that β-chloro-D-alanine supports oxygen consumption by vesicles at a rate of 5.3 nmol of oxygen per min per mg of protein. The D isomer then is an oxidizable substrate for the D-alanine dehydrogenase. This conclusion was reinforced when assays were performed using dichlorophenolindophenol rather than oxygen as a respiratory chain electron acceptor.

We next performed an inactivation experiment similar to the type reported by Manning et al. (26); incubation of vesicles with 40 mM β-chloro-D-alanine for 3 hours in an ice bath (low temperatures were used because after 3 hours at 37° all transport is lost, even in control vesicles). The vesicles then were centrifuged and washed to remove excess unreacted chloroalanine. At this point they were tested for alanine racemase activity with L-alanine by oxygen electrode assay. Indeed, no detectable racemase activity remained (5% could have been detected). Control vesicles carried through the procedure still retained racemase activity (76% of the original activity). Specificity of the irreversible racemase inactivation was proved by the fact that the same amount of D-alanine dehydrogenase activity was retained in both chloroalanine-treated and control vesicles.

At this juncture, active transport capacity of these vesicles was assayed as shown in Fig. 7. The D-alanine- and L-alanine-stimulated L-[14C]proline uptake in control vesicles is as expected (cf. Fig. 1). As predicted, in the treated vesicles, L-alanine-stimulated uptake of L-[14C]proline uptake was assayed as shown in Fig. 7. The D-alanine- and L-alanine-stimulated L-[14C]proline uptake in control vesicles is as expected (cf. Fig. 1). As predicted, in the treated vesicles, L-alanine-stimulated L-[14C]proline uptake is negligible. Surprisingly though, D-alanine also could not longer drive proline transport, despite the fact that the D-alanine dehydrogenase was fully active. Even the endogenous transport activity was greatly reduced. These phenomena are fully reproducible.

In connection with this inactivation of vesicle transport capacity, it is noted that oxidation of the D isomer of β-chloroalanine by the dehydrogenase should yield β-chloropyruvic acid, a product containing a reactive α-haloketone moiety. Such functionalities have repeatedly been demonstrated to be active species in alkylation of enzyme residues, leading often to irreversible loss of activity as covalent modification proceeds (27).

To test the possibility that a reactive oxidation product of β-chloro-D-alanine was responsible for the loss of D-alanine stimu
FIG. 7. The effects of β-chloro-n-alanine on proline transport in Escherichia coli B membrane vesicles. Vesicles, 500 μl (5 mg of membrane protein), were suspended in water to give a final volume of 1 ml (50 mM potassium phosphate, pH 7.5). The mixture was incubated for 3 hours with 40 mM β-chloro-n-alanine in an ice bath. Ten milliliters of cold 0.1 M potassium phosphate, pH 7.5, then were added and the vesicles were pelleted by centrifugation, 25 min at 45,000 × g. The pellet was resuspended and homogenized to a final volume of 500 μl with 0.1 M potassium phosphate buffer. The membranes were assayed for n-alanine dehydrogenase and alanine racemase activity by oxygen electrode. Transport of L-proline in the presence of various energy sources was also looked at. A control incubation of vesicles, replacing the β-chloro-n-alanine with water, was run at the same time under identical conditions. L-Proline uptake in the β-chloro-n-alanine-treated membranes stimulated by n-alanine (O-O) and L-alanine (A-A) was measured. L-Proline transport in the control stimulated by n-alanine (O-O), L-alanine (A-A), and no energy source (+m) also was determined.

Finally, it was noted briefly above in a discussion of Table II that β-chloro-n-alanine could drive L-[14C]proline uptake, provided 10 mM dithiothreitol was present. Since the D isomer of β-chloroalanine is a substrate for the D-alanine dehydrogenase, it ought to drive solute uptake. Fig. 9 indicates a stimulation of proline transport during the first 5 min of uptake but the rate levels off and accumulation of proline drops below the endogenous level by 15 min, suggesting inactivation of transport. Inclusion of 10 mM dithiothreitol allows active L-[14C]proline uptake to proceed. These observations will be described in full detail else-

FIG. 8. The effect of β-chloro-n-alanine on proline transport after protection with dithiothreitol. A 3-hour incubation of vesicles in ice was set up as described in Fig. 7. In addition to 40 mM β-chloro-n-alanine, dithiothreitol was added to a final concentration of 15 mM. After washing the membranes, L-proline transport was assayed in the presence of n-alanine (O-O), L-alanine (A-A), and no energy source (+m).

FIG. 9. The uptake of proline in Escherichia coli B membrane vesicles stimulated by β-chloroalanine. In order to determine whether β-chloroalanine could drive transport, 25 μl of vesicles (0.25 mg of membrane protein) were assayed as described before. 20 mM β-chloro-n-alanine was added along with L-[14C]proline and uptake was measured (O-O). In a second experiment, 10 mM dithiothreitol (final concentration) was added immediately before the addition of β-chloro-n-alanine and the time course of L-proline transport was measured (O-O). L-[14C]Proline uptake stimulated by 20 mM β-chloro-L-alanine also was assayed (O-O). The level of L-[14C]proline transport in the absence of an energy source was found to be the same, 0.33 nmol per mg of protein at 10 min with or without 10 mM dithiothreitol in the assay medium.
where, along with data indicating that authentic β-chloropyruvic acid can cause irreversible inactivation of amino acid transport.*

Effects of β-Chloro-L-alanine on Alamine-driven Transport—The data of Table III indicate that β-chloro-L-alanine also can stimulate oxygen uptake by the vesicles. One then might expect that the L isomer is racemized to the D isomer by alanine racemase and that it might eventually cause racemase inactivation analogously to the D isomer. In a 3-hour incubation in ice of vesicles with 40 mM L isomer, 50% of the racemase activity was lost (versus 100% with the D isomer). D-Alanine dehydrogenase activity was unaffected. In contrast to the above result from β-chloro-D-alanine incubation, D-alanine-driven proline uptake was intact.

Another contrast between L and D isomers surfaces in Fig. 9; 20 mM β-chloro-L-alanine drives proline uptake (0.045 nmol/min/mg of protein, cf. Table II), at a rate greater than that supported by L-alanine, without any detectable transport inactivation. With D isomer as electron donor, complete inactivation of proline transport had occurred within 15 min. If β-chloro-L-alanine is enzymatically racemized to β-chloro-D-alanine which is then dehydrogenated to β-chloropyruvic acid, there should be no such marked differences.

A possible resolution for the discrepancy is that alanine racemase does not in fact racemize β-chloro-L-alanine but instead an α,β elimination of HCl occurs. This is a well documented reaction pathway for chloroalanine with other pyridoxal enzymes such as aspartate transaminase (28) and β-aspartate decarboxylase (29). The aminocrotonate product would rapidly tautomerize to the imine, then hydrolyze to pyruvate and ammonia as the observed products. This route is currently under investigation. It does predict that pyruvate must then be oxidized by the membrane vesicles to account for both oxygen uptake and active transport seen on exogenous addition of β-chloro-L-alanine.

Pyruvate Oxidation and Coupling to Active Transport in Membrane Vesicles—To test the above hypothesis, 20 mM pyruvate, freshly distilled and neutralized to avoid any lactate contamination, was added to a suspension of membrane vesicles; oxygen uptake was detectable at a rate of 7.03 nmol of oxygen/min/mg of protein. A lower level of pyruvate oxidase activity (1.02 nmol of oxygen/min/mg of protein) was observed when vesicles were prepared from E. coli B grown on glucose.

Pyruvate also can serve as an energy source to drive amino acid active transport in these membrane vesicle preparations, as indicated in Fig. 10A with 20 mM pyruvate. The vesicles from alanine-glycerol E. coli B accumulate L-[14C]proline at rates equivalent to that with L-alanine as an energy source. The rate of L-[14C]proline uptake is much reduced in glucose-grown vesicles, correlating with lower levels of pyruvate oxidase activity.

It is extremely unlikely that lactate contamination in the pyruvate could account for the above results for the following reasons. The proton NMR spectrum of the freshly distilled and neutralized pyruvate methyl group is a singlet at 2.3 ppm (6), while the lactate methyl would appear as a doublet at 1.4 ppm (6). A 5% contamination would have been easily detectable. In data not shown, we determined Km values for L lactate and for pyruvate as energy sources for L-[14C]proline active transport. The Km for L-lactate was 4.4 mM, in agreement with previous values (18), while that measured for pyruvate is 1 to 2 mM, again arguing against a contaminant as the actual energy source. Lastly, we have noted that exogenous 2-hydroxy-3-butoenate (vinylglycolate), after a short preincubation with vesicles, irreversibly blocks pyruvate-stimulated uptake but not D- or L-lactate-driven transport.†

Two further controls for the pyruvate effect were also performed. First, pyruvate contamination in L-serine and β-chloro-L-alanine stock solutions must be ruled out to ensure that the alanine racemase is actually using these L isomers as substrates (for racemization or elimination). Second, the observed coupling of active transport to D-amino acid dehydrogenation must be determined to not be solely due to subsequent oxidation and coupling of the α-keto acid products.

Stock solutions of L-serine and both enantiomers of β-chloro-alanine were assayed for pyruvate contamination by enzymatic assay with NADH and lactate dehydrogenase. No contamination was detected. Controls indicated that as little as 0.01 μmol of pyruvate in the presence of 5 μmol of the amino acids was easily detectable.

Pyruvate is produced from D-alanine oxidation by the membrane dehydrogenase and 20 mM exogenous pyruvate can drive L-[14C]proline uptake at 1.07-fold the rate seen with 20 mM exogenous D-alanine; it is thus moot at present what per cent contribution subsequent pyruvate oxidation provides to D-alanine-stimulated active transport. On the other hand d-phenylalanine (Fig. 2B) also drives solute uptake during oxidation to phenylalanine.

*G. Kaczorowski, L. Shaw, R. Laura, and C. Walsh, manuscript in preparation.

†L. Shaw, G. Kaczorowski, and C. Walsh, unpublished observations.
pyruvate. This α-keto acid cannot drive solute uptake. Fig. 10B shows no stimulation of L-[14C]proline transport in response to 10 mM phenylpyruvate. It is very likely that the keto acid oxidation products from the other D-amino acids of Table II also are not substrates for the membrane pyruvate oxidase and will not drive active transport. The specificity of the pyruvate oxidase will be analyzed carefully.

DISCUSSION

The experiments of this paper confirm the findings of Jenkins and his colleagues (5, 6) that growth of E. coli B on D-alanine and glyceral results in induction of two membrane-bound enzymes, an alanine racemase and a D-alanine dehydrogenase. We have now shown that these enzymes can function in isolated cytoplasmic membrane vesicles to couple substrate oxidation to solute active transport. This is the first instance of amino acids serving as oxidizable energy sources for maintenance of a concentration gradient across a biological membrane. It is possible that D-alanine and other D-amino acids may have a physiological role in bacteria in addition to their involvement in cell wall biosynthesis. They may be significant energy sources for active transport.

The D-alanine dehydrogenase joins, as the first amino acid dehydrogenase, a growing class of bacterial membrane-bound dehydrogenases, which are thought to be flavoenzymes. Upon reoxidation of the reduced coenzyme, electrons are funneled into the respiratory chain and then to oxygen as terminal electron acceptor. At some point the potential drop of these electrons in transit is coupled mysteriously to solute active transport.

This dehydrogenase, as noted earlier (5), is not absolutely specific for D-alanine, but will oxidize a wide variety of other D-amino acids at lower rates.

Jenkins and co-workers (5, 6) argued for the absence of any L-alanine dehydrogenase activity in these membranes on the basis of complete inhibition of L-alanine oxidation by aminooxyacetate, an O-substituted hydroxylamine which is a specific pyridoxal phosphate inhibitor, and purportedly a block for alanine racemase activity in crude dialyzed extracts of E. coli. This compound was found by them to be bacteriocidal and seemed most directly explicable by the fact that racemase inactivation would interrupt the supply of D-alanine needed for cell wall synthesis. Presumably, for coherent active site modification of pyridoxal phosphate enzymes by β-chloroalanine, derives from the work of Mosino and Okamoto (28) on aspartate transaminase and Meister's group on β-aspartate dehydroxylase (29). In each case the enzyme carries out β elimination to the enamine which is then attacked by either an active site lysine (28) or an active site glutamate (29).

We have confirmed the fact that β-chloro-D-alanine will inactivate membrane-bound alanine racemase 100% after 3 hours at ice bath temperatures. However, the L isomer which one might expect to be recognized equally well by the racemase, caused only 50% inactivation after the identical ice bath incubation. This inequity of D and L isomers of β-chloroalanine carries over into absolute differentials in transport uncoupling (Fig. 9) where only the D isomer is deleterious.

An alternate candidate for racemase inactivator arises from the observation that β-chloro-D-alanine is oxidized by the membranous D-alanine dehydrogenase. The expected oxidation product, β-chloropyruvate, has an α-haloketone moiety and as such, the requisite chemical reactivity for alkylation of membrane proteins, including the racemase. Such haloketones have served as Trojan horse reagents with serine proteases and other enzymes (27) and bromopyruvate itself is a classical protein alkylation reagent (35). Thus the bacteriocidal effect of β-chloro-D-alanine observed by Manning et al. (26) could be due primarily to its oxidation by the α-specific dehydrogenase. As noted above, experiments in progress, with authentic β-chloropyruvate may allow distinction of these possibilities. The uncoupling effect produced during membrane vesicle oxidation of β-chloro-D-alanine is not limited to D-alanine-driven transport. As will be detailed elsewhere, D-lactate stimulated uptake of several solutes is also abolished. The failure of β-chloro-L-alanine to uncouple transport would be attributed to the lack of chloropyruvate produced. This should occur only if β-chloro-L-alanine is not racemized by the alanine racemase to β-chloro-D-alanine, but rather, as noted above, converted to pyruvate directly by the racemase in a β-elimination process.

Finally, it is noted that these initial experiments with β-chloro-D-alanine complement our previous studies of specific inactivators of bacterial transport processes generated by membrane-bound enzymes. The acetylendic suicide substrate 2-hydroxy-3-butyronate irreversibly inactivates the membrane flavin-linked lactate dehy-
drogenases, specifically shutting off d-lactate-stimulated solute active transport in membrane vesicles (13). d-Alanine dehydrogenase is unaffected by this molecule. Vinlyglycolate, a nonactivating substrate for these same lactate dehydrogenases (and alanine dehydrogenase) specifically and irreversibly blocks hexose active transport driven by the P-enolpyruvate-dependent phosphotransferase system (23, 24). Now, β-chloro-n-alanine is a third enzyme substrate whose oxidation generates intravesicularly a product molecule which can uncouple substrate oxidation from active transport.

REFERENCES

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