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THE EFFECTS OF *N*-ETHYLMALEIMIDE ON ACTIVE AMINO ACID TRANSPORT IN *ESCHERICHIA COLI*

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Summary

N-Ethylmaleimide (MalNEt) binds covalently and without specificity to accessible sulfhydryl residues in proteins. In some cases specificity has been imposed on this reaction by manipulating reaction conditions, yielding information concerning both enzyme mechanism and the identity of specific proteins (for example C.F. Fox and E.P. Kennedy (1965) Proc. Natl. Acad. Sci. U.S. 54, 891—899) and R.E. McCarty and J. Fagan (1973) Biochemistry 12, 1503—1507). We have examined the effects of MalNEt on the active accumulation of nine amino acids by *Escherichia coli* strains ML 308-225 and DL 54. Whole cells have been used in order that transport systems both dependent on and independent of periplasmic binding proteins could be studied under various conditions of energy supply for transport. Our results suggest that the systems transporting ornithine, phenylalanine and proline are those most likely to undergo inactivation by direct reaction of MalNEt with the transport apparatus, rather than merely via side effects such as interruption of their energy supply. The inhibition of proline transport is specifically enhanced by the presence of proline, competitive inhibitors of proline transport, or carbonylcyanide *p*-trifluoromethoxyphenylhydrazone during MalNEt treatment. The other eight systems tested showed no analogous effects.

Introduction

The sulfhydryl reagent *N*-ethylmaleimide (MalNEt) has frequently been used to study active transport in *Escherichia coli*. Capable of penetrating the plasma

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Abbreviations: MalNEt, *N*-ethylmaleimide; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

membrane, MalNEt reacts covalently with free sulfhydryl residues and, less readily, with other amino acid functional groups [1,2]. In whole cells MalNEt has been shown to inhibit sugar phosphotransferase systems [3—5] and various systems for the active transport of sugars [6—10], amino acids [11,12] and dicarboxylic acids [13], but not the glycerophosphate acyltransferase system [4]. In two cases direct reaction of MalNEt with proteins catalyzing active transport has been demonstrated and used to identify them for purification [6,13].

As a protein group-specific reagent MalNEt can inhibit a number of metabolic pathways, including respiration and glycolysis, when applied to whole cells [1,14]. The enzymes inhibited by MalNEt treatment of *E. coli* in vivo have not been fully enumerated, but the hydrolytic activity of the Mg^{2+}-ATPase is known not to be inhibited [15]. While the effect of MalNEt on oxidative phosphorylation in *E. coli* has not been determined, ATP synthesis via the corresponding enzyme in chloroplasts (CF 1) is inhibited by MalNEt under certain conditions [16,17].

The purpose of this investigation was to identify those amino acid transport systems suitable for further study using MalNEt. Whole cells were used in order that both osmotic shock-sensitive and -resistant transport systems could be studied and compared and so that a variety of energy-producing pathways could be used to drive transport. Our basic approach has been to treat cells with MalNEt under a single set of conditions and then to measure their transport activity under various conditions of energy supply. We have also examined the effects of MalNEt treatment in the presence of transport substrate or of energy poisons.

Materials and Methods

Chemicals. ^{3}H- and ^{14}C-labelled amino acids were purchased from New England Nuclear Corp. and prepared for use in transport assays as previously described [18]. MalNEt and 2,4-dinitrophenol were purchased from Sigma. Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a gift from Dr. Efraim Racker. Resazurin was a gift from Dr. Jane Gibson. All other reagents were analytical grade.

Bacterial strains. The parent strain, *E. coli* ML308-225, and the Mg^{2+}-ATPase-deficient mutant derived from it, DL 54 [19], were donated by Dr. R.D. Simoni (Stanford University). All strains were grown in a synthetic phosphate-buffered medium [20] (Medium A) supplemented with 0.5% (v/v) glycerol as previously described [21] with the exception that for cells assayed using succinate as energy source, 1% sodium succinate was used in place of glycerol as carbon source for growth.

Transport assays. Initial rates of aerobic transport were measured as previously described [18].

In order to modify this procedure to allow the measurement of rigorously anaerobic transport, an oxidation-reduction indicator system (2 ng/ml of resazurin plus 0.1 mg/ml of cysteine) was used to monitor oxygen concentration in dummy assays. The reaction mixture containing Medium A, chloramphenicol (80 μg/ml), and energy substrate was simultaneously boiled and

bubbled with N_2 to remove dissolved oxygen. An appropriate volume of reaction mixture was transferred into a small test tube through a serum cap and further bubbled with nitrogen for 5 min. Simultaneously, the cell suspension to be assayed was bubbled with N_2 for 5 min. Cells were added to the mixture in the reaction tube and allowed to incubate for 5 min at 23°C before the reaction was initiated by the addition of radioactively labeled substrate. Samples of 0.2 ml were taken at 15 and 40 s, filtered and counted as usual. All transfers were performed with Hamilton syringes.

Measurement of ATP levels. ATP was extracted and measured as described previously [18].

Oxygen uptake measurements. Oxygen uptake measurements were made with a Gilson oxygraph using a sample volume of 1.2 ml.

Protein assay. The protein content of cell suspensions was determined by a micro-modification of the method of Lowry et al. [22] with bovine serum albumin as a standard.

MalNEt treatments. Reaction flasks containing Medium A, cells (0.5 mg per ml), chloramphenicol (80 μg/ml), glucose (11 mM), and inhibitors or transport substrates when used were preincubated for 5 min at 37°C. MalNEt was added and the reaction was allowed to proceed for 10 min before it was stopped by adding a 5-fold molar excess of β-mercaptoethanol. The cells were centrifuged, washed three times with Medium A and resuspended in 20 ml of Medium A per g wet cell weight. For experiments on the effects of arsenate during the MalNEt treatment, Medium B [21]-washed cells were used and Medium B replaced Medium A in the treatment mix. After treatment, the cells were washed and resuspended in Medium A as above.

For every experiment two assays were performed on control cell preparations. The untreated control was exposed to neither MalNEt nor β-mercaptoethanol but was prepared, incubated and washed with the treated cells. A second, "zero-time control" preparation was made by adding a mixture of both MalNEt and β-mercaptoethanol after the preincubation period, incubating and washing the cells as above. In the experiments reported the transport rates of zero time and untreated controls were within experimental error of each other. The transport rate of the zero time control was taken as an uninhibited rate of transport.

Expression and reliability of transport data. In our hands the standard error for replicate amino acid transport assays using a single batch of cells is less than 5% of the mean activity. However, the transport activity of different, though identically prepared, cell batches varies much more widely as is shown in Table I. We have therefore expressed residual transport activity in MalNEt-treated cells as a fraction of the uninhibited rate, and the data in Fig. 1—4 represent averages of two or more sets of values determined in this manner.

The rates of transport supported by endogenous energy reserves in cells grown and treated as described were always small in comparison with those rates determined in the presence of exogenous energy supplies.

Results

Fig. 1 illustrates the inhibition of the nine transport systems on treatment of the cells for 10 min with MalNEt concentrations from 0 to 1 mM. At a MalNEt

TABLE I

REPRODUCIBILITY OF TRANSPORT ASSAYS

Transport was assayed in strain ML 308-225, using glucose as energy source, as previously described [18]. Transport is reported in nmol/min per mg of cell protein. The number of different cell batches assayed to yield these data is given in parentheses after the mean value in the table.

Amino Acid	Mean rate of transport	Range of transport rates	Standard error of mean
Phenylalanine	4.5 (5)	3.3—6.5	1.3
Proline	6.4 (6)	4.6—11.3	2.5
Glycine	4.6 (5)	1.7—7.5	2.8
Cysteine	60.0 (5)	21.0—105.0	30.0
Serine	14.0 (5)	8.9—17.9	4.0
Glutamine	7.6 (6)	4.8—11.4	2.2
Arginine	7.4 (5)	4.2—12.0	3.0
Histidine	1.9 (5)	0.9—2.6	0.6
Ornithine	7.3 (5)	5.1—10.9	2.9

concentration of 0.5 mM, respiration supported either by glucose or by D-lactate is inhibited 15% in strain ML 308-225 and 30% in strain DL 54. ATP levels are unaltered by 0.5 mM MalNEt treatment of strain ML 308-225, supplied with glucose.

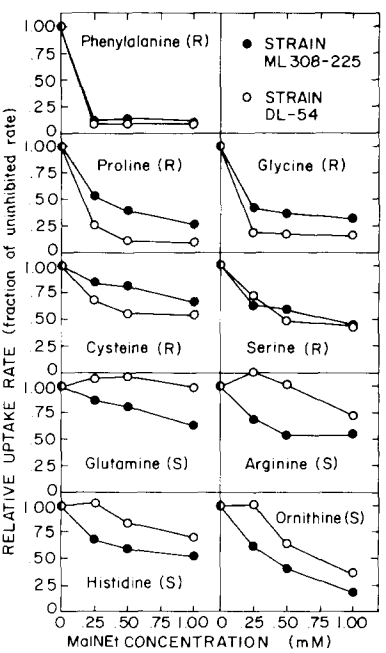

Fig. 1. The inhibition of nine amino acid transport systems in strains ML 308-225 (●———●) and DL 54 (○———○) by treatment for 10 min with varying concentrations of MalNEt. The cells were treated and transport was measured as described under Materials and Methods using glucose as the transport energy source. The graphs represent the averaged values from five experiments in the case of strain ML 308-225 and from two experiments in the case of strain DL 54. R designates osmotic shock-resistant transport, S the osmotic shock-sensitive systems.

Osmotic shock-sensitive transport

Among the four osmotic shock-sensitive transport systems studied the inhibition of glucose supported transport in strain ML 308-225 by 0.5 mM MalNEt varied from 20 to 60% with increasing inhibition in the order glutamine, arginine, histidine, and ornithine. In every case the inhibition was less in strain DL 54 under the same treatment and assay conditions (Fig. 1). By comparing D-lactate supported uptake in strain ML 308-225 with glucose supported uptake in strain DL 54, one can assess the relative abilities of the respiratory and glycolytic pathways to support osmotic shock-sensitive uptake in MalNEt-treated cells. Fig. 2 shows accordingly that transport supported via respiration (ML 308-225, D-lactate) is much more susceptible to MalNEt inhibition than is transport supported via glycolysis (DL 54, glucose). Furthermore, the use of succinate as the respiratory substrate in strain ML 308-225 gives results similar to D-lactate (data not shown) while glycerol, which enters the later stages of the glycolytic pathway as dihydroxyacetone phosphate, is similar to glucose in its ability to support uptake in strain DL 54 (Fig. 2). Thus the variations in MalNEt inhibition of each system generated by using alternate pathways of energy supply are probably due to differential effects of MalNEt on these pathways of energy metabolism.

Attempts were also made to vary the degree of transport inhibition by introducing substrate or inhibitors during MalNet treatment. Cells treated with MalNEt in the presence of glutamine (10 μM), histidine (2 μM), ornithine (10 μM), FCCP (5 μM), or sodium arsenate (0.5 mM) showed no change in the degree of inhibition for transport of any of these substrates over that observed for MalNEt treatment alone.

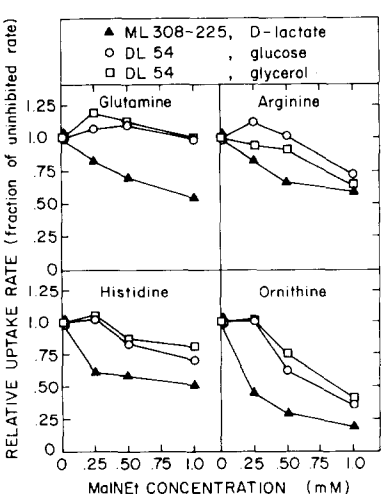

Fig. 2. The variation in osmotic shock-sensitive transport activity supported by three energy generating pathways in cells treated with varying MalNEt concentrations for 10 min. The cells were treated and transport was measured as described under Materials and Methods. The curves represent D-lactate-supported transport in strain ML 308-225 (▲———▲), D-glucose-supported transport in strain DL 54 (○———○), and glycerol-supported transport in strain DL 54 (□———□).

Osmotic shock-resistant transport

As Fig. 1 demonstrates, the osmotic shock-resistant transport systems are inhibited to varying degrees by MalNEt in strain ML 308-225 and this inhibition is either unaltered or increased in strain DL 54. In strain ML 308-225 D-lactate, glucose- (Fig. 3) and succinate- (not shown)-supported uptake show similar levels of inhibition by MalNEt. Glycerol-supported respiration is much more strongly inhibited by MalNEt than is glucose-supported respiration (82 and 41%, respectively in DL 54 at 1 mM MalNEt), probably because both glycerol kinase and the membrane bound glycerol-3-phosphate dehydrogenase contain sensitive sulfhydryl groups. Yet glycerol-supported uptake rates for the osmotic shock-resistant systems in strain DL 54 (data not shown) were not significantly different from those supported by glucose (Fig. 1).

In an attempt to differentiate inhibition of energy metabolism from inhibition of the transport apparatus for these systems we compared the MalNEt effects on transport supported by an electrochemical gradient generated via respiration or via the hydrolysis of glycolytic ATP (Fig. 3). Osmotic shock-resistant uptake in strain ML 308-225 supported aerobically by D-lactate (respiratory pathway) is inhibited by MalNEt to the same degree as uptake supported anaerobically by glucose (glycolytic pathway), although D-lactate supported respiration is only very slightly inhibited (21% at 1 mM MalNEt).

The apparent reduction in sensitivity to MalNEt inhibition shown by anaer-

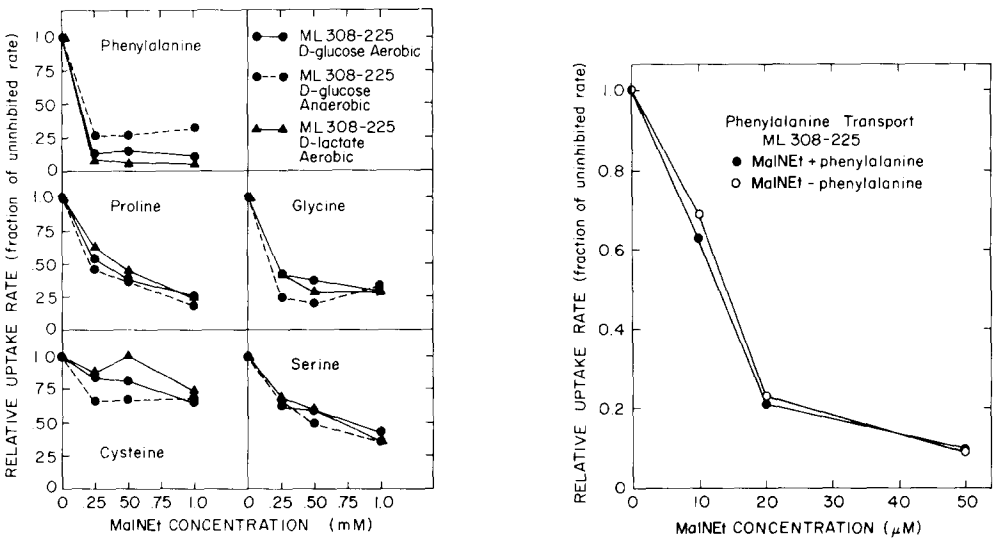

Fig. 3. The variation in osmotic shock-resistant transport activity supported by three energy-generating pathways in cells of strain ML 308-225 treated with varying concentrations of MalNEt for 10 min. The cells were treated and transport was measured as described under Materials and Methods. The curves represent aerobic D-glucose-supported uptake (●———●), anaerobic D-glucose-supported uptake (●- - - - -●), and aerobic D-lactate-supported uptake (▲———▲).

Fig. 4. The inhibition of L-phenylalanine transport by MalNEt. Strain ML 308-225 was treated and transport was measured as described under Materials and Methods using glucose as the transport energy source. The curves represent the relative transport activity of cells treated with MalNEt in the presence (●———●) and absence (○———○) of L-phenylalanine (10 μM).

obic phenylalanine transport in Fig. 3 in fact results from the unusual lability of the phenylalanine system. The uninhibited rates from which these data are derived were 4.5 and 4.7 nmol/min per mg cell protein, respectively, for aerobic glucose and D-lactate supported uptake and only 0.5 nmol/min per mg cell protein for anaerobic uptake supported by glucose. Thus the absolute, MalNEt-inhibited rates of anaerobic phenylalanine transport are similar to those determined aerobically and the difference in relative inhibition is probably not significant. The rate of phenylalanine uptake could be restored to 80% of its original level if cells that had been made anaerobic for 15 min were bubbled with oxygen for 10 min prior to measuring transport.

In addition to its specific inactivation by anaerobiosis, the phenylalanine system was unusually sensitive to MalNEt inhibition (Figs. 1 and 3). Fig. 4 shows that phenylalanine transport is 90% inactivated at 50 μM MalNEt, and that half-maximal inactivation occurs at roughly 15 μM MalNEt, a 10-fold higher sensitivity to MalNEt than was observed for any other system.

Attempts to vary the degree of transport inhibition by introducing transport substrate or inhibitors of energy transduction during MalNEt treatment met with some success. Treatment of strain ML 308-225 with 0.25 mM MalNEt in the presence of phenylalanine (10 μM), proline (10 μM), glycine (10 μM), cysteine (190 μM), serine (10 μM), sodium arsenate (0.5 mM) or FCCP (5 μM) did not alter the extent to which MalNEt inhibited the glucose-supported uptake of phenylalanine, glycine, cysteine or serine. Nor was there any alteration in the inhibition of phenylalanine transport if phenylalanine (10 μM) was added during treatment of the cells with 10, 20 or 50 μM MalNEt (Fig. 4). The inhibition of proline transport was enhanced notably, however, by proline or by FCCP in the inhibition mixture.

TABLE II

THE ENHANCEMENT OF MalNEt INHIBITION BY TRANSPORT SUBSTRATE OR UNCOUPLER

Transport was assayed as previously described [18] in strain DL 54, using glucose as energy source. The numbers shown are relative values calculated with respect to the uptake rate of cells treated for 10 min at 37°C with a mixture of MalNEt, β-mercaptoethanol and the stated addition as described under Materials and Methods. The concentrations of the additions in the treatment mixture were: dinitrophenol, 1 mM; FCCP, 5 μM; amino acids and analogues, 100 μM.

Conditions of MalNEt treatment	Relative transport activity		
	Proline	Glycine	Glutamine
MalNEt (0.25 mM)	0.38	0.52	1.34
MalNEt (0.5 mM)	0.26	0.43	1.25
MalNEt (0.25 mM), FCCP	0.09	n.d.	n.d.
MalNEt (0.5 mM), FCCP	0.04	0.47	1.26
MalNEt (0.25 mM), dinitrophenol	0.32	n.d.	n.d.
MalNEt (0.5 mM), dinitrophenol	0.21	0.27	0.80
MalNEt (0.25 mM), L-proline	0.06	0.48	1.65
MalNEt (0.25 mM), 4-hydroxy-L-proline	0.27	n.d.	n.d.
MalNEt (0.25 mM), L-thiazolidine-4-carboxylic acid	0.05	n.d.	n.d.
MalNEt (0.25 mM), L-azetidine-2-carboxylic acid	0.06	n.d.	n.d.
MalNEt (0.25 mM), L-glutamine	0.40	0.43	1.51
MalNEt (0.25 mM), L-glycine	0.34	0.38	1.44

n.d., not determined.

Table II shows that the enhancement of inhibition is specific for proline transport and that it is also produced by the proline analogues L-thiazolidine-4-carboxylic acid and L-azetidine-2-carboxylic acid which are competitive inhibitors of proline transport [23]. 4-Hydroxy-L-proline, which does not inhibit proline transport in our hands, also does not yield the same strong enhancement of MalNEt inhibition. Unlike FCCP, dinitrophenol yields only a slight enhancement of MalNEt inhibition that is shared by the transport systems for glutamine, proline, and glycine (other systems not tested).

Discussion

The objective of these experiments has been to identify those amino acid transport systems whose protein components are inactivated by reaction with MalNEt. A major aspect of this problem has been to resolve the generalized MalNEt inhibition of energy metabolism or other metabolic activities from its inactivation of those components specifically concerned with energy utilization and amino acid translocation during active transport. This difficulty is intensified by our ignorance of the mechanism and stoichiometries of energy coupling to transport.

The results in Figs. 1, 2 and 3 may be summarized as follows. Among the osmotic shock-sensitive systems the smallest extent of MalNEt inhibition was always seen for transport energized via glycolysis, for example by measuring glucose- or glycerol-supported transport in strain DL 54 (Figs. 1 and 2). Under these conditions glutamine transport was not inhibited, showing a reproducible stimulation of up to 20%. Arginine and histidine transport were slightly inhibited by MalNEt under the same conditions indicating either partial inhibition of the transport process or more stringent energy requirements than those for glutamine transport. Finally, ornithine transport is substantially inhibited at 1 mM MalNEt (58% inhibition), making the ornithine system the best candidate for MalNEt inactivation of a transport component. Although the amino acid composition of the binding protein involved in ornithine transport has not been determined, most periplasmic binding proteins are devoid of sulfhydryl groups. The MalNEt sensitivity of ornithine transport may therefore provide the key to the first biochemical identification of a membrane-bound protein involved in osmotic shock-sensitive transport.

Among the osmotic shock-resistant systems, in contrast with those sensitive to osmotic shock, glucose- (or glycerol-) supported transport was inhibited to the same extent or more strongly in strain DL 54 than in strain ML 308-225 (Fig. 1). The supply of energy via the wide variety of pathways depicted in Figs. 1 and 3 (including D-lactate-supported respiration, which is notably MalNEt resistant) yielded essentially no variation in the inhibition of transport by MalNEt. This observation is consistent either with MalNEt inactivation at the level of the transport apparatus, or with inactivation due to side effects of MalNEt treatment not specifically effecting particular energy-generating pathways.

Phenylalanine transport is uniquely sensitive to MalNEt inactivation (Fig. 4), as well as to inhibition by anaerobiosis. The latter effect may be due to the interaction with the transport proteins of endogenous reducing agents present

at elevated levels in cells subjected to our very stringent conditions of anaerobiosis. In contrast Sprott et al. [24,25] have shown a singular resistance of phenylalanine transport to anaerobiosis in *E. coli* strain ML 308, though perhaps under less strict conditions of anaerobiosis. The small, residual transport activity for phenylalanine, both on MalNEt treatment and during anaerobiosis, may be due to the relatively weak osmotic shock-sensitive transport system for phenylalanine described by Brown [26]. Thus the extreme lability of the phenylalanine transport system makes it the second candidate for MalNEt inactivation at the level of the transport apparatus.

For all nine transport systems attempts were made to vary the effect of MalNEt on transport by introducing transport substrate or energy poison during the MalNEt treatment. The enhanced inhibition of proline transport by MalNEt in the presence of transport substrate or FCCP is analogous to the previously reported effects on D-glucuronate transport [8] and the glucose phosphotransferase system [5]. Each observation may result from the exposure of a critical sulfhydryl group during a protein conformational change as a result of substrate translocation. The results of our investigation support the view that the observed inhibition of proline transport is a specific effect on the transport apparatus and may provide a procedure for the identification and purification of the proline transport protein(s). Recent work (Grunwald, G.B., unpublished) has confirmed that proline transport in cytoplasmic membrane vesicles is very sensitive to MalNEt inhibition (100% inhibition at 100 μM MalNEt), but has shown that proline itself no longer enhances transport inhibition in that system. Perhaps inhibition in this case results from reaction of MalNEt with newly exposed components of the membrane, overwhelming more specific effects on the transport system itself. If so, the previously observed inhibition of transport by sulfhydryl reagents in membrane vesicles [10] may result from inactivation of their energy supply, resulting in both failure to couple metabolic energy to uptake and failure to catalyze efflux [27–31]. Since dinitrophenol does not specifically enhance MalNEt inhibition while FCCP does cause a striking enhancement that is specific for proline transport, further investigation will be required to determine whether the enhancement is due to the uncoupling activity of FCCP or to some other property.

These results provide very limited support for the earlier observation by Berger and Heppel [18] that osmotic shock-sensitive transport systems are less subject to MalNEt inhibition than are osmotic shock-resistant systems. The small general differences may be due to differential inhibition of their two modes of energy supply, and the data do not permit us to draw any more detailed mechanistic distinctions.

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