Biochimica et Biophysica Acta, 1015 (1990) 87–95
Elsevier

BBABIO 43112

Reactive exofacial sulfhydryl-groups on the arginine-ornithine antiporter of *Lactococcus lactis*

Arnold J.M. Driessen and Wil N. Konings

Department of Microbiology, University of Groningen, Haren (The Netherlands)

(Received 22 May 1989)

Key words: Arginine-ornithine antiporter; Cation exchange; Sulfhydryl group; (*L. lactis*)

The effect of various sulfhydryl (SH)-specific reagents on arginine-ornithine antiport activity in membrane vesicles of *Lactococcus lactis* was studied. Little or no inhibition of arginine-ornithine exchange was observed with maleimides and arsenicals. $HgCl_2$ and the organic mercurials *p*-chloromercuribenzene sulfonate (*p*CMBS), *p*-chloromercuribenzoate (*p*CMB), and *O*-(3-hydroxymercuri-2-methoxypropyl)carbamylphenoxyacetate completely inactivated the antiporter. This effect could not be attributed to vesicle disruption. Inactivation of arginine-ornithine exchange by *p*CMBS could be reversed by dithiothreitol. It was reflected by a decreased V_{max} with no change in the K_t for arginine uptake, and correlated with a reduction of the number of arginine binding sites. The poorly penetrating *p*CMBS had at low concentrations only access to the reactive SH-group(s) from the outer surface of the membrane, while the permeant *p*CMB reacts with SH-group(s) at both membrane surfaces. Arginine and ornithine, if present on the outer surface of the membrane, protected the arginine-ornithine antiporter against *p*CMBS inactivation. Membrane-impermeable oxidizing agents have no effect on the exchange activity, indicating that the antiporter is not regulated by the redox potential of the environment. It is concluded that the arginine-ornithine antiporter contains one or more reactive exofacial SH-groups which are presumably located in or near the substrate binding site.

Introduction

The arginine-ornithine antiporter is a transport system that is involved in the uptake and excretion of metabolites of the arginine deiminase pathway in a variety of lactic acid bacteria [1–7]. The conversion of arginine into ornithine by the arginine deiminase pathway is coupled to the synthesis of ATP [7–10], and can thereby serve as an additional source of metabolic energy. Arginine uptake mediated by this antiporter is thought to result from a tight coupling with the downhill efflux of ornithine. Observations which are consistent with the operation of this cationic exchanger are: (i) the demonstration of obligatory coupled arginine and ornithine movements across the cytoplasmic membrane which occur independently of the protonmotive force, and (ii) both transportable substrates arginine and ornithine mutually compete for binding and transport [1,6,7]. The exchange beteen arginine and ornithine is electroneutral and not influenced by the electrical potential across the membrane ($\Delta\psi$). Kinetic analysis of homologous and heterologous exchange modes suggests that the antiporter operates according to a Ping-Pong mechanism [6]. This model assumes that the binding site for the substrate is alternately exposed first at one and then at the other surface of the membrane.

There is no information available with respect to functional amino acid residues that are essential for activity. In this report we describe the effect of various sulfhydryl (SH)-specific reagents on the arginine-ornithine exchange and arginine binding capacity of the antiporter in membrane vesicles derived from *Lactococcus lactis*. The results demonstrate that the SH-reagent *p*-chloromercuribenzene sulfonate, and other mercurials inactivate the arginine-ornithine antiporter. This inactivation is completely reversed by dithiothreitol treatment. An unmodified SH-residue is necessary for arginine binding as demonstrated directly by protection and binding experiments. The reactive SH-group is most likely located at the outer surface of the cytoplasmic membrane.

Abbreviations: *p*CMBS, *p*-chloromercuribenzene sulfonate; *p*CMB, *p*-chloromercuribenzoate; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SH, sulfhydryl.

Correspondence: A.J.M. Driessen, Laboratorium voor Microbiologie, Rijksuniversiteit Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands.

Methods

Growth conditions and isolation of membrane vesicles. *Lactococcus lactis* ML_3 was grown to late exponential phase at 30°C in a pH-controlled (pH 6.4) 5-l fermenter on a synthetic medium [2] supplemented with 1% (w/v) galactose and 25 mM L-arginine. Right-side-out membrane vesicles were prepared by osmotic lysis [11]. Inside-out membrane vesicles were obtained by direct lysis of protoplasts by a single passage through a French pressure cell as described [6,12]. Membranes were stored in liquid nitrogen until use.

Incubations with redox and sulfhydryl-specific reagents. The effect of redox and SH-specific reagents was studied by incubation of the membrane vesicles (1.2 mg of protein) at 25°C with various amounts of reagents in 1 ml of 50 mM potasisum phosphate (pH 7.0). After 10 min, the reaction was arrested by a 40-fold dilution into ice-cold 50 mM potassium phosphate (pH 7.0) supplemented with 500 μM ornithine. Membrane vesicles were collected by centrifugation (48 200 × *g* for 30 min, 4°C), and equilibrated with the dilution buffer for at least 2 h at 25°C. Loaded membranes were concentrated (48 200 × *g* for 30 min, 4°C) to a final protein concentration of 20–25 mg/ml. In case of cadmium (Cd^{2+}), potassium phosphate was replaced by 50 mM potassium-Hepes (pH 7.0).

Transport assays. For transport measurements, 2 μl of the concentrated membrane suspension (usually 40–50 μg of protein) was diluted into 200 μl of magnetically stirred 50 mM potassium phosphate (pH 7.0) containing 0.47 μM [^{14}C]arginine, unless indicated otherwise. At times indicated, the reaction was arrested by dilution into 2 ml of 0.1 M LiCl, and filtration over cellulose-nitrate membrane filters (BA 85; 0.45 μm; Schleicher & Schuell, Dassel, F.R.G.). Filters were washed once with 2 ml of 0.1 M LiCl and dried. Radioactivity retained by the membrane filters was corrected for background binding of arginine. Estimates of the initial rate were made after 5 s in triplicate. All transport measurements were performed at 18°C.

For kinetic measurements, external ornithine was removed by washing the loaded membrane vesicles at 4°C with 40 ml of 50 mM potassium phosphate (pH 7.0). The concentrated membrane suspension was chilled on ice to prevent excessive leak of ornithine [6], and used within 1 h.

Protonmotive-force driven uptake of leucine was measured as described [13].

Arginine binding measurements. Binding of arginine to membrane vesicles was determined by centrifugation as described previously [6]. Membane vesicles treated and untreated with *p*CMBS were frozen into liquid nitrogen and subsequently slowly thawed at room temperature. This freeze-thaw cycle was repeated twice in order to obtain leaky membrane structures which allow a rapid attainment of the arginine binding equilibrium. Membranes were washed and concentrated (48 000 × *g* for 30 min, 4°C). Binding of [^{3}H]arginine was measured in a final volume of 0.1 ml containing 5 mg of membrane vesicle protein suspended into 50 mM potassium phosphate (pH 7.0). The [^{3}H]arginine (37 kBq) concentration was 50 nM. For the estimation of the apparent dissociation constant (K_d) and maximal binding level (B_{max}), the [^{3}H]arginine concentration was varied between 20 nM and 50 μM. The suspensions were equilibrated for 20 min at 18°C, and subsequently centrifuged for 10 min in a Beckmann Airfuge at full speed. Under these conditions, essentially all membrane protein was found in the pellet. Samples of 3 times 20 μl of the supernatant were taken and counted for radioactivity. Membrane runs were performed with [^{3}H]arginine at a final concentration of 5 mM, and the amount of label associated with the membrane vesicles was taken as non-specific binding [6].

Crossed immunoelectrophoresis and localization experiments. The sidedness of the membrane vesicles was examined by localization experiments of the catalytic site of the BF_0F_1-ATPase using the immunoabsorption technique [14]. Immunoglobulins were used that were raised against membrane vesicles of *L. lactis* subsp. *cremoris* Wg_2 and showed a large degree of cross-specificity with membranes of *L. lactis* ML_3. Membrane vesicles (5 mg of protein/ml) suspended in 25 mM barbital · HCl (pH 8.0) were diluted with an equal volume of 5% (v/v) Triton X-100 or buffer. The suspensions were incubated for 1 h at 25°C with constant agitation. For immunoabsorption experiments, immunoglobulins (about 8 mg protein) and intact or solubilized membrane vesicles (0–0.6 mg protein) were mixed in 25 mM barbital · HCl (pH 8.0) in a final volume of 0.5 ml. The suspensions were incubated for 1 h at 25°C, and immunoprecipitates and membrane fragments were removed by centrifugation (48 200 × *g* for 30 min, 4°C). Crossed immunoelectrophoresis was performed as described [15] with 400 μl of the supernatant in the second dimension gel. In the first dimension 30 μg of 2.5% (v/v) Triton X-100 solubilized membrane protein was applied to the wells. The gels were run at 3.5 V/cm for 90 min in the first dimension and at 1.5 V/cm in the second dimension. After electrophoresis, immunoprecipitates were stained for ATPase activity [16]. The BF_0F_1-ATPase complex forms a characteristic immunoprecipitate in the crossed immunoelectrophoresis gels. Therefore, Coomassie Brilliant Blue staining [15] was used in most experiments to identify the BF_0F_1-ATPase complex. The reciprocal area of the immunoprecipitate was plotted against the amount of membrane vesicles protein used in the immunoabsorption experiment, and the accessibility of the BF_0F_1-ATPase to the antibody from the outer surface of the membrane was estimated from the relative slopes of

these plots recorded in the presence and absence of Triton X-100.

Other analytical procedures. The generation of a pH gradient (ΔpH, interior acid) upon addition of ATP was followed by the fluorescence quenching of 9-aminoacridine [17]. The reaction mixture contained (final concentration): 50 mM potassium phosphate (pH 7.0), 2 mM $MgSO_4$, 100 nM valinomycin, 5 μM 9-aminoacridine and membrane vesicles (0.2 mg/ml). The reaction was started by the addition of 5 mM ATP (Mg^{2+} salt). Fluorescence of 9-aminoacridine was measured at excitation and emission wavelengths of 405 and 455 nm, respectively. Since the addition of ATP causes a decrease in the level of 9-aminoacridine fluorescence, a qualitative estimate of the pH gradient was made upon the addition of 20 mM NH_4Cl. A collapse of the pH gradient corresponded to an increase of the level of 9-aminoacridine fluorescence.

Protein was determined by the Lowry method [18] in the presence of 0.2% (v/v) SDS [19] using bovine serum albumin as standard.

Materials

L-[U-^{14}C]Arginine (11 TBq/mol), L-[2,3,4,5-^{3}H]-arginine (2.4 TBq/mol), and L-[U-^{14}C]leucine (12.4 TBq/mol) were obtained from Amersham Corp. (Buckinghamshire, U.K.) L-[2,3-^{3}H]Ornithine (1.1 TBq/mmol) was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). *N*-[1-Pyrene]maleimide was from Fluka AG (Buchs, F.R.G.). Eosine-5-maleimide, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid and didansyl-L-cystine were obtained form Molecular Probes, Inc. (Eugene, OR, U.S.A.). Phenylarsineoxide, 2-(3,6-disulfo-2-hydroxy-1-naphthylazo)benzene arsonic acid (thorin), *N*-[1-naphthyl]maleimide, NEM, *p*-chloromercuribenzene sulfonate (*p*CMBS), *p*-chloromercuribenzoate (*p*CMB), *O*-(3-hydroxymercuri-2-methoxypropyl)carbamylphenoxyacetate (mersalyl), oxidized (GSSG) and reduced (GSH) forms of glutathione, 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin), and dithiothreitol (DTT) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Solutions of SH-specific and redox reagents were prepared just before use in buffer solutions, ethanol or dimethylformamide. Final solvent concentations did never exceed 1% (v/v), and appropriate solvent controls were included in the experiments. All other chemicals were of reagent grade and acquired from commercial sources.

Results

Effect of sulfhydryl-group reagents on the arginine-ornithine antiporter

The effect of various SH-modifying reagents on the activity of the arginine-ornithine antiporter was examined in right-side-out membrane vesicles of *Lactococcus lactis*. Membrane vesicles were incubated with these compounds at different concentrations and the residual arginine-ornithine exchange activity was estimated from

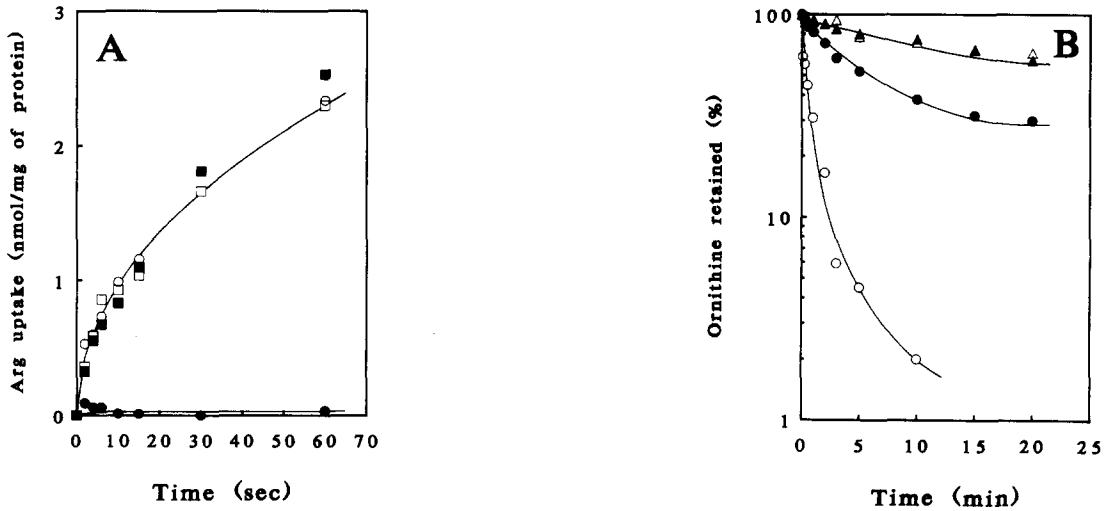

Fig. 1. Effect of *p*CMBS and DTT on the uptake of arginine (A) and efflux of ornithine (B). Membrane vesicles (1.2 mg of protein/ml) suspended in 50 mM potassium phosphate (pH 7.0) were incubated in the absence (open symbols) or presence (closed symbols) of 100 μM *p*CMBS for 10 min at 25 °C. Samples of treated and untreated membrane vesicle were diluted 40-fold into an ice-cold solution containing 50 mM potassium phosphate (pH 7.0) and 500 μM ornithine. Subsequently, membrane vesicles were washed free of reagent in the presence or absence of 5 mM DTT, and equilibrated with 50 mM potassium phosphate (pH 7.0) containing 500 μM L-[2,3-^{3}H]ornithine. Arginine-ornithine antiport activity (A, by arginine uptake; B, by ornithine release) was measured by diluting [^{3}H]ornithine-loaded membrane vesicles 100-fold into 50 mM potassium phosphate (pH 7.0) containing 25 μM L-[^{14}C]arginine. (A) Uptake of [^{14}C]arginine by untreated (○); DTT-washed (□); *p*CMBS-treated (●); and *p*CMBS-treated, DTT-washed (■) membrane vesicles. (B) Release of [^{3}H]ornithine by untreated (○); and *p*CMBS-treated (●) membrane vesicles. Also shown is the release of ornithine by untreated (△); and *p*CMBS-treated (▲) membrane vesicles when diluted into 50 mM potassium phosphate (pH 7.0) in the absence of external arginine.

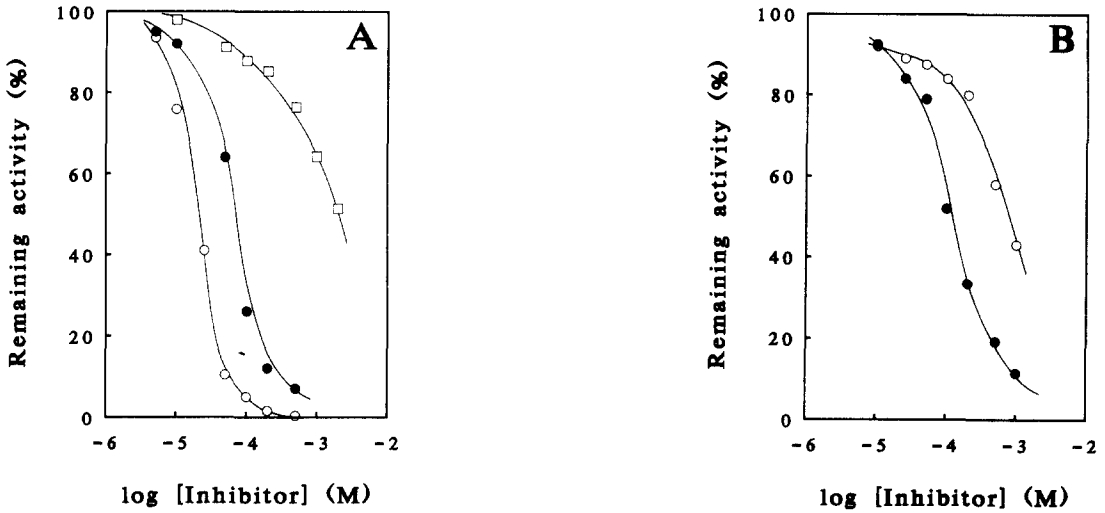

Fig. 2. *p*CMBS, *p*CMB and NEM inactivation of arginine-ornithine exchange in right-side-out (A) and inside-out membrane vesicles (B). Membrane vesicles (1.0–1.2 mg of protein/ml) were incubated for 10 min at 25 °C in 50 mM potassium phosphate (pH 7.0) containing increasing concentrations of *p*CMBS (○), *p*CMB (●) and NEM (□), washed free of reagent, and equilibrated in 50 mM potassium phosphate (pH 7.0) containing 500 μM ornithine. Arginine uptake by ornithine-loaded membrane vesicles was assayed as described under Materials and Methods, and plotted as percentage of the control activity. The control value for arginine uptake by right-side-out and inside-out membrane vesicles were 48 and 57 pmol/mg of protein per s, respectively.

the rate of arginine uptake by ornithine-loaded membrane vesicles. Arginine transport and ornithine efflux was completely blocked by the mercurials *p*-chloromercuribenzene sulfonate (*p*CMBS) (Fig. 1A and 2A), *p*-chloromercuribenzoic acid (*p*CMB) (Fig. 2A), *O*-(3-hydroxymercuri-2-methoxypropyl)carbamylphenoxyacetate (mersalyl), and $HgCl_2$. IC_{50} values for the inactivation of arginine transport are shown in Table I. At pH 8.0, lipophilic thiol-specific alkylating reagents like *N*-ethylmaleimide (NEM) (Fig. 1A), *N*-[1-naphthyl]maleimide and *N*-[1-pyrene]maleimide displayed a low reactivity, while the membrane impermeable eosine-5-maleimide and 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid did not react at all at the indicated concentrations. The inactivating effect of *p*CMBS (Fig. 1), *p*CMB and mersalyl (not shown) was completely reversed by dithiothreitol (DTT) and β-mercaptoethanol, while these compounds alone were without effect. *p*CMBS (up to 500 μM) had no effect whatsoever on the rate of protonmotive-force driven leucine uptake (not shown). These results indicate that inactivation of the arginine-ornithine antiporter is due to a specific reaction with SH-groups, and not due to the disruption of the membrane vesicles.

TABLE I

Half-maximal inhibition ocnstants for sulfhydryl-modifying reagents

IC_{50} values for the inactivation of arginine-ornithine exchange by various SH-modifying reagents were determined as described in the legend to Fig. 2.

Reagent	IC_{50} (nmol per mg of protein)	IC_{50} (μM)
N-Ethylmaleimide	1300 [a]	1560
N-[1-Naphthyl]maleimide	290 [a]	350
N-[1-Pyrene]maleimide	350 [1]	420
Eosine maleimide	200 [a,b]	–
4-Acetamido-4′-maleimidyl-stilbene-2,2′-disulfonic acid	500 [a,b]	–
$HGCl_2$	9	11
*p*CMB	29	35
*p*CMBS	14	17
Mersalyl	22	26
Iodoacetic acid	5000 [b]	–
Phenylsarsene oxide	350 [b]	–
Cd^{2+}	1000 [b]	–
Thorin	1500 [b]	–

[a] pH 8.0.
[b] Less than 10% inhibition at the indicated amount of reagent.

Evidence for the presence of vicinal thiol groups in a protein can be obtained with the dithiol-specific reagent 2-(3,6-disulfo-2-hydroxy-1-naphthylazo)benzene arsonic acid (thorin) or the lipophilic phenylarsine oxide [20,21]. These compounds may cause the formation of an intramolecular disulfide bridge by the generation of a cyclic dithiol arsenite. The stability of this adduct depends on the spatial location of the thiols. High concentrations of either of these compounds failed to inhibit arginine-ornithine exchange activity (Table I). Cd^{2+}, a metal ion which is known to bind strongly to dithiols [22] had no effect at a concentration of 2 mM.

Localization of the reactive sulfhydryl-group

The sidedness of the interaction of mercurials with the reactive SH-group(s) was investigated by comparing the effects of the permeant *p*CMB and poorly permeant *p*CMBS on arginine-ornithine exchange in right-side-out

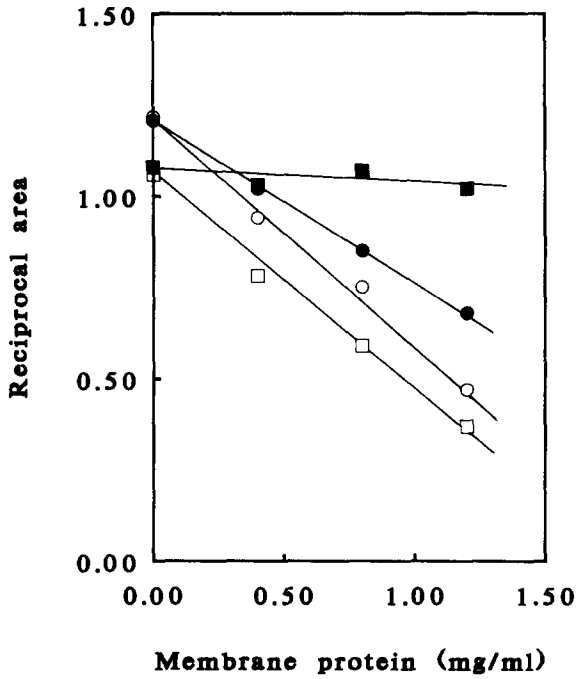

Fig. 3. Accessibility of the catalytic site of the BF_0F_1-ATPase in right-side-out and inside-out membrane vesicles to immunoglobulins in the absence and presence of Triton X-100. The peak areas in cm^2) of immunoprecipitates of BF_0F_1-ATPase activity after immunoabsorption of immunoglobulins with various concentrations of membrane vesicle protein were determined as described under Materials and Methods. Absorption experiments were performed with intact (closed symbols) and Triton X-100 solubilized (open symbols) membrane vesicles obtained by osmotic lysis (■,□) and french press treatment (●,○).

and inside-out membrane vesicles. Inside-out membrane vesicles were obtained after a single passage of protoplasts through a French press. Immunoabsorption experiments performed in the presence and absence of the detergent Triton X-100 (Fig. 3) indicate that a large fraction of these membrane vesicles is indeed inside-out with respect to the accessbility of the catalytic site of the BF_0F_1-ATPase. About 77% of immunoprecipitable F_1 was exposed to immunoglobulins from the outside. Membrane vesicles obtained by osmotic lysis are mostly right-side out. Only 12% of the F_1 was accessible from the external surface (Fig. 3). In contrast to right-side-out membrane vesicles, these inside-out membrane vesicles generated a pH gradient, inside acid, when supplied with ATP (not shown). *p*CMBS is a poor inhibitor of arginine-ornithine exchange in inside-out membrane vesicles (Fig. 2B) compared to right-side-out membrane vesicles (Fig. 2A). IC_{50} values for inactivation of the antiporter by *p*CMBS in inside-out and right-side-out membrane vesicles were 280 and 14 nmol per mg protein, respectively. The permeant *p*CMB showed similar reactivities with inside-out and right-side-out membrane vesicles, with IC_{50} values of 55 and 29 nmol per mg protein, respectively. These results demonstrate that *p*CMB is much more reactive in inside-out membrane vesicles than *p*CMBS (Fig. 2B), whereas little difference in reactivity of these organomercurials is observed with right-side-out membrane vesicles (Fig. 2A). We conclude that the reactive SH-group(s) is exposed to the external surface of the membrane.

Effect of pCMBS on the kinetics of exchange and the binding of arginine

The effects of *p*CMBS on the kinetic parameters of arginine-ornithine exhcange by right-side-out membrane vesicles are shown in Table II. When pretreated, *p*CMBS caused a reduction in the maximal velocity (V_{max}) without a major change in the affinity constant (K_t) of arginine uptake. Arginine binding can be readily measured by a centrifugation assay [6]. In order to assess the effects of *p*CMBS on the binding of arginine to the antiporter, membrane vesicles treated with *p*CMBS were subjected to three freeze-thaw cycles in order to increase the accessibility of the substrate binding sites. A control sample exhibited the binding (B_{max}) of about 70 pmol arginine/mg of protein (Fig. 4) with a dissociation constant (K_d) of 10.5 μM. Treatment of the membrane vesicles with increasing concentrations of *p*CMBS caused a proportional decrease of the binding of arginine measured at a concentration of 50 nM. The reduction in the number of binding sites correlated with the decrease in arginine-ornithine exchange activity (Fig. 4). Assuming a one-to-one stoichiometry of arginine binding to the antiporter [6], the data in Fig. 4 suggest that the turnover number of the antiporter (V_{max}/B_{max} ratio) will be approx. 4–5 s^{-1}. This value is in agreement with estimates of the turnover number reported previously [6]. These results indicate that inhibition of arginine-ornithine exchange activity by *p*CMBS is the result of a reduction of the number of active carriers.

TABLE II

Effect of pCMBS on the kinetic parameters of arginine-ornithine exchange

Membrane vesicles were treated with various amounts of *p*CMBS as described in the legend to Fig. 2. Kinetic parameters of arginine-ornithine exchange were determined by diluting membrane vesicles loaded with 200 μM ornithine 50-fold into 50 mM potassium phosphate (pH 7.0) containing 0.47 to 10.2 μM [^{14}C]arginine. Rates of arginine uptake were estimated from the amount of label accumulated within 5 s. Kinetic data was analyzed by Eady-Hofstee plots.

Amount of *p*CMBS (nmol per mg of protein)	K_t (μM)	V_{max} (pmol per mg (of protein per s)
0	6.2	297
16	5.9	159
32	6.3	88
64	6.9	23

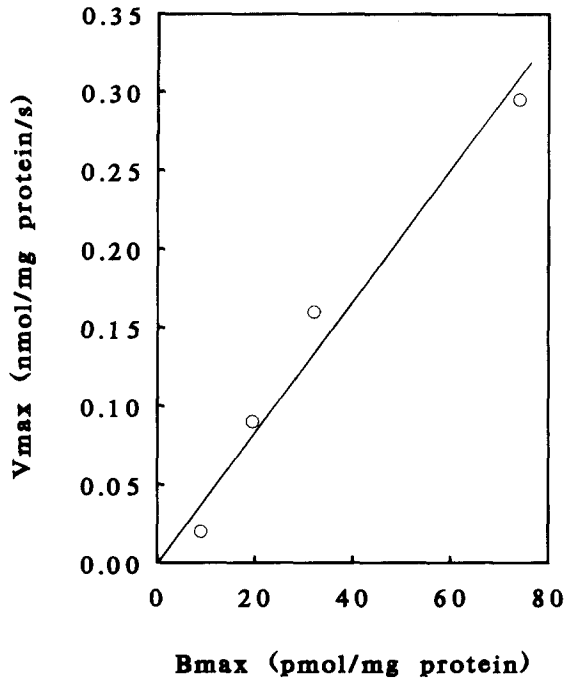

Fig. 4. Correlation between the activity of the arginine-ornithine antiporter and the number of arginine binding sites titrated with *p*CMBS. Membrane vesicles were treated with 0, 16, 32, and 64 nmol *p*CMBS/mg of protein, and the binding of L-[2,3,4,5-^{3}H]arginine was measured as described under Materials and methods. The initial free arginine concentration was 50 nM. The binding capacity (B_{max}) was calculated using the experimentally determined K_d for arginine binding of 10.5 μM. It is assumed that the K_d is not affected by *p*CMBS. B_{max} is plotted as a function of the maximal arginine uptake rate observed at each *p*CMBS concentration. V_{max} data shown was obtained from Table II.

External and not internal arginine protects the arginine-ornithine antiporter against pCMBS inactivation

The effect of the substrates of the antiporter on the process of *p*CMBS inactivation was investigated. The presence of 1 mM arginine (Table III) at the time of *p*CMBS treatment largely prevented inactivation of the antiporter. The efficiency of protection decreased in the following order: L-arginine > D-arginine > L-ornithine ≫ L-lysine (not shown). This parallels the specificity of transport [6,7]. No protection was observed with L-leucine or L-glutamic acid. Protection is not the result of complex formation between *p*CMBS and these amino acids. When mixed in solution no change in absorbance at 250 nm was observed which would otherwise indicate the formation of a mercaptide [23].

The sidedness of the protective effect of arginine against *p*CMBS inactivation was examined in more detail. The effect of *p*CMBS on the activity of the antiporter was studied under three conditions; (i) with arginine only present in the external medium; (ii) with arginine only present in the intravesicular space; and (iii) with arginine present on both sides of the membrane. Only when the antiporter is saturated with arginine at the external surface of the membrane, a significant level of protection of the SH-group is observed (Table III). A low level of protection is found when arginine is only present at the internal surface of the membrane. However, as a result of the loading and dilution procedure, a low concentration of arginine (about 20 μM) was also present on the external surface of the membrane. This amount of arginine almost fully accounts for the observed level of protection with arginine only present in the intravesicular space. These results suggest that the antiporter is only susceptible to *p*CMBS inactivation when the unloaded substrate binding site faces the external surface of the membrane. The reactive SH-group is presumably located in or near the substrate binding site of the arginine-ornithine antiporter.

Effect of redox-reagents on the arginine-ornithine antiporter

The activity of several transport systems is believed to be controlled by the redox state of dithiols in these carriers [24,25]. To test if the reactive SH-group(s) of the arginine-ornithine antiporter responds to changes in the redox potential of the solution, the effect of oxidizing and reducing reagents was investigated. Pretreatment of the membrane vesicles with the membrane-impermeable oxidant ferricyanide ($Fe(CN)_6^{3-}$) (5 mM) led to a reduction of the exchange activity by about 30% (not shown). Inhibition appeared to be rather unspecific, since a similar inhibition was exerted by the reductant ferrocyanide ($Fe(CN)_4^{2-}$), while the inhibition by $Fe(CN)_6^{3-}$ was not relieved by subsequent addition of excess DTT (10 mM). The disulfide L-cystine (5 mM), its fluorescent derivative didansyl-L-cystine (2 mM), and the oxidized form of glutathione (GSSG),

TABLE III

Substrate protection of the arginine-ornithine antiporter against pCMBS inactivation

Membrane vesicles (10 mg protein/ml) incubated with or without 1 mM arginine were diluted 50-fold into a solution containing 50 mM potassium phosphate (pH 7.0), 50 μM *p*CMBS and arginine at the indicated concentration. After 10 min, membranes were diluted 10-fold into 50 mM potassium phosphate (pH 7.0), supplemented with 500 μM ornithine, washed once and equilibrated with the same buffer. The 5 s uptake of arginine was assayed as described under Materials and Methods at a final concentration of 0.47 μM. Each result is the mean of triplicate estimated with indicated ± S.E.

Arginine (mM)		*p*CMBS	Rate of arginine uptake (pmol per mg of protein per s)	Protection (%)
inside	outside			
0	0	−	58 ± 7	−
0	0	+	5 ± 2	−
1	1	+	53 ± 5	91
0	1	+	42 ± 8	70
1	0.02	+	25 ± 3	38
0	0.02	+	18 ± 3	25

which react with thiols to form mixed disulfides, had no effect when tested up to a concentration of 5 mM (not shown). Similar results were obtained with reducing reagents such as DTT, β-mercaptoethanol, L-cysteine and the reduced form of glutathione (GSH). Unlike the membrane-impermeable oxidants, the lipophilic 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin) is a very efficient inhibitor of the antiporter with an IC_{50} value of about 120 nmol/mg of protein (approx. 150 μm). Inhibition was reversed by the addition of an excess DTT. In the presence of plumbagin, a marked decrease in the rate of protonmotive-force driven leucine uptake was observed. However, qualitative measurements of the magnitude of the imposed ψ, using the lipophilic cation tetraphenylphosphonium, indicated that plumbagin (when used at a concentration of 200 μM) has no pronounced effect on the ψ. Since the arginine-ornithine antiporter is not susceptible to membrane-impermeable oxidizing reagents, it is concluded that the reactive SH-group(s) is not a primary target for redox control.

Discussion

The results presented in this paper demonstrate that sulfhydryl (SH)-specific reagents, in particular organomercurials, are effective inhibitors of arginine-ornithine exchange. The difference in the ability of *p*CMBS and *p*CMB to block arginine uptake by right-side-out and inside-out membrane vesicles suggests that the reactive SH-group of the antiporter is exposed to the external surface of the cytoplasmic membrane. The finding that *p*CMBS blocks the binding of arginine to the antiporter, while the presence of arginine on the external surface of the membrane alters the reactivity of the SH-group towards *p*CMBS, suggests that the reactive SH-group is present near but not necessarily in the substrate binding site. It should be realized that inhibition may be caused by a substantial change in the conformation of the carrier resulting from the reaction of the external SH-group(s). This may arise from steric hindrance by the mercurial group of substrate binding or translocation-related conformational changes. Moreover, the reaction of *p*CMBS with the antiporter may involve one or more essential SH-groups.

Care should be taken with respect to the hypothesis that the reactive SH-group(s) plays a dynamic role in the energetics of translocation. Based on the substrate protection against NEM inactivation, an active cysteine was postulated to be involved in substrate or cation binding in the lactose [26] and proline [27] carrier of *Escherichia coli*. Replacement of the critical cysteine by oligonucleotide-directed site-specific mutagenesis by a serine or other amino acid residues, conferred both transport systems with a resistance against NEM inactivation without an appreciable effect on the activity [28–30]. It was therefore concluded that the NEM-reactive cysteine residues in the lactose and proline carrier are not important for dynamic events during a translocation cycle, but are located in close proximity to the substrate binding site. Our results do not indicate a definite role of the reactive SH-group in either binding of the substrate or the energetics of translocation.

Evidence has been presented that the activities of the lactose and proline carriers in *E. coli* are controlled by the redox potential of the bulk solution [24,25]. This appears not to be the case with the arginine-ornithine antiporter. No significant effect on the activity was observed when the redox state of the antiporter was changed with strong oxidizing ($Fe(CN)_6^{3-}$) and reducing (DTT) reagents. It furthermore appears that the antiporter is rather insensitive to the oxidation by molecular oxygen.

The lipophilic quinone plumbagin was found to be a strong inhibitor. Plumbagin either oxidizes a thiol group buried in a more hydrophobic part of the antiporter, or reacts with the organomercurial reactive SH-group(s) by adduct formation [31].

An interesting observation is that the antiporter is relative insensitive to NEM, while organomercurials readily inhibit exchange activity at low concentrations. A similar phenomena has been noted with the mitochondrial carriers for dicarboxylates, 2-oxoglutarate, and neutral amino acids [32]. The 2-oxoglutarate carrier is, however, strongly inhibited by the fluorescent membrane-impermeable eosine-5-maleimide [33], suggesting that eosine maleimide and NEM react with different SH-groups. Site-specific differences between these two maleimides were also found with the mitochondrial ATP/ADP carrier [34,35]. With respect to the reactivity of cysteine residues towards organomercurials and maleimides, it should be realized that these reagents not necessarily react with the same SH-groups [21,23]. It has been suggested that the reactivity of cysteine residues towards NEM strongly depends on the polarity of the environment. For instance, only one out of six cysteine residues of the mitochondrial phosphate carrier is reactive towards NEM when the carirer is in its native conformation [36]. The high reactivity of this residue, i.e., Cys^{42} has been attributed to electrostatic effects of two neighboring basic groups, Lys^{41} and Arg^{43} [37]. These basic groups would facilitate the dissociation of the sulfhydryl-proton, and therefore increase the reactivity of the cysteine with NEM, which involves the addition of the mercaptide ion to the olefinic double bond of the maleimide [23,38]. The other cysteine residues exist as disulfides or are sterically inaccessible to NEM.

Numerous examples can be found in literature in which a transport system is identified on SDS-polyacrylamide gels by the use of a differential labeling method using radioactive NEM [27,39–41]. Membranes are incubated with non-radioactive NEM in the pres-

ence of a protective substrate. After removal of these compounds the deprotected carrier is allowed to react with only enough radioactive NEM to label specifically the protein. A similar strategy can be followed with the arginine-ornithine antiporter which can be protected by arginine against inactivation by *p*CMBS. However, in contrast to NEM, mercurials are not suitable for this procedure, since they tend to dissociate and associate randomly with SH-groups in the sample during electrophoresis [42]. On the other hand, an advantage of the inhibitory effect of *p*CMBS is its potent applicability in rapid sampling techniques, using inhibitor (*p*CMBS)-stop experiments [6]. The inhibitor must be efficient, rapid and block the transport system without unwanted side-effects. In this respect, some, but not all, organomercurials cause an uncoupling of the inwardly and outwardly directed solute flux mediated by the mitochondrial aspartate/glutamate carrier [43]. These compounds cause a somewhat unspecific flux through the carrier, that could be reversed by dithiothreitol. This weakly specific 'leak' induced by organomercurials was not observed with the arginine-ornithine antiporter (see Fig. 1B).

The interaction with organomercurials can be advantageous in the purification of the carrier. Membranes solubilized with detergent can be passed through an organomercurial agarose column and the carrier can be eluted with DTT (see also Ref. 38). The arginine-ornithine antiporter is readily solubilized with *n*-octyl-β-D-glucopyranoside from a protein aggregate that is obtained after cholate extraction of membrane vesicles (Tolner, B. and Driessen, A.J.M., unpublished results). When this extract is passed through an organomercurial agarose column, the arginine-ornithine antiporter and other proteins stay behind. The antiporter can be eluted by low concentrations of DTT, and resonstituted in an active form into proteoliposomes with a specific exchange activity nearly 20-fold enriched in comparison to the membrane vesicles. Only a few protein bands remain on a SDS polyacrylamide gel with molecular masses of 55–64 kDa. A major obstacle we encountered with the use of organomercurial agarose columns is the great difference in binding capacity between various lots of this product. This results in unwanted variations in the degree of purification.

Currently, efforts are made to identify and clone the gene coding for the arginine-ornithine antiporter. Once this goal has been realized, cysteine residues are likely candidates for oligonucleotide-directed site-specific mutagenesis experiments. This should allow a further definition of the functional role of these groups. In conclusion, our findings suggest that the arginine-ornithine antiporter of *Lactococcus lactis* contains one or more reactive SH-groups on the external surface of the cytoplasmic membrane which are located in or near the substrate binding site.

References

1 Driessen, A.J.M., Poolman, B. and Konings, W.N. (1987) Proc. Natl. Acad. Sci. USA 84, 6093–6097.
2 Poolman, B., Driessen, A.J.M. and Konings, W.N. (1987) J. Bacteriol. 169, 5597–5604.
3 Thompson, J. (1987) J. Bacteriol. 169, 4247–4253.
4 Driessen, A.J.M., Van Leeuwen, C. and Konings, W.N. (1989) J. Bacteriol. 171, 1453–1458.
5 Driessen, A.J.M., Smid, E.J. and Konings, W.N. (1988) J. Bacteriol. 170, 4522–4537
6 Driessen, A.J.M., Molenaar, D. and Konings, W.N. (1989) J. Biol. Chem. 264, 10361–10370.
7 Driessen, A.J.M. and Konings, W.N. (1989) in The Bacteria. Bacterial Energetics (Krulwich, T.A., ed.), Academic Press, Inc., New York, in press.
8 Crow, V.L. and Thomas, T.D. (1982) J. Bacteriol. 150, 1024–1032.
9 Cunin, R., Glansdorf, N., Pierárd, A. and Stalon, V. (1986) Microbiol. Rev. 50, 314–152.
10 Simon, J.P., Wargnies, B. and Stalon, V. (1982) J. Bacteriol. 150, 1085–1090.
11 Otto, R., Lageveen, R.G. and Konings, W.N. (1982) J. Bacteriol. 149, 733–738.
12 Houng, H.S., Lynn, A.R. and Rosen, B.P. (1986) J. Bacteriol. 169, 1040–1044.
13 Driessen, A.J.M., Kodde, J., De Jong, S. and Konings, W.N. (1987) J. Bacteriol. 169, 2748–2754.
14 Owen, P. and Kaback, H.R. (1978) Proc. Natl. Acad. Sci. USA 75, 3148–3152.
15 Smyth, C.J., Singel, J., Salton, M.J.R. and Owen, P. (1978) J. Bacteriol. 133, 306–319.
16 Owen, P. and Salton, M.R.J. (1975) Proc. Natl. Acad. Sci. USA 72, 3711–3715.
17 Rottenberg, H. (1977) Methods Enzymol. 55, 547–569.
18 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
19 Dulley, J.R. and Grieve, P.A. (1975) Anal. Biochem. 64, 136–140.
20 Stevenson, K.J., Hale, J. and Perhem, R.N. (1978) Biochemistry 17, 2189–2192.
21 Webb, J.L. (1966) Enzyme and Metabolic Inhibitors, Academic Press, New York.
22 Graber, B.P. and Fluharty, A.L. (1968) Q. Rep. Sulfur Chem. 3, 318–335.
23 Riordan, J.F. and Vallee, B.L. (1967) Methods Enzymol. 11, 541–547.
24 Konings, W.N. and Robillard, G.T. (1982) Proc. Natl. Acad. Sci. USA 79, 5480–5484.
25 Poolman, B., Konings, W.N. and Robillard, G.T. (1983) Eur. J. Biochem. 135, 41–46.
26 Kaback, H.R. (1987) Biochemistry 26, 2071–2076.
27 Hanada, K., Yamato, I. and Anraku, Y. (1985) FEBS Lett. 191, 278–282.
28 Neuhaus, J.M., Soppa, J., Wright, J.K., Riede, I., Blocker, H., Frank, R. and Overath, P. (1985) FEBS Lett. 185, 83–88.
29 Sakar, H.K., Menick, D.R., Viitanen, P.V., Poonian, M.S. and Kaback, H.R. (1986) J. Biol. Chem. 261, 8914–8918.
30 Yamato, I. and Anraku, Y. (1988) J. Biol. Chem. 263, 16055–16057.
31 Nickerson, W.J., Falcone, G. and Strauss, G. (1963) Biochemistry 2, 537–545.
32 LaNoue, K.F. and Schoolwerth, A.C. (1979) Ann. Rev. Biochem. 48, 871–922.
33 Palmieri, F., Bisaccia, F., Genchi, G., Indiveri, C. and Zara, V. (1988) in Molecular Basis of Biomembrane Transport (Palmieri, F. and Quagliariello, E., eds.), 163–174, Elsevier Biomedical Division, Amsterdam.
34 Munding, A., Drees, M., Beyer, K. and Klingenberg, M. (1987) Biochemistry 26, 8637–8644.

35 Houštěk, J. and Pedersen, P.L. (1985) J. Biol. Chem. 260, 6288–6295.
36 Kolbe, H.V.J., Costello, D., Wong, A., Lu, R.C. and Wohlrab, H. (1984) J. Biol. Chem. 259, 9115–9120.
37 Kolbe, H.V.J. and Wohlrab, H. (1985) J. Biol. Chem. 260, 15899–15906.
38 Foss, O. (1961) in Organic Sulfur Compounds (Karasch, N., ed.), Vol. 1, pp. 83–96, Pergamon Press, New York.
39 Fox, C.F. and Kennedy, E.P. (1965) Proc. Natl. Acad. Sci. USA 54, 891–899.
40 Macpherson, A.J.S., Jones-Mortimer, M.C., Horne, P. and Henderson, P.J.F. (1983) J. Biol. Chem. 258, 4350–4356.
41 Kolbe, H. (1986) Biochim. Biophys. Acta 853, 115–134.
42 Kolbe, H.V.J. and Kadenbach, B. (1981) Hoppe-Seyler's Z. Physiol. Chem. 362, 1583–1590.
43 Diriks, T. and Krämer, R. (1988) Abstracts of the Fifth European Bioenergetic Conference, Short Reports, p. 209, Aberystwyth, Wales.