The arginine-dependent extreme acid resistance response of Escherichia coli operates by decarboxylating arginine. AdiC, a membrane antipporter, catalyzes arginine influx coupled to efflux of the decarboxylation product agmatine, effectively exporting a proton in each turnover. Using the adIC coding sequence under control of a tetracycline promoter in an E. coli vector, we expressed and purified the transport-protein with a yield of ~10 mg/liter bacterial culture. Glutaraldehyde cross-linking experiments indicate that the protein is a homodimer in detergent micelles and lipid membranes. Purified AdiC reconstituted into liposomes exchanges arginine and agmatine in a strictly coupled, electrogenic fashion. Kinetic analysis yields $K_m \approx 80 \mu M$ for Arg, in the same range as its dissociation constant determined by isothermal titration calorimetry.

Enteric bacteria mount stress responses that allow them to survive in acidic conditions as they pass through the stomach (pH 2–4) on their way to the intestine (1, 2). Escherichia coli resists strong acid challenges by activating multicomponent systems that pump protons out of the cytoplasm as rapidly as they leak in, thereby maintaining a steady-state intracellular pH of ~5.0 (3). Two well studied acid resistance systems use proton extrusion pumps powered by amino acid decarboxylation: one for glutamate and the other for arginine. This report concerns the arginine system, outlined in Fig. 1. Arg enters the cell through a membrane transporter and is then decarboxylated by an acid-activated arginine decarboxylase. This reaction consumes a proton, which ends up on the 1-position of the product 1-amino-4-guanidino-n-butane, commonly denoted agmatine (Agm).2 Agm leaves the cell, carrying this “virtual proton” also belongs to the subfamily of decarboxylation-driven “vector, we expressed and purified the transport-protein with a yield of ~10 mg/liter bacterial culture. Glutaraldehyde cross-linking experiments indicate that the protein is a homodimer in detergent micelles and lipid membranes. Purified AdiC reconstituted into liposomes exchanges arginine and agmatine in a strictly coupled, electrogenic fashion. Kinetic analysis yields $K_m \approx 80 \mu M$ for Arg, in the same range as its dissociation constant determined by isothermal titration calorimetry.

This report describes the overexpression, purification, and quaternary structure of AdiC, along with an initial description of its membrane transport behavior. The protein forms a stable homodimer in detergent micelles and phospholipid membranes. Exchange transport of Arg and Agm is tightly coupled, electrogenic, and acid-activated. The unusually high expression level of this membrane protein makes it an attractive system for detailed structure-function analysis.

MATERIALS AND METHODS

Expression, Purification, and Reconstitution—The coding sequence of the adIC gene of E. coli was inserted into the XbaI/HindIII cassette of vector pASK-IBA2 (11) behind a ribosome-binding sequence (TAACGAGGGGAAAAA), as described for a Cl− transporter (12). A hexahistidine tag followed by a thrombin recognition sequence and linker (HHHHHHSGGLVPRGS) was interposed between the initiator methionine and the natural AdiC sequence. Transformed BL21(DE3) cells were grown in Terrific Broth at 37 °C to $A_{600}$ of 1.5 and induced with 0.2 mg/liter anhydrotetracycline for 3 h. Cell pellets, suspended in 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, were sonicated on ice with protease inhibitors (1 mg/ml leupeptin/pepsatin; 1 mM phenylmethylsulfonyl fluoride), and membrane protein was extracted in 40 mM decylmaltoside (DM) at 4 °C overnight, and AdiC was eluted by 400 mM imidazole in DM. After concentrating to 0.5–1 ml, the sample was treated with thrombin (Roche Applied Science, 0.1 units/mg of AdiC) at 4 °C overnight, and AdiC was purified on a Superdex 200 size exclusion column in DM with 5 mM DM. This column was calibrated (13) with the elution times in DM solutions of functionally active integral membrane protein standards of similar molecular sizes: MthK, 250 kDa (14), 10.5 ml; CLC-ec1–F$_{AB}$ complex (15), 200 kDa, 11.1 ml; MloK1 (16), 150 kDa, 11.3 ml; CLC-ec1, 100 kDa, 12.5 ml; KcsA (17), 74 kDa, 13.3 ml. Bound detergent and lipid make these membrane proteins elute systematically ahead of soluble-protein standards of similar molecular size: catalase, 232 kDa, 12.3 min; F$_{AB}$ 100 kDa, 15.9 min bovine serum albumin, 67 kDa, 13.8 min. The AdiC peak was collected into an Amicon concentrator with 50-kDa cutoff and concentrated to 5–10 mg/ml. Molar...
extinction coefficient was estimated from the AdiC sequence as 86,000 m$^{-1}$ cm$^{-1}$ ($A_{280}$ of unity equivalent to 11.6 $\mu$M, 0.54 mg/ml), a value confirmed by substrate binding stoichiometry via isothermal titration calorimetry.

Arg-transporting proteoliposomes were formed from a micellar solution consisting of 34 mM Chaps in WB, 20 mg/ml phospholipid (E. coli polar lipid, Avanti), and AdiC (typically 1–5 $\mu$g/ml of lipid); detergent was removed by extensive dialysis against external buffer (EB, 150 mM KCl, 25 mM Mes-KOH, pH 6.0). For experiments in which pH was varied, 25 mM citrate/25 mM phosphate was substituted for Mes. Proteoliposomes represent protonation states required for this scheme to work as an outwardly directed virtual proton pump. The coupled reactions are driven in the direction of the arrows by the favorable free energy of decarboxylation.

FIGURE 1. Arginine-dependent acid resistance scheme. The standard model of arginine-dependent extreme acid resistance mechanism in E. coli (2) is shown. Arginine enters the cell through the AdiC antiporter, and agmatine is formed by the AdiA decarboxylase. The proton consumed in this reaction is exported on agmatine by AdiC. Charges indicated on the AdiC substrates represent protonation states required for this scheme to work as an outwardly directed virtual proton pump. The coupled reactions are driven in the direction of the arrows by the favorable free energy of decarboxylation.

RESULTS

Purification and Homodimeric Architecture—A construct of AdiC bearing an N-terminal hexahistidine tag was expressed in E. coli, solubilized in detergent (DM), and purified by a conventional three-step procedure involving Co-affinity chromatography, thrombin cleavage of the His tag, and final cleanup by gel filtration. The procedure is illustrated in Fig. 2A, which shows the completeness of His tag removal and high purity of the final preparation of AdiC, a single band on SDS-PAGE gels running somewhat faster than the position expected from its polypeptide sequence (46.8 kDa), as has been seen with other membrane proteins. In gel filtration on a calibrated Superdex 200 column, micellar AdiC runs as a symmetrical, monodisperse peak (Fig. 2B), but it co-elutes (12.4 ml) with the 100-kDa standard CLC-ec1, well ahead of the position expected (~14 ml) from its monomer molecular size and also ahead of the 74-kDa standard KcsA (13.3 ml). Thus, AdiC appears to behave as a 94-kDa homodimer in detergent solution. However, this inference is shaky since membrane proteins typically bind detergent and lipids, unknown extra mass that affects the gel filtration profile. This ambiguity is mitigated empirically by the use of membrane proteins for calibration, but it is not eliminated since it is still possible that AdiC may bind much more detergent than do the standards.

In light of these uncertainties, which apply to all hydrodynamic size determination methods for membrane proteins, we turned to an alternative, independent approach to scrutinize the oligomeric state of the protein: glutaraldehyde cross-link-
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FIGURE 2. Purification of AdiC. A, SDS-PAGE analysis of steps in the AdiC purification procedure, with lanes as follows. Shown is a Coomassie Blue-stained gel of whole cells, before induction (lane 1) and after induction (lane 2); cobalt column eluate (lane 3); gel filtration eluate after thrombin cleavage (lane 4); protein standards, with molecular sizes (kDa) indicated (lane 5). B, preparative scale Superdex 200 profile of AdiC running at 1 ml/min, with arrows indicating positions and molecular sizes (kDa) of membrane protein standards (see “Materials and Methods”).

FIGURE 3. Glutaraldehyde cross-linking of AdiC. SDS-PAGE analysis of cross-linking of AdiC in detergent or phospholipid membranes is shown. A, Coomassie Blue-stained gel of AdiC in detergent, molecular size markers as in Fig. 2 (lane 1), before glutaraldehyde (lane 2), after glutaraldehyde in 10 mM DM (lane 3), and after glutaraldehyde in 0.5% SDS (lane 4). B, silver-stained gel of AdiC reconstituted in liposomes at limiting low density, 0.1 μg/mg, control (lane 1), and after glutaraldehyde (lane 2).

Does AdiC form a homodimer in membranes? To address this biologically pertinent question, we cross-linked functionally reconstituted AdiC, taking advantage of liposomes as nano-vessels in which individual transporters can be held in isolation from each other. If AdiC is reconstituted at high protein density, 20 μg/ml, where many transporters inhabit each liposome, a menagerie of high oligomers is seen with much of the protein unable to enter even the stacking gel (data not shown), as expected from intermolecular cross-linking of proteins sharing the same liposome. However, a completely different gel pattern is seen if AdiC is reconstituted at 200-fold lower density, where the number of protein molecules in the sample is much less than the number of liposomes. In this situation, most of the liposomes are devoid of protein, and those containing AdiC carry only a single transporting unit (13, 22). In these “lonesome transporter” conditions, glutaraldehyde quantitatively shifts the monomer band to the dimer position without the appearance of any higher bands (Fig. 3B). These results are starkly incompatible with AdiC being monomeric and thus establish the membrane-embedded protein as a homodimer. This conclusion is notable as most well studied MFS transporters function as monomers, but it is not unprecedented since a few cases of multimeric MFS proteins have been described (23, 24).

Arginine Transport—Purified AdiC was reconstituted into lipid vesicles preloaded with the physiological exchange partner, Agm, at a high concentration, 5 mM. The addition of 50 μM [14C]Arg to the external solution evokes robust uptake observable within a few seconds and approaching a steady level in 10 min (Fig. 4). The large outwardly directed Agm gradient drives Arg into the liposomes; the magnitude of active accumulation can be appreciated by noting that at maximal uptake, ~30% of the Arg in the system ends up inside the liposomes, which take up only ~2% of the volume. A rough calculation indicates that at maximal uptake, internal Arg is concentrated to ~1.5 mM, external Arg is depleted to ~35 μM, and internal Agm falls to ~3.5 mM. Somewhat faster uptake is seen under [14C]Arg-Agm exchange conditions. No Arg uptake occurs in liposomes in which transport substrate is absent or in liposomes loaded with d-Arg.
Arg-Agm exchange increases with the concentration of AdiC in the liposomes (Fig. 5A). At limiting low density, ∼0.1 μg/mg of lipid, where most of the liposomes are protein-free and those liposomes that do contain transporters carry only a single copy, uptake is low but readily discernable. As AdiC density increases, so does uptake, which tends toward saturation in both rate and final extent above ∼3 μg/mg. For the initial rate, this probably reflects the limited time resolution of our transport assay (10 s), whereas saturation of final uptake is expected if all liposomes are reconstituted with protein at these high densities.

As a component of an *E. coli* acid resistance response, AdiC operates below pH 5, and this biological imperative shows itself in the pH dependence of transport (Fig. 5B). The protein supports respectable antiport around neutral pH, but the uptake rate is much greater at pH 4. (Liposome leakiness precludes measurements below pH 4.)

**Electrogenicity of Arg-Agm Exchange—** *E. coli* uses AdiC to bring Arg into the cell as Agm is simultaneously excreted. This function demands a one-for-one transport stoichiometry, as has been confirmed in whole-cell studies (4). In our standard assay conditions at pH 6, Arg and Agm differ in charge (+1 and +2, respectively), and so the reaction is expected to be electrogenic, generating outward positive current and thus inside negative voltage. The resulting electrical imbalance would hinder the uptake reaction unless charge buildup were relieved. However, under the conditions of Fig. 4, with symmetrical KCl, the K⁺ ionophore valinomycin, which would dissipate any such inhibitory membrane potential, fails to enhance Arg-Agm exchange (data not shown). This negative result has two possible explanations. Either the transporter is unexpectedly electroneutral, or the K⁺ and Cl⁻ ions in the system, despite their low membrane permeabilities, manage to electrically compensate by virtue of their vast excess over the transported substrates. We tested these alternatives by imposing large membrane potentials, either positive or negative, using K⁺ gradients in the presence of valinomycin and substituting a large impermeant anion, glutamate (13, 25), for Cl⁻. Arg-Agm exchange was examined in liposomes loaded with Tris-glutamate and immersed in K-glutamate (high voltage, positive inside) and in liposomes with these ionic conditions reversed (high negative voltage). In the absence of valinomycin, both conditions show Arg uptake (Fig. 6), with liposomes formed in Tris-glutamate somewhat more active than those formed in K-glutamate, for unknown reasons. Valinomycin now alters transport dramatically, exactly as expected for electrogenic exchange. Initial rates are accelerated by positive voltage at least 10-fold (beyond the time resolution of the assay) and inhibited at least 10-fold by negative voltage. Steady-state uptake levels respond similarly,
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Although not by the same factors as the initial rates, control experiments (not shown) confirm that valinomycin has negligible effect on [14C]Arg-Arg self-exchange, which is necessarily electroneutral. These results demonstrate that AdiC-mediated Arg-Agm exchange is electrogenic. As a supplementary point, we note that if Cl− is used as the anion in similar K+ gradient experiments (not shown), the valinomycin effect on transport is less impressive than with glutamate; this result suggests that the background permeability of Cl− in our system accounts for the aforementioned failure of valinomycin to stimulate transport in symmetrical KCl solutions.

Transport Kinetics and Substrate Specificity—To gain a preliminary kinetic overview of AdiC, we measured the initial steady-state rate of [14C]Arg-Arg exchange as a function of external Arg concentration, with saturating Arg inside the liposomes (Fig. 7A). The rate follows a Michaelis-Menten relation (Fig. 7B), with $K_m$ of 80 µM, in reasonably good agreement with the half-saturation concentration (83 µM) for Arg uptake into whole E. coli cells acid-shocked at pH 2.5 (6); when these rates are normalized to the amount of protein in the assay, $V_{max}$ is on the order of 1 s$^{-1}$, a surprisingly low value, even at the submaximal pH used in these experiments.

A few substrate analogues were qualitatively assessed for transport competence (Fig. 7C). After accumulation of [14C]Arg to a steady level, test compounds were added to the external solution at a high concentration (5 mM); the ensuing Arg efflux reflects coupled influx of the test compound. l-Arg, the natural substrate, stimulates almost complete efflux by the first time point; in contrast, d-Arg shows no transport whatever, as expected from its inability to support l-Arg/d-Arg exchange in the direct uptake assay (Fig. 4). Lysine and ornithine show intermediate behavior, and 1,5-diaminopentane, an Agm analogue in which an amino group substitutes for the guanidinium, is transported well. This small survey serves as a guide for future detailed studies on transport specificity.

It is an enzymological truism that the Michaelis-Menten constant of a substrate does not represent equilibrium binding, and this dictum is all the more applicable in a membrane transport protein, where the reaction involves multiple conformations and substrates are distinguished vectorially. However, the equilibrium binding parameters for substrates, analogues, and inhibitors are nevertheless desirable as these can give information on energetic determinants of substrate recognition. We therefore used ITC of AdiC in detergent solution to investigate binding of its two natural substrates. For both Arg and Agm, substrate titrations produce easily measured signals of heat absorption, showing that binding is enthalpically unfavorable, and thus entropy-driven, with Arg showing substantially larger binding enthalpy than Agm. The titration data are fit well by isotherms saturating at one binding site per AdiC monomer (Fig. 8), with equilibrium dissociation constants of ~100 µM for Arg and ~30 µM for Agm. Full thermodynamic parameters are reported in Table 1.

**DISCUSSION**

The Arg-Agm exchanger AdiC, a key component in a strong acid resistance pathway of E. coli, belongs to the enormous MFS superfamily of coupled transporters. MFS proteins labor at an impressively diverse range of biological tasks in prokaryotes (7), including proton-coupled accumulation of nutrients and extrusion of xenobiotics, obligatorily linked substrate-product exchange, and membrane energization by virtual proton pumping. In E. coli cells, AdiC-mediated Arg-Agm exchange across the inner membrane is undetectable at neutral pH but is acutely activated by acid challenge (4–6). However, this does not mean that AdiC itself is acid-activated since in cellular conditions, transport is strictly coupled to Agm generation by arginine decarboxylase, an acid-activated enzyme (26). We were therefore motivated to study AdiC in isolation, removed from the complexities of the cytoplasm. To our surprise, we found that this membrane protein is overexpressed in E. coli, that it can be readily purified to homogeneity in monodisperse form, and that it is stable in detergent solution for several weeks at 4 °C. Approximately 10 mg of purified AdiC is routinely obtained per liter of bacterial culture, a circumstance that eases the pathway toward understanding the workings of the transporter.

AdiC retains in vitro its essential biological function: electrogenic exchange of Arg for Agm at low pH. Electrogenicity is unsurprising under our usual assay conditions at pH 6, where Arg(+1) and Agm(+2) differ in charge, but it is noteworthy in view of the cellular conditions prevailing during acid shock. At pH 2, for instance, the carboxyl group of Arg ($pK_a \sim 3.5$) is over 95% protonated, and consequently, both substrates are almost always in the +2 form. Nevertheless, an electrogenic mechanism that in acidic conditions selects the rare Arg(+1) form is biologically necessary since electroneutral exchange of Arg(+2) for Agm(+2), being proton-neutral, would be useless for acid resistance (4). It is satisfying that this inference from physiological purpose, an electrogenic mechanism for Arg-Agm exchange, is experimentally verified in the reduced, purified system.
AdiC is a homodimer in phospholipid membranes and probably in detergent micelles as well. This conclusion is based on several lines of evidence, gel filtration in detergent being merely suggestive and cross-linking at limiting dilution in liposomes being compelling; quantitative cross-linking to a dimer in liposomes that contain only a single transporting unit cannot be reasonably understood otherwise. We have to emphasize that this result implies nothing at all about the exchange mechanism or the functional relevance of dimeric associa-

FIGURE 7. Kinetics and specificity of transport. A, Arg dependence of initial rate. Arg-Arg exchange was examined at the indicated external Arg concentration (μM) in the early part of the time course, at a protein density of 1 μg of AdiC/mg of lipid. Initial rates were estimated from linear fits (solid lines) of the data between 10 and 60 s of uptake. Each point represents triplicate samples containing 0.5 μg of AdiC. B, kinetic parameters of Arg-Arg exchange. Initial rates, calculated above, are plotted versus substrate concentration. The solid curve shows the Michaelis-Menten fit, with $K_m = 80 \mu M$, $V_{max} = 5$ pmol/s. C, transport by substrate analogues. After 30 min of Arg-Arg exchange, 5 mM indicated substrate was added (arrow) to assess its ability to expel accumulated Arg from the liposomes. cont, control.

FIGURE 8. Equilibrium binding of substrates. Isothermal titration calorimetry was used to measure equilibrium binding isotherms of Arg or Agm to AdiC. Independent site binding isotherms (solid lines) were calculated from heat absorbed per injection as a function of substrate concentration, with parameters given in Table 1. AdiC is a homodimer in phospholipid membranes and probably in detergent micelles as well. This conclusion is based on several lines of evidence, gel filtration in detergent being merely suggestive and cross-linking at limiting dilution in liposomes being compelling; quantitative cross-linking to a dimer in liposomes that contain only a single transporting unit cannot be reasonably understood otherwise. We have to emphasize that this result implies nothing at all about the exchange mechanism or the functional relevance of dimeric associa-
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TABLE 1
Equilibrium binding parameters derived from isothermal titration calorimetry
Parameters reported are the values derived from the fits of the experimental ITC traces shown in Fig 8. $\Delta H^0$ and $\Delta S^0$, enthalpy and entropy of substrate binding.

| Substrate | $K_D$ (µM) | Number of binding sites per AdiC monomer | $\Delta H^0$ (kcal/mol) | $\Delta S^0$ (cal/mol-K) |
|-----------|------------|---------------------------------------|-------------------------|--------------------------|
| Arginine  | 93         | 1.03                                  | 4.6                     | 34                       |
| Agmatine  | 29         | 1.03                                  | 0.4                     | 22                       |

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