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Direct Evidence for Substrate-induced Proton Release in Detergent-solubilized EmrE, a Multidrug Transporter*

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A novel approach to study coupling of substrate and ion fluxes is presented. EmrE is an H^+-coupled multidrug transporter from *Escherichia coli*. Detergent-solubilized EmrE binds substrate with high affinity in a pH-dependent mode. Here we show, for the first time in an ion-coupled transporter, substrate-induced release of protons in a detergent-solubilized preparation. The direct measurements allow for an important quantitation of the phenomenon. Thus, stoichiometry of the release in the wild type and a mutant with a single carboxyl at position 14 is very similar and about 0.8 protons/monomer. The findings demonstrate that the only residue involved in proton release is a highly conserved membrane-embedded glutamate (Glu-14) and that all the Glu-14 residues in the EmrE functional oligomer participate in proton release. Furthermore, from the pH dependence of the release we determined the p*K* of Glu-14 as 8.5 and for an aspartate replacement at the same position as 6.7. The high p*K* of the carboxyl at position 14 is essential for coupling of fluxes of protons and substrates.

Coupling of ion and substrate fluxes allows for concentrative transport across biological membranes, a process highly relevant to human physiology and disease. Obligatory coupling of fluxes at the mechanistic level implies that one of the steps in the catalytic cycle cannot occur in the absence of one of the reactants. In an effort to identify the molecular basis of coupling, binding and release of substrates and coupling ions has been studied using several approaches. These include measurements of transient electrical responses (*e.g.* Ref. 1), substrate-induced changes in accessibility or reactivity of specific residues (*e.g.* Ref. 2), and binding of inhibitors to the membrane-bound or detergent-solubilized proteins (*e.g.* Ref. 3). However, despite the important information provided by this type of studies, most of them are either indirect or they are done in the membrane where it may be difficult to isolate the binding reaction from other steps in the catalytic cycle.

In this work we measure directly for the first time substrate-induced proton release in a detergent-solubilized transporter. EmrE, a proton-coupled multidrug transporter from *Escherichia coli*, is used as the experimental model system (4, 5). It is a small transporter, 110 amino acids long, that extrudes various drugs in exchange for protons, thereby rendering bacteria resistant to these drugs (4, 5). The protein functions as an oligomer and has been characterized, purified, and reconstituted in a functional form (6). The detergent-solubilized EmrE is quite unique in its ability to bind substrates with high affinity; therefore, it provides an experimental system to study also proton release. In addition, the size of the protein allows identification of the residue involved in this reaction.

EmrE has only one membrane-embedded charged residue, Glu-14, which is conserved in more than 100 homologous proteins (7, 8). Carboxylic residues embedded in the membrane were shown to be important for activity in various ion-coupled transporters. In some cases, these carboxyls are involved in substrate recognition and binding; in others, they are part of the coupling ion binding site (reviewed in Refs. 9 and 10). EmrE is unique in that a single carboxyl is involved in recognition of both substrate and the coupling ion (11). Substrate binding increases dramatically between pH 6.5 and 8.0, suggesting that deprotonation of the site is required (9, 12). Conversely, release of substrate from the protein is accelerated at low pH values, suggesting that protonation is required for efficient release (12). When Glu-14 is replaced with Asp, substrate binding and release are practically independent of pH in the range 6.5–8.0 (12). This is most likely because the Asp at position 14 has a lower p*K*, and therefore the protein is largely deprotonated above pH 6.0.

The findings described above have been substantiated for the transport cycle through experiments with proteoliposomes reconstituted with purified wild type and mutant proteins (13). Based on these results, we have proposed that the mutual dependence for interaction with EmrE is the molecular basis of coupling between protons and substrates fluxes (9, 10).

Direct observation of the substrate-induced proton release allows for quantitation of the reaction. The stoichiometry and the pH dependence of the reaction were measured in the wild type protein as well as in two other mutants: a protein with a single carboxyl at position 14 and one with a carboxyl that displays a lower p*K*. We conclude that the glutamyl residue at position 14 in the wild type protein is the only amino acid involved in proton release and that all the Glu-14 residues in the oligomer participate in the release reaction. The p*K* of the carboxyls involved was measured from the pH dependence of the release reaction. The high p*K* of the carboxyl at position 14 is essential for coupling fluxes of protons and substrates.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli TA15 (14) was used for EmrE expression. Cells were transformed with plasmid pGP1–2, which encodes for the regulated expression of T7 polymerase under the inducible control of the λ P_L promoter (15). The plasmids used for EmrE gene expression are pT7–7 (15) derivatives with the hexahistidine tag using a Myc epitope as linker (12). Throughout this paper, for simplicity, Myc- and His-tagged protein is named EmrE. The E14D-EmrE and E25C/D84C-EmrE (SC-EmrE, single carboxyl construct) mutants were previously characterized (11, 12).

Overexpression and Purification of EmrE—E. coli TA15 cells that

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bear plasmids pGP1–2 and pT7–7 containing His-tagged EmrE constructs were grown at 30 °C in minimal medium A supplemented with 2.5 μg/ml thiamine, 0.5% glycerol, 100 μg/ml ampicillin, and 50 μg/ml kanamycin. When the culture reached A_{600} = 1, the temperature was elevated rapidly to 42 °C to allow for T7 polymerase expression for 15 min, and then the temperature was decreased back to 30 °C. Two hours later the cells were harvested by centrifugation and washed once with buffer containing 150 mM NaCl, 15 mM Tris, pH 7.5, 250 mM sucrose before storage at −70 °C. For membrane preparation, cells were resuspended in the same buffer containing 2.5 mM $MgSO_4$, 1 mM dithiothreitol, 15 μg/ml DNaseI, and 1 mM phenylmethylsulfonyl fluoride (5 ml of buffer/g cells). Membranes were prepared by disrupting the cells using a Microfluidics microfluidizer processor (M-110EHi). Nondisrupted cells were discarded by centrifugation (4225 × *g* for 5 min at 4 °C), and the membranes were collected by centrifugation at 240,000 × *g* for 90 min at 4 °C. The membrane pellet was washed and resuspended in 150 mM NaCl, 15 mM Tris, pH 7.5, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (membrane protein concentration ~20 mg/ml), frozen in liquid air, and stored at −70 °C. Wild type EmrE and the mutants were purified by solubilizing membranes (~5 mg of protein/ml) in 1% DDM,[1] 150 mM NaCl, 15 mM Tris-HCl, pH 7.5, 50 mM imidazole, pH 7.5, and 1 mM phenylmethylsulfonyl fluoride for 40 min at 25 °C. Unsolubilized material was removed by centrifugation at 240,000 × *g* for 45 min at 4 °C. In the case of SC-EmrE, β-mercaptoethanol (15 mM) was added throughout purification to prevent cysteine crosslinking.

The solubilized protein was loaded on HiTrap™ chelating HP column (Amersham Biosciences) mounted on Akta Explorer (Amersham Biosciences) and washed with 0.08% DDM, 150 mM NaCl, 15 mM Tris-HCl, and 50 mM imidazole, pH 7.5 till A_{280} of the flowthrough decreased below 0.05. EmrE was eluted with a gradient of up to 300 mM imidazole. Major peak fractions were pooled, and the protein solution was brought to ~10 mg/ml EmrE (according to A_{280}). Concentrations of imidazole and Tris were decreased below 1 mM through a series of dilution-concentration cycles with 0.08% DDM and 150 mM NaCl solution using a 30-kDa cutoff Centriprep (Amicon[R] Bioseparations). The protein stock was aliquoted, frozen in liquid air, and stored at −70 °C.

H^+ Assay—Measurements were carried out at 20 °C in a glass cell with magnetic stirring, and a stream of argon was applied to decrease the effect of CO_2 on pH. pH was monitored using pHC4000–8 microelectrode (Radiometer Analytical) connected to a PHM240 pH meter (MeterLab™, Radiometer, Copenhagen, Denmark). Data were recorded using WINview CP Series RS232 data acquisition software (SuperLogics). The solution containing 0.4–4 μM EmrE (7–70 μg), 150 mM NaCl, and 0.08% DDM was brought to the pH of interest by addition of diluted NaOH or acetic acid. To assay proton release, TPP^+ (4 nmol or other substrate in saturating amounts) was added and followed after 60–100 s by 5 nmol NaOH that was used as an internal standard to calculate the absolute amount of the protons released. On this small range of pH changes, the relation between amount of released (consumed) protons and the solution pH was assumed linear. The calculation of the actual changes after substrate or NaOH addition was done from the digital recorded traces using linear regression to compensate for pH drifts.

DCCD Inhibition—The protein stock was diluted to 4 μM in the assay solution, titrated to pH 6.5 with diluted NaOH, and incubated with 200 μM DCCD for 2 h in glass tubes with magnetic stirring before the measurement.

RESULTS

Substrate-induced H^+ Release in Wild Type and Mutants—Addition of saturating concentrations of the high affinity substrate TPP^+ to unbuffered detergent-solubilized EmrE (pH 7.0) induces a rapid acidification of the medium of about 0.15 pH units (Fig. 1*A*). The absolute amount of protons released is calculated from calibration with an aliquot of NaOH and is about equimolar to the EmrE monomers. To show that this is a property related to the protein function, EmrE was pretreated with DCCD, a hydrophobic carbodiimide that covalently reacts with Glu-14 and prevents substrate binding and transport (11). As shown in Fig. 1*B*, DCCD also blocks the TPP^+-induced H^+ release. Furthermore, when the E14C-EmrE mutant, a protein that does not bind substrate (12), replaces the wild type protein no acidification is detected upon addition of TPP^+ (Fig. 1*C*).

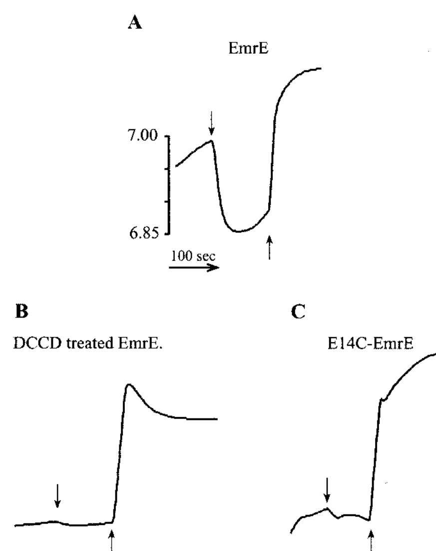

FIG. 1. **Substrate-induced proton release in detergent-solubilized functional EmrE.** pH was monitored as described under "Experimental Procedures." The reactions contained 4.8 nmol (72 μg) of wild type EmrE (*A* and *B*) or E14C-EmrE in a solution containing 0.08% DDM and 150 mM NaCl. TPP (4 nmol, 3.3 μM) was added at the *down arrows*. *B*, wild type protein was pretreated with DCCD as described under "Experimental Procedures." The pH scale corresponds to trace A and is shown only to give an idea of the magnitude of the changes. To quantitate the changes, NaOH (5 nmol) was added in each of the reactions (*up arrow*) to calibrate the amount of protons causing the change in pH. The traces shown are from representative experiments that were repeated at least three times.

To further characterize the reaction, other substrates of EmrE were tested for their ability to induce proton release. This is shown in Fig. 2, *A–D* for saturating concentrations of ethidium, a substrate with a lower affinity, tetraphenyl antimonium ($TPSb^+$), acriflavine, and dequalinium salts. The saturation is demonstrated in Fig. 2*A* where a further aliquot of substrate does not bring on release. In addition, the two substrates compete for the same site because addition of TPP^+ after $TPSb^+$ does not provoke release. We conclude that the H^+ release reaction described above reflects a step in the catalytic cycle of EmrE that involves deprotonation of one or more residues in the EmrE protein.

Stoichiometry of H^+ Released per Monomer in Wild Type and in a Protein with a Single Carboxyl—The amount of protons released increased linearly with the increase of TPP concentration and saturates at about ~ 3–5 μM (data not shown). The concentration of TPP^+ required to elicit maximal response is in the same concentration range as EmrE. Albeit this concentration is well above the affinity of EmrE to TPP^+ at pH 7.0 (about 50 nM), it is necessary to saturate the high EmrE concentration used in these experiments.

At a saturating concentration of TPP^+, the dependence of proton release on protein concentration is linear in the range tested, 0.33–4.0 μM (0.4–4.8 nmol, Fig. 3*A*, *diamonds*). The results demonstrate that at these conditions ~0.8 protons are released per EmrE monomer. The same stoichiometry was observed when ethidium, another substrate of EmrE, was used to elicit proton release (Fig. 3*A*, *circles*).

Wild type EmrE contains three carboxyls: Glu-14 in the membrane domain and Glu-25 and Asp-84 in the loops. In previous work SC-EmrE, a mutant with a single carboxyl residue at position 14, was shown to display binding and transport

[1] The abbreviations used are: DDM, *n*-dodecyl-β-maltoside; TPP^+, tetraphenylphosphonium; DCCD, *N*,*N*-dicyclohexylcarbodiimide; EmrE, EmrE tagged with Myc epitope and six His residues; SC-EmrE, EmrE with only one carboxyl at position 14.

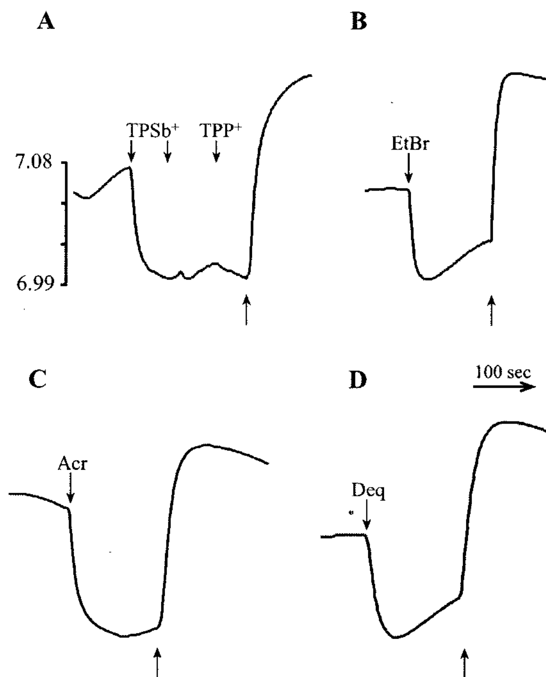

FIG. 2. **Various substrates induce similar proton release.** The reaction was as in Fig. 1 except that the *down arrows* indicate addition of two consecutive additions of 4.8 nmol of tetraphenyl antimonium ($TPSb^+$) followed by 4 nmol TPP^+ (*A*); ethidium Br, 130 nmol (*B*); acriflavine, 130 nmol (*C*); and dequalinium, 6.5 nmol (*D*). The *up arrow* is as in Fig. 1.

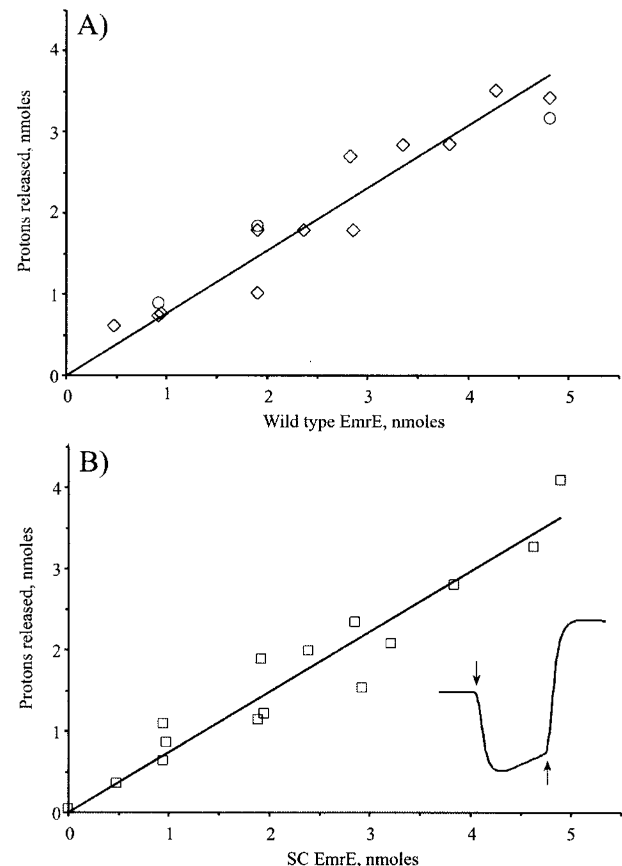

FIG. 3. **The stoichiometry of substrate-induced proton release.** *A*, proton release for wild type-EmrE (*diamonds* and *circles*) is plotted against the amount of protein added per assay. pH was monitored as described under "Experimental Procedures." Proton release was measured at pH 7.0 upon addition of 4 nmol TPP^+ (*diamonds* and *squares*) or 138 nmol EtBr (*circles*). *B*, proton release for varying amounts of SC-EmrE (*squares*) was assayed as described for wild type-EmrE. The *inset* shows an example of the time course with 4.8 nmol protein. The *two lines* shown correspond to trend lines obtained from linear regression of wild type-EmrE data that yields ~0.77 protons/EmrE monomer with R^2 ~0.92 (*A*) and SC-EmrE data (*B*) that yields ~0.74 protons/EmrE monomer with corresponding R^2 ~0.92. Release data for SC-EmrE were corrected according to the TPP^+ binding activity per protein that was slightly lower than the wild type-EmrE. SC-EmrE binds ~1.2 times less TPP^+ than wild type-EmrE at 200 nM TPP^+, measured as described elsewhere (11). When expressed per protein, without correction for TPP binding activity, the ratio of protons/SC-EmrE was 0.62. The experimental points shown for the wild type protein were collected from three different experiments. In addition, this experiment was performed twice following the fluorescence of the pH indicator 8-hydroxypyrene-1,3,6-trisulfonic acid (Pyranine; Molecular Probes Inc., Eugene, Oregon) (M. Soskine, Y. Adam, and S. Schuldiner, unpublished results). For SC-EmrE the experimental data is from two experiments.

abilities very similar to those of the wild type protein (11), and it was suggested that Glu-14 is the only carboxyl needed for coupling of proton and substrate fluxes. To test directly the contention that the protons are released from Glu-14, the protein was purified and challenged with substrate. The SC-EmrE protein displays proton release of a magnitude similar to that of the wild type protein. As shown in Fig. 3*B*, the dependence of proton release on protein concentration was linear in the range tested and the stoichiometry in these experiments was 0.74 H^+/mol SC-EmrE, only slightly lower than that displayed by the wild type protein.

The pH Dependence of H^+ Release Allows Estimation of pK of Glu-14—The studies described above were performed at pH 7.0, where the response to substrate was maximal. The magnitude of release varied significantly at different pH values. The results in Fig. 4*A* demonstrate a bell shape response with a maximum at around pH 7.0. A very small response is detected at pH 5.2, which increases with increase in pH up to about pH 7.0–7.2 and then decreases to nearly undetectable values at pH 9.0 and above. The pH dependence is virtually identical when SC-EmrE is used (Fig. 4*B*, *squares*), suggesting that the pH dependence reflects mainly the affinity of Glu-14 to protons (Fig. 5, *step 1*) relative to the affinity of TPP^+ (Fig. 5, *step 2*). Because all the release is from Glu-14, we conclude that at pH 7.0 and below the carboxyl is practically fully protonated in the absence of substrate and becomes fully deprotonated upon addition of saturating substrate concentrations. The decrease in proton release at alkaline pH values is not due to decreased TPP^+ binding because this increases with increasing pH and plateaus up to very high pH values (11, 12). We propose that the decrease is due to a lower fraction of protonated Glu-14 and that this is a reflection of the p*K* of this residue. This is a spontaneous reaction, independent of the presence of substrate (Fig. 5, *step 1*). From the decrease in substrate-dependent release, we can therefore estimate a p*K*. The point at which the ratio of proton released per monomer is half maximal is around pH 8.3–8.5 (Fig. 4*A*). On the other side of the scale, at acidic pH values, we propose that the lower response is due to the increase in proton concentration that hinders release from a carboxyl with very high and unusual affinity to protons. Indeed, this suggestion is borne out by two results. 1) The TPP^+ concentration used in these experiments (3 μM) is saturating at pH 7 and above. At lower pH values, TPP^+ concentrations as high as 1 mM are needed to induce maximal response (Fig. 4*A*, *inset*). 2) Correspondingly, when the affinity to protons changes such as in E14D-EmrE (carboxyl with lower p*K*), the protein is capable of robust H^+ release at pH values as low as 4.5 (Fig. 4*B*, *filled circles*). The response increases and peaks between pH 5.8 and 6.0 and decreases thereafter at increasing pH values. Following the reasoning discussed above, we estimate the p*K* of the carboxyl in the E14D-EmrE mutant as around 6.5, the

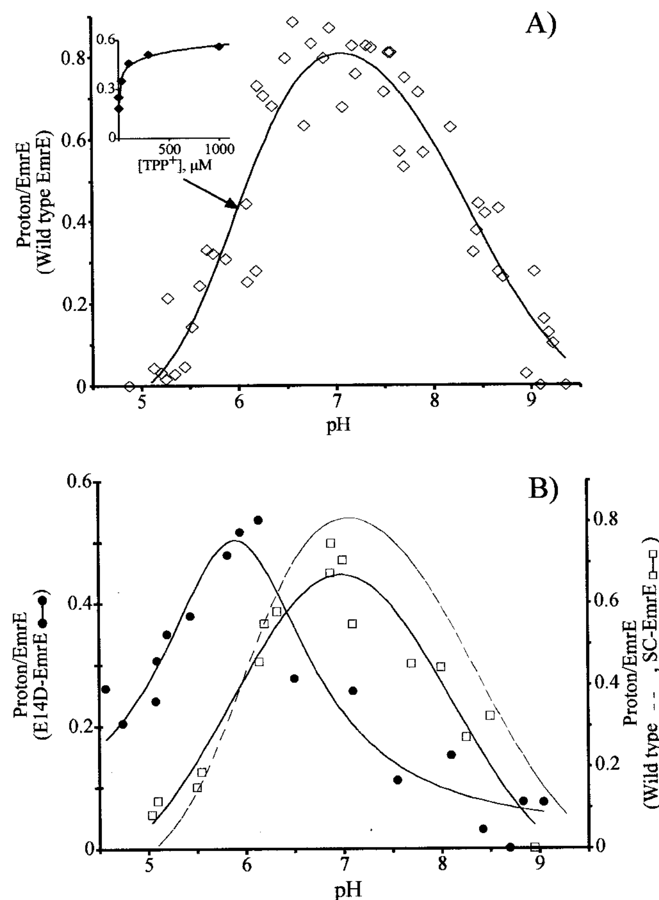

FIG. 4. **pH dependence of substrate-induced proton release.** *A*, release of protons from wild type-EmrE was assayed in the following fashion: the reaction mixture contained 4.8 nmol of the protein (72 μg) in 0.08% DDM and 150 mM NaCl solution that was brought to desired pH with diluted NaOH or acetic acid before addition of 4 nmol of TPP^+ (3.3 μM) followed by addition of 5 nmol of NaOH (internal standard, used to calculate absolute amount of the protons released). The ratio of protons released per protein was plotted against the pH measured before addition of TPP^+. Results from four different repeats are shown in the same graph to illustrate the reproducibility of the experiments. The *inset* shows the concentration dependence of the release reaction at pH 6.0 with TPP^+ concentrations 1–1000 μM. *B*, E14D-EmrE (*filled circles*) and SC-EmrE (*squares*) were challenged with substrate at various pH values as described for the wild type-EmrE. Results from two different repeats are shown. The trend line of wild type EmrE (*dashed line*) is shown for reference.

point where the release decreases to 50% of the maximal value. This may be an overestimate because the magnitude of the maximal release was about 0.5 mol/mol EmrE, lower than the values observed with wild type EmrE. This is possibly due also to competition between protons and substrate and other nonspecific effects of the low pH that may prevent the protein from reaching its maximal activity.

DISCUSSION

In this report, we describe substrate-induced proton release in a detergent-solubilized preparation of EmrE, a multidrug transporter from *E. coli*. Detergent-solubilized EmrE binds substrate with high affinity in a pH-dependent fashion (12). The above findings and further characterization of the response in a single carboxyl mutant (11) implied that deprotonation of Glu-14 is necessary to allow substrate binding. The experiments described here directly demonstrate the above contention and allow quantitation and characterization of the H^+ release reaction. Thus, the stoichiometry of the release and the pH dependence were measured in the wild type protein as

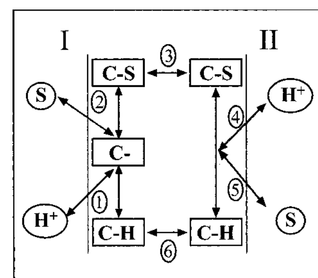

FIG. 5. **Kinetic model of the transport cycle.** EmrE (*C*) releases H^+ (*step 1*) prior to substrate binding (*step 2*). It then translocates the substrate to the trans face of the membrane (*step 3*). Proton binding (*step 4*) accelerates substrate release (*step 5*). When the binding site is protonated (*step 6*) or occupied by substrate (*step 3*) it can face either side of the membrane.

well as in a fully active mutant with a single carboxyl at position 14 (SC-EmrE) and a mutant with a carboxyl with a lower p*K* (E14D-EmrE).

In SC-EmrE there are only two other residues besides Glu-14 that could act as potential proton donors or acceptors: Lys-22 and His-110. His-110 is the carboxyl-terminal amino acid and was previously modified to Cys or completely removed with no impairment whatsoever of the activity (13). Lys-22 is fully conserved in the SMR family (7), but replacement with Arg yielded a protein with activity practically indistinguishable from the wild type (13). Even the Cys replacement was functional though its activity was lower than that of the wild type (13). Therefore, the results presented here unequivocally demonstrate that proton release is solely from the carboxyl of the glutamyl residue at position 14. In addition, a replacement with Asp at position 14 results in a mutant with a lower p*K* and modified pH dependence of all the reactions tested. We can therefore conclude that the pH dependence of EmrE is dictated solely by the unusually high p*K* of the carboxyl at position 14.

Substrate binds (Fig. 5, *step 2*) only to the deprotonated EmrE species (9, 10). Substrate binding at high pH does not induce release because deprotonation (Fig. 5, *step 1*) occurs spontaneously as dictated by the p*K* of Glu-14. At lower pH values substrate binding shifts the equilibrium as dictated by simple mass laws.

Binding to a protein that has already lost its proton would seem not productive from the point of view of coupling of substrate and proton fluxes. However, the finding that the rate of substrate release is accelerated at high proton concentrations (12) suggests that release of the substrate at the trans side of the membrane (Fig. 5, *step 5*) is dependent on prior binding of a proton to the site (*step 4*). This fact provides another site for obligatory coupling between substrate and proton interaction with the protein.

In the case of the E14D mutant, it binds and releases substrate in a practically pH-independent mode in the range 6.5–8.8 and thus cannot efficiently couple the reaction to the proton gradient. It catalyzes facilitated diffusion type of transport at rates that are about 60% of the rates of the wild type protein (13). These findings hint that in the case of E14D the unloaded, unprotonated transporter can return at a rate high enough to support the uncoupled reaction. Thus, a fundamental requirement for coupling seems to be a residue (in our case Glu-14) with a highly distinct and properly tuned p*K*. Change in p*K* of carboxyls in proteins is usually either because of a low dielectric environment or charge proximity that can result in ion pairing or charge clusters. In the case of the EmrE oligomer, there is evidence that the Glu-14 residues of two neighbor monomers are very close to each other (16, 17). Aspartyl residues in the same position would be farther apart and thereby

display p*K*s closer to the p*K* of a carboxyl in aqueous environment.

Previous studies show that TPP^+ binds to the oligomer with a stoichiometry of 2–3 monomers/TPP^+ (12, 18). The fact that 0.8 H^+ are released per monomer demonstrates that binding of one substrate molecule to the oligomer brings on release of protons from all the Glu-14 residues in the oligomer. This finding suggests that all the Glu-14 residues are functionally equivalent in the oligomer. Apparent negative dominance is observed when mixing an inactive E14C mutant with wild type protein (19). However, later studies showed that this is the result of a 20-fold decrease in the affinity to the substrate (20). That the binding site is contributed by each of the subunits was also demonstrated by experiments in which the hetero-oligomer with Cys residues only in the inactive monomer was challenged with *N*-ethylmaleimide (NEM), a sulfhydryl reagent. NEM inhibited the activity of this hetero-oligomer even though it does not have any effect on the activity of the wild type (20).

Previous studies suggested that protons and substrate share the same binding domain and that Glu-14 is an essential residue in this domain (8–10, 21). The simplest interpretation of our data is based on the scheme described in Fig. 5 where the substrate binds only to the non-protonated species and pushes the equilibrium away from protonation. In such a mechanism, substrate does not necessarily change the p*K* of the carboxyl upon binding as was previously suggested (9). The experimental approach described in this study and our interpretation of the results could be applied to a symporter as well with only slight modifications. In a symporter, substrate would bind exclusively to the protonated form of the transporter. Therefore, at the appropriate pH, substrate binding to an H^+ symporter will cause removal of H^+ from the medium.

EmrE is specially suited to the experiments described in this work because of the ability of the detergent-solubilized protein to bind substrates and protons. The detergent-solubilized preparation allows for direct observation of individual steps in the transport cycle. The availability of a scalar system simplifies the quantitation and characterization but needs careful substantiation for the transport reaction through experiments with proteoliposomes as performed with EmrE (13). The only difference we were able to detect between the properties of the membrane-bound and the detergent-solubilized protein is the inability of the latter to recognize methyl viologen, one of the substrates of EmrE (12). Although EmrE is a very sturdy and stable protein, the detergent requirement for maintenance of function is quite strict and is fulfilled only by a handful of detergents, DDM being the best among the ones tested.[2] Another advantage of EmrE is the low number of titratable groups, a fact that lowers the buffer capacity of the system and allows for a high signal to noise ratio. Finally, the size of the protein allows for an accurate assignment of the residue involved in proton binding and release. EmrE has only one membrane-embedded residue, Glu-14, and it is highly conserved. Our previous studies have suggested that Glu-14 is part of the substrate-binding domain and that there is an overlap between the proton- and substrate-binding domains. The simplest interpretation of the results presented here is that substrates and protons compete for the same site.

Many studies of H^+-coupled transporters have elegantly and unequivocally documented vectorial transport of protons as part of the transport cycle. These studies, including our own, have always implied the binding and release of protons in the catalytic cycle of the transporter. Here we provide for the first time direct experimental evidence for this reaction in a detergent-solubilized protein. Exploration of this reaction has provided a tool for its quantitation, determination of p*K* of the groups involved, and identification of the residues involved directly in proton release. It will now be possible to observe this reaction in shorter time spans and observe subtle influences in the rate of release by modifying residues that may be in the pathway of the proton on its way from and to Glu-14, the critical acceptor/donor.

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[2] M. Aharon and S. Schuldiner, unpublished results.

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