Three two-component transporters with channel-like properties have monovalent cation/proton antiport activity

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Properties of four two-component bacterial transport systems of the cation/proton antiporter-2 (CPA2) family led to suggestions that this CPA2 subset may use a channel rather than an antiport mechanism [see Booth IR, Edwards MD, Gunasekera B, Li C, Miller S (2005) in *Bacterial Ion Channels*, eds Kubalski A, Martinac B (Am Soc Microbiol, Washington, DC), pp 21–40]. The transporter subset includes the intensively studied glutathione-gated K^+ efflux systems from *Escherichia coli*, KefGB, and KefFC. KefG and KefF are ancillary proteins. They are peripheral membrane proteins that are encoded in operons with the respective transporter proteins, KefB and KefC, and are required for optimal efflux activity. The other two-component CPA2 transporters of the subset are AmhMT, an NH_4^+ (K^+) efflux system from alkaliphilic *Bacillus pseudofirmus* OF4; and YhaTU, a K^+ efflux system from *Bacillus subtilis*. Here a K^+/H^+ antiport capacity was demonstrated for YhaTU, AmhMT, and KefFC in membrane vesicles from antiporter-deficient *E. coli* KNabc. The apparent K_m for K^+ was in the low mM range. The peripheral protein was required for YhaU- and KefC-dependent antiport, whereas both AmhT and AmhMT exhibited antiport. KefFC had the broadest range of substrates, using $Rb^+ \approx K^+ > Li^+ > Na^+$. Glutathione significantly inhibited KefFC-mediated K^+/H^+ antiport in vesicles. The inhibition was enhanced by NADH, which presumably binds to the KTN/RCK domain of KefC. The antiport mechanism accounts for the H^+ uptake involved in KefFC-mediated electrophile resistance *in vivo*. Because the physiological substrate of AmhMT in the alkaliphile is NH_4^+, the results also imply that AmhMT catalyzes NH_4^+/H^+ antiport, which would prevent net cytoplasmic H^+ loss during NH_4^+ efflux.

AmhMT | KefC | YhaTU | K^+/H^+ antiport | ammonium transport

Cation/proton antiporters (CPAs) play major physiological roles in pH, volume, and cation homeostasis in both eukaryotic cells and their organelles and in prokaryotes (1, 2). In bacteria, CPAs typically function as secondary active transporters that couple the efflux of diverse cations to the inward movement of H^+. This antiport is energized by the proton-motive force across the membrane, alkali and negative inside relative to outside, that is generated by proton pumping during respiration or ATP hydrolysis (3). At least 10 families of membrane-transport proteins within the sequence-based transporter classification database include such secondary CPAs (4, 5). These antiporters have established roles in resistance to diverse cytotoxic cations, alkali resistance, electrophile resistance, osmoregulation, magnetosome formation, and endospore germination (2, 6–9). The CPA2 family contains both eukaryotic and prokaryotic transporters. It is unique among the CPA families in having a subset of four bacterial transporters that have ambiguity associated with their catalytic mechanism (10). The four transporters of this CPA2 subset are: (*i*) KefC and KefB, well-studied glutathione (GSH)-gated K^+ efflux proteins from *Escherichia coli* that play a role in electrophile resistance; (*ii*) AmhT, an NH_4^+ and K^+ transporter from alkaliphilic *Bacillus pseudofirmus* OF4 that has a physiological role in NH_4^+ homeostasis; and (*iii*) YhaU, a related K^+ efflux system from *Bacillus subtilis* that may also transport NH_4^+ (10–12) (Fig. 1). The similarity of these four proteins to CPA2 transporters with documented cation/proton antiport activity was recognized because of the discovery of KefB and KefC (originally called TrkB and TrkC) (13–15). However, the possibility of a channel mechanism was also raised early on (16, 17) because of the gating properties and fast *in vivo* rates of K^+ efflux. A channel mechanism has remained a major theme in recent work on these proteins, whereas no antiport capacity has been demonstrated (11, 12, 18, 19).

Two considerations led us to further investigate antiport capacity for AmhT and YhaU and then add KefC to the study. First, evidence has emerged for all four proteins of this CPA2 subset that ancillary hydrophilic proteins modulate their cation flux activities. The 18.5- to 21-kDa ancillary proteins are encoded upstream of the membrane-transport gene in operons from which they are expressed together. The ancillary proteins apparently interact with the transporters as peripheral membrane proteins (11, 12, 20). The two *Bacillus* ancillary proteins, AmhM and YhaT, exhibit 28% sequence identity and 56% similarity to one another. They possess domains called either KTN (K^+ transport nucleotide binding) or RCK (regulating the conductance of K^+) (21–25), which are predicted to be ligand-binding. By contrast, nucleotide-binding KTN/RCK domains (26, 27) are part of the membrane proteins KefC and KefB (10). The sequences of the KefF and KefG ancillary proteins of these systems are not related to the sequences of AmhM or YhaT but are related to each other (40% identical, 55% similarity) and have homology to quinone oxidoreductases (10, 22). The existence of ancillary proteins that modulate activity of this CPA2 transporter subset raised the possibility that antiport activity is a property of the protein pair, rather than the CPA2 membrane protein alone, and therefore had been missed in earlier assays of the membrane proteins alone (11, 12). Second, biological considerations made cation/proton antiport a more attractive mechanism than channel activity for the KefFC and KefGB systems as well as for AmhMT. In *E. coli*, electrophiles release KefFC and KefGB from inhibition by bound GSH, thus eliciting their K^+ efflux activity. Electrophile resistance, however, is not mediated by K^+ efflux *per se* but by H^+ uptake that depends on K^+ efflux. In models presented to date, the H^+ uptake is hypothesized to occur by an unknown pathway secondary to channel-mediated K^+ efflux (28, 29). That gap would be ad-

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Abbreviations: AO, acridine orange; CPA, cation/proton antiporter; GSH, glutathione; NEM, *N*-ethylmaleimide; ΔpH, transmembrane pH gradient.

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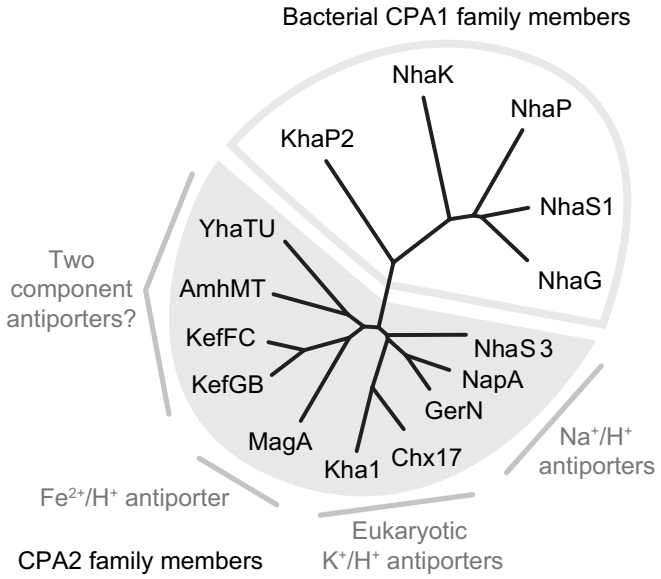

Fig. 1. CPA2 and bacterial CPA1 family members whose activities have been assessed. Multiple alignment of the indicated proteins was carried out by clustal W analysis (50). The neighbor-joining bootstrap method (1,000 counts) was used to obtain the phylogenetic tree data in phylip format (51). These data were displayed with the TreeView program (52) version 1.6.6 and then modified for presentation by Adobe Photoshop CS2. The two-component subset is presented with the ancillary protein, followed by the CPA2 membrane protein.

dressed if KefGB and KefFC use a K^+/H^+ antiporter mechanism in which K^+ efflux and H^+ uptake are mechanistically coupled. An NH_4^+/H^+ antiport mechanism for alkaliphile AmhMT would similarly make more physiological sense than NH_4^+ efflux alone. Cytoplasmic pH homeostasis at extremely high pH depends on H^+ capture and retention (2, 30). Efflux of NH_4^+ by a channel mechanism would result in cytoplasmic H^+ loss, whereas NH_4^+/H^+ would not. The *in vitro* assays reported here support a monovalent cation/proton antiport mechanism for YhaTU, AmhMT, and KefFC while also revealing distinctions among these two-component CPA2 systems.

Results

CPA2 Transporters YhaTU and AmhMT, as well as AmhT Alone, Exhibit K^+/H^+ Antiport Activity. Antiporter activity of the two *Bacillus* CPA2 systems of interest was assessed in a triple antiporter mutant of *E. coli*, strain KNabc. This host is deficient in K^+/H^+ as well as $Na^+(Li^+)/H^+$ and Ca^{2+}/H^+ antiport (31–33). Tris·HCl was used instead of the Tris-Hepes used in earlier assays (11, 12), and higher concentrations of K^+ were tested in view of recent reports of several bacterial K^+/H^+ antiporters with low affinity for cation (32, 34). In assays of the *B. subtilis* YhaTU system, neither the transporter protein YhaU nor the ancillary protein YhaT exhibited K^+/H^+ antiport activity at pH 8.5 when expressed individually. However, vesicles in which both YhaT and YhaU were expressed exhibited distinct K^+/H^+ antiport activity upon addition of 200 mM KCl (Fig. 2*A*). YhaTU also exhibited Rb^+/H^+ antiport activity, but neither Na^+/H^+ nor Li^+/H^+ antiport was observed (data not shown). The K^+/H^+ antiport activity of YhaTU was highest at pH 9.0, and no activity was observed at pH 7.5 (Fig. 2*B*). When assayed at pH 9.0 as a function of K^+ concentration, the apparent K_m value for K^+ of YhaTU was 7.2 mM (Fig. 2*C*). To test whether the presence of K^+ uptake systems in the *E. coli* KNabc strain impacted the antiport activity observed, assays were conducted with vesicles from transformants with the same plasmids in K^+-uptake-deficient *E. coli* TK2420, Δ(*kdp*ABC)*trk*D1Δ*trk*A (35). After correction for a low level of background activity in *E. coli* TK2420, the same antiport pattern and levels were observed in this host as in *E. coli* KNabc (data not shown).

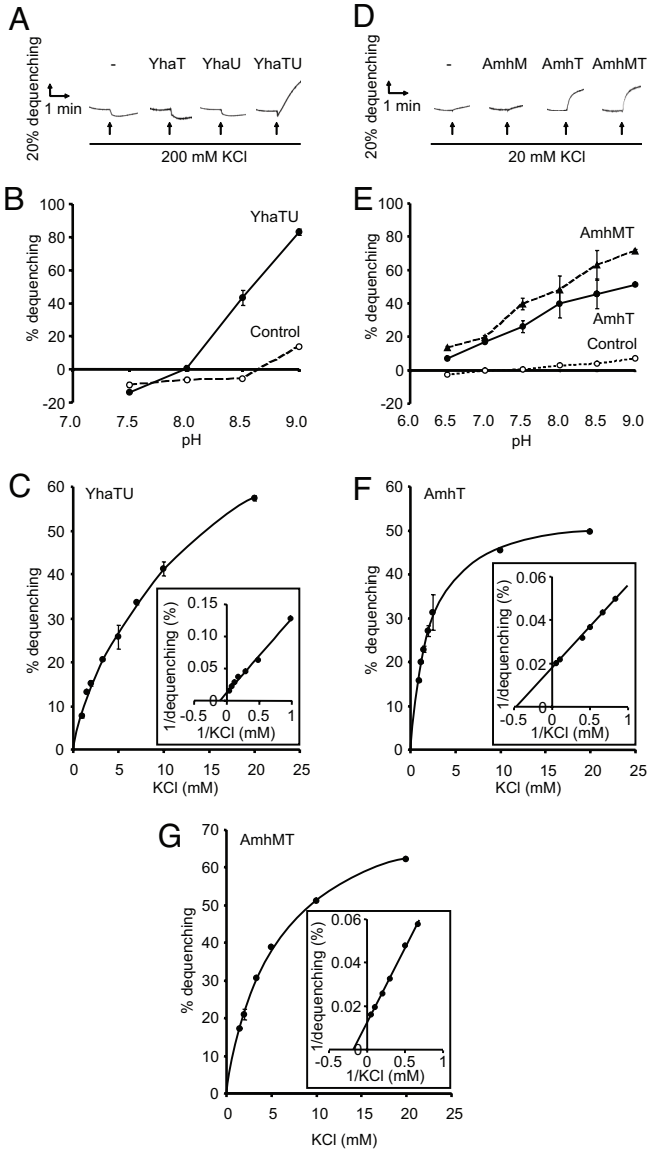

Fig. 2. K^+/H^+ antiport activity profiles of YhaTU, AmhT, and AmhMT in everted vesicles from transformants of *E. coli* KNabc. (*A–C*) Data for YhaT, YhaU, and YhaTU antiport. (*A*) Percentage dequenching of AO fluorescence when 200 mM K^+ was added to everted vesicles (pH 8.5) that had achieved a steady-state ΔpH, acid in, by respiration; the percentage dequenching is an assessment of K^+/H^+ antiport activity supported by control vector pKK223–3 (−) or vector expressing YhaT, YhaU, or YhaTU. (*B*) Control or YhaTU K^+/H^+ antiport activity as a function of pH. (*C*) Michaelis–Menten analysis for the K^+/H^+ antiport activity of YhaTU (pH 9.0). (*Inset*) Double reciprocal plot. (*D–G*) Data for AmhM, AmhT, and AmhMT antiport. (*D*) Antiport assays were conducted as in *A* except that 20 mM K^+ was added to vesicles (pH 8.5) of control (−) and pKK223–3 expressing AmhM, AmhT, or AmhMT. (*E*) Control, AmhT, and AmhMT K^+/H^+ as a function of pH. (*F* and *G*) Michaelis–Menten analyses for K^+/H^+ of AmhT and AmhMT (pH 8.5). (*Insets*) Double reciprocal plots. Dequenching traces are representative data; other data are presented with error bars that show the standard deviation.

K^+/H^+ antiport activity was also observed in assays of the AmhMT system from *B. pseudofirmus* OF4 in *E. coli* KNabc. The initial assays were conducted at 20 mM K^+ because earlier

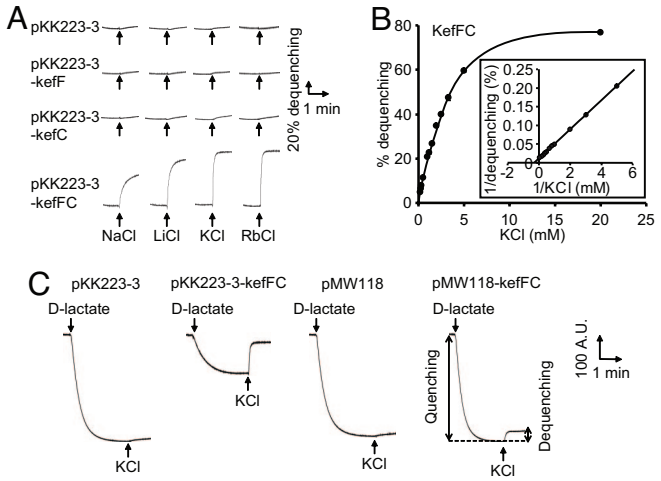

Fig. 3. Cation/H^+ antiport activity profile of KefFC. (*A*) Percentage dequenching in assays of pKK223–3 (control), pKK223–3-KefF, pKK223-KefC, and pKK223–3-KefFC. The indicated cations were added to 20 mM (pH 8.5). (*B*) Michaelis–Menten analysis of the K^+/H^+ antiport activity of KefFC (pH 8.5). (*Inset*) Double reciprocal plot. The error bars represent the standard deviation. (*C*) Representative traces of the D-lactate-dependent quenching (in arbitrary units of fluorescence) and K^+-dependent dequenching in vesicles from *E. coli* KNabc transformed with pKK223–3 (control), pKK223–3-KefFC, pMW118 (control), and pMW118-KefFC.

physiological studies suggested that this system functioned at low cation concentration (11). Both the membrane protein component AmhT alone and AmhMT together exhibited K^+/H^+ antiport activity (Fig. 2*D*), in contrast to YhaTU, which required both components for antiport activity (Fig. 2*A*). No activity was observed with AmhM alone. Both AmhT and AmhMT exhibited Rb^+/H^+ antiport activity but no $Na^+(Li^+)/H^+$ antiport activity (data not shown). AmhT- and AmhMT-mediated K^+/H^+ antiport activity was observed at pH 6.5 and was optimal at ≈pH 8.5 after correction for the background in the control (Fig. 2*E*). AmhT and AmhMT exhibited apparent K_m values for K^+ of 3.1 and 4.5 mM, respectively (Fig. 2 *F* and *G*). Thus, both two-component CPA2 systems from *Bacillus* catalyzed $K^+(Rb^+)/H^+$, and it was of interest to examine one of the homologous *E. coli* systems.

***E. coli* KefFC Exhibits $K^+(Rb^+)(Na^+)(Li^+)/H^+$ Antiport Activity.** To examine whether antiport capacity extended to *E. coli* KefFC, the most intensively studied bacterial CPA2 system, KefFC was expressed from pKK223–3 and studied in *E. coli* KNabc, compared with the empty vector and plasmids expressing only KefC or KefF. KefFC not only exhibited $K^+(Rb^+)/H^+$ antiport, but also showed a capacity for $Li^+(Na^+)/H^+$ antiport. Neither KefF nor KefC alone exhibited significant antiport with any of the cation substrates (Fig. 3*A*). A high level of KefFC-dependent K^+/H^+ antiport activity was observed over a pH range of 6.5–9.0 with no obvious optimum. When pH dependence was assayed in transformants expressing KefFC from the low copy vector pMW118 and corrections were made for the low background antiport activity of this strain at high pH, the pH optimum for KefFC-dependent antiport was ≥9.0 (data not shown). The apparent K_m for K^+ was examined for KefFC in the pKK223–3 transformant and found to be 3.8 mM at pH 8.5 (Fig. 3*B*).

Although KefFC-dependent antiport was clearly demonstrated by the assays, it was puzzling that the initial respiration-dependent AO quenching in the everted vesicles from pKK223–3-KefFC was always much lower than the quenching observed in the vector control vesicles (Fig. 3*C*). The initial quenching reflects the magnitude of the transmembrane pH gradient (ΔpH) generated by respiration net of H^+ leaks and any antiport that is occurring with contaminating cations. Vesicles with either KefF or KefC alone showed quenching that was the same as quenching in the control so neither protein created an intrinsic leak (data not shown). Instead, it seemed likely that KefFC-dependent antiport activity was occurring during the period after D-lactate addition and before KCl addition, using contaminating cations in the assay mix. This finding would account for a lower quench before addition of substrate cation in the assay protocol. Such an effect should be eliminated or reduced if KefFC were less highly overexpressed. To test this theory, assays were conducted on everted vesicles from *E. coli* KNabc expressing KefF, KefC, and KefFC from pMW118, a much lower copy plasmid than pKK223–3. As anticipated, the initial quenching was now comparable to that seen in the control vesicles, whereas the antiport activity assessed by percentage dequenching was much lower than that observed in vesicles from the pKK223–3-KefFC transformant (Fig. 3*C*). Even with the pMW118-KefFC transformant, significant $K^+(Rb^+)/H^+$ antiport activity was observed [Fig. 3*C* and supporting information (SI) Fig. 5] and modest Li^+/H^+ antiport activity was evident, whereas only a hint of Na^+/H^+ antiport activity was observed (SI Fig. 5). The next experiments used GSH, the *in vivo* inhibitor of KefFC-mediated K^+ flux, to test whether its addition would also increase quenching in respiring pKK223–3-KefFC vesicles and decrease the amount of antiport activity observed.

GSH Inhibits the K^+/H^+ Antiport of KefFC, and NADH Increases Inhibition. KefFC activity of *E. coli* cells is held in check by the cytoplasmic pool of GSH, with GSH binding directly to KefC (see Fig. 4*A*). This GSH-mediated inhibition is released when the organism is exposed to an electrophile such as endogenously produced methylglyoxal or an added electrophile such as *N*-ethylmaleimide (NEM). The electrophile forms adducts with the bound GSH and releases the inhibition of KefFC. The adducts may also activate K^+ efflux (16, 17, 29, 36). Antiporter-bound GSH is substantially lost during the preparation of everted membrane vesicles (14). If the low respiration-dependent quenching in pKK223–3-KefFC vesicles reflects antiport activity that uses contaminating cations, addition of GSH would be expected to increase the quenching, making it closer to the control level. GSH addition also would be expected to inhibit the antiport activity observed after the substrate cation is added (i.e., reducing the percentage dequenching upon K^+ addition). Because the transporter was overexpressed in the assay system, the effects of GSH were assayed with concentrations of GSH within and beyond the physiological range of ≤10 mM (37). The results showed that concentrations of GSH well within the physiological range increased the respiration-dependent acridine orange (AO) fluorescence quenching in the pKK223–3-KefFC vesicles. Higher concentrations of GSH doubled the size of the respiration-dependent quench relative to that observed in the absence of GSH (Fig. 4*B*). GSH also caused an ≈45% reduction in antiport activity (percentage dequenching) (Fig. 4*C*). The effects of GSH on quenching and dequenching depicted in Fig. 4 were tested by using a protocol in which ATP hydrolysis established the ΔpH that energized antiport (see *Methods*). Comparable effects were observed in vesicles energized with electron donors (NADH or Tris-D-lactate) (data not shown). The results indicate that GSH-suppressible antiport activity accounts for a significant portion, but not all, of the reduced respiration-initiated quenching in pKK223–3-KefFC relative to control vesicles. That activity is presumably supported by contaminating levels of cations in the buffer that can serve as antiporter substrates. Furthermore, the results demonstrated the inhibitory effect of GSH on the antiport activity of KefFC.

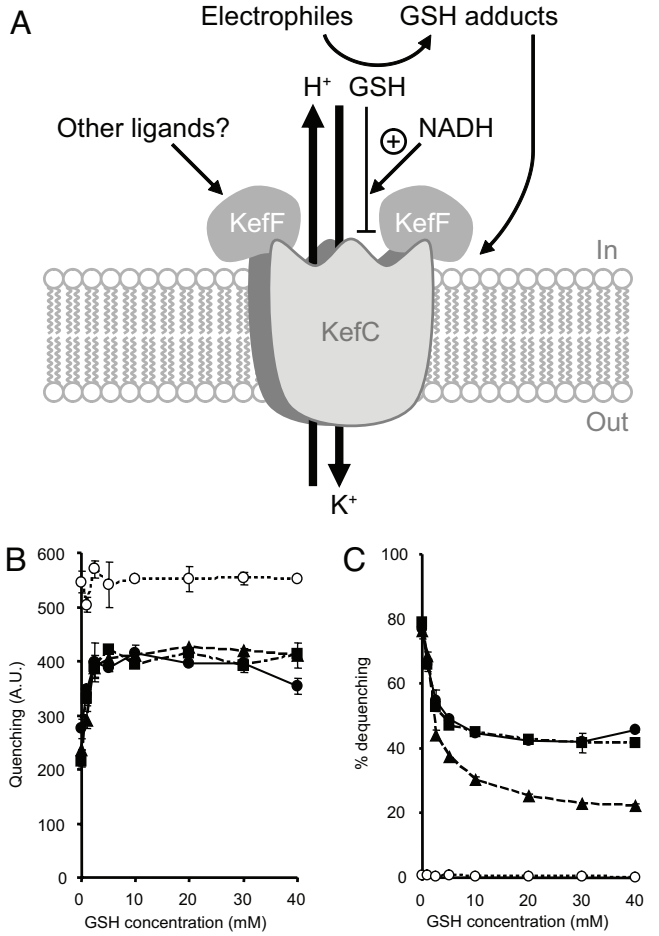

Fig. 4. Diagrammatic model of KefFC function and assays of GSH, NADH, and NAD^+ effects on net ΔpH generation (quenching) and antiport activity (percentage dequenching). (*A*) Schematic diagram of KefFC as a K^+/H^+ antiporter. Based on studies of other transporters (21, 23–25), KefC is likely to be a homooligomer to which an oligomeric KefF binds on the cytoplasmic side. Antiport activity is inhibited by GSH, which probably binds to KefC. NADH, presumably binding to the KTN/RCK domain of KefC, increases that inhibition. Although not studied here, electrophiles release the GSH inhibition, and the adducts formed between GSH and the electrophiles may activate antiport (35). Additional modulators that bind to KefF also are anticipated (see *Discussion*). (*B* and *C*) Net ΔpH generation (quenching) and antiport activity (percentage dequenching) was assayed in ATP-energized everted vesicles of *E. coli* KNabc transformed with a control vector or pKK223–3-KefFC; the effects of GSH with and without NAD^+ or NADH were determined. Assays were conducted at pH 7.5, and AO fluorescence was monitored by using 580 nm as the emission wavelength. All everted membrane vesicles were preincubated with 100 mM sodium cyanide for 10 min at room temperature before being diluted into the assay buffer and energized by addition of Tris-ATP as described (see *Methods*). The effects of the indicated concentrations of GSH on vesicles expressing KefFC (filled symbols) were assayed relative to a control with no additions (open circles, dashed lines). GSH additions were assayed in the presence or absence of 200 μM NADH or NAD^+. Shown are the effects of the additions on AO quenching (i.e., ΔpH generation) (*B*) and percentage dequenching (i.e., K^+/H^+ antiport activity) (*C*). The filled symbols represent the KefFC results with different GSH additions only (filled circles), GSH and NADH (filled triangles), or GSH and NAD^+ (filled squares). The error bars represent the standard deviations.

The KTN domain of KefC is expected to bind NADH, which could provide another regulatory feature for the KefFC transporter (26, 27). The *in vitro* antiport assay system using ATP hydrolysis to generate the ΔpH made it possible to test whether NAD^+ or NADH affects KefFC activity. The vesicles were pretreated with cyanide so that added NADH was not oxidized. The effects of added NAD^+ or NADH were examined in the absence and presence of GSH at pH 7.5. The addition of 200 μM of NAD^+ or NADH had no significant effect on quenching (i.e., on ΔpH generation) (Fig. 4*B*), nor did NAD^+ have any effect on antiport activity (dequenching) regardless of whether GSH was present (Fig. 4*C*). By contrast, NADH had a significant synergistic effect on GSH-mediated inhibition of antiport (Fig. 4*C*). NADH did not decrease dequenching in the absence of GSH, but in the presence of both GSH and NADH the maximal inhibition of antiport activity was ≈79% compared with the 45% inhibition by GSH alone (this synergy is shown schematically in Fig. 4*A*). Attempts were made to reverse the GSH inhibition of antiport by addition of NEM so that the *in vitro* system could be used to explore the reported *in vivo* effects of NEM and NEM-GSH adducts on GSH inhibition of KefFC (36) (Fig. 4*A*). These attempts were not successful because NEM caused dequenching in control vesicles regardless of whether an electron donor such as Tris-D-lactate or ATP was used to initiate quenching (data not shown). The NEM effects were not studied further, but the experiments demonstrate an inhibitory effect of GSH on KefFC-dependent antiport activity that was consistent with *in vivo* observations (10). They further show an effect of NADH on GSH-mediated inhibition of antiport activity that suggests a role for the KTN domain of KefC.

Discussion

The fluorescence-based assays of antiport activity showed that CPA2 cation/H^+ antiporters of the two-component subset catalyze monovalent cation/H^+ antiport. The YhaU and KefC membrane-transport components exhibited antiport activity only when expressed as YhaTU and KefFC pairs (Figs. 2*A* and 3*A*). Membrane protein AmhT alone exhibited antiport activity, but AmhMT conferred somewhat different properties (e.g., a higher apparent K_m for K^+) (Fig. 2 *D–G*). Because the ancillary protein of each of these protein pairs is encoded directly upstream of the membrane-transport protein and the two proteins are expressed together, it is likely that antiport is a major physiological activity of all three of these transporter pairs. However, membrane proteins YhaU and KefC both catalyzed K^+ efflux *in vivo* in the absence of their ancillary proteins (12, 20), leaving open the possibility that these proteins display channel-like activity in the absence of the ancillary protein. Antiport and pore-like properties also probably coexist in AmhT in the absence of AmhM. In earlier physiological experiments with the AmhMT system in the native alkaliphile host, there were conditions in which an *amhM* mutant of *B. pseudofirmus* OF4 exhibited more ammonium flux than the wild-type strain that contained both AmhM and AmhT (11). Channel-like properties may extend to CPA2 antiporters beyond the two-component subset (e.g., GerN) (Fig. 1) (38), but kinetic studies of the purified GerN as well as the two-component CPA2 systems studied here are needed to confirm these indications. NhaA, the extensively characterized, highly kinetically competent Na^+/H^+ antiporter of *E. coli* (2, 39), has been assigned to the CPA2 cluster of pro- and eukaryotic antiporters by Brett *et al.* (1). NhaA displays a unique fold with features that also are found in the chloride channel family (39). The chloride channel family has become a paradigm for a transporter family in which there are both channels and antiporters and in which some members "straddle" the demarcation between these two modes (40, 41). Comparable straddling has been noted in other transporter families and suggests the evolution of transporters from precursor channels (4).

The antiport capacity of the two-component subset of CPA2 proteins resolves questions about their functions *in vivo*. The antiport mechanism provides a direct mechanistic basis for the H^+ influx that mediates electrophile resistance when GSH-dependent inhibition of K^+ efflux by KefFC is released by

electrophiles (Fig. 4*A*) (6). KefGB is likely to function similarly, but probably differs from KefFC with respect to specific properties of substrate range, pH optimum, or kinetics. Perhaps in addition to supporting electrophile resistance, GSH-inhibited KefFC are partially activated at cytoplasmic pH values that approach the pK_a of glutathione, reported to be 9.2–9.4 in solution (42). Activation of antiport activity by KefFC at elevated pH could offer emergency antiport capacity to support toxic cation or alkali resistance at pH values above the optimal range of NhaA (2). The K^+/H^+ antiport capacity of KefFC is consistent with predictions from earlier work by Rosen and colleagues (43) of an *E. coli* K^+/H^+ antiporter that participates in cytoplasmic pH regulation and has a broad substrate range. It may also account for the residual K^+/H^+ antiport activity observed at high pH in a *chaA* mutant of *E. coli* (32), which was a level that exceeds the level expected from the multifunctional MdfA antiporter (34).

Studies of AmhMT in whole cells showed that NH_4^+ is the physiological substrate, although K^+ efflux is also catalyzed (11). Therefore, the current results imply that AmhMT is a $NH_4^+(K^+)/H^+$ antiporter. Use of a ΔpH-based assay precluded *in vitro* evaluation of NH_4^+ as a substrate for AmhMT as well as YhaTU and KefFC in the current studies. NH_4^+ addition would abolish the ΔpH and cause dequenching without a carrier. It will be of interest to test NH_4^+ as a substrate by using other assay protocols. If *E. coli* KefFC is a $K^+(Rb^+)(NH_4^+)/H^+$ antiporter (with more modest Na^+/H^+ antiport capacity), KefFC-mediated exchange reactions of the transporter could account for the K^+/NH_4^+ antiport reported in *E. coli* but not identified with specific gene products (44).

Finally, the two-component CPA2 subset appears to be integrated into the physiology of the cell by regulatory effectors, some of which are still to be identified (Fig. 4*A*) (10). NADH showed significant synergy with GSH in suppressing the antiport activity of KefFC, thereby providing a linkage between the NADH/NAD^+ ratio and the antiport activity of KefFC. The ancillary protein KefF is likely to bind additional small modulating ligands that reflect aspects of the metabolic state. KefF has an oxidoreductase domain (Fig. 4*A*) (10, 22) similar to that found in the ancillary protein of the two-component antiporter CzcOD (45). Integration of the activity of a Shaker channel family member with metabolism has similarly been suggested to be mediated by the redox state of NADPH by the aldo-keto-reductase activity of its β-subunit (46).

In summary, the experiments demonstrate antiport activity for YhaTU, AmhMT, and KefFC, three two-component bacterial transport systems of the CPA2 family that also have channel-like properties. The studies confirm the inhibitory effect of GSH on KefFC activity that was demonstrated by others *in vivo* and demonstrate a synergistic effect of NADH on that inhibition. The findings also raise the possibility of NH_4^+ as an additional antiport substrate for one or more of these transporters.

Methods

Bacterial Strains, Plasmids, Chemicals, and Growth Conditions. The *E. coli* strains used in this study were DH5αMCR (Gibco-BRL, Gaithersburg, MD), XL-1 blue MRF′ (Promega, Madison, WI), and Na^+/H^+ antiporter-deficient KNabc (Δ*nhaA* Δ*nhaB* Δ*chaA*) (33). The plasmids used for gene cloning were low copy number pMW118 (Nippon Kayaku, Tokyo, Japan) and high expression vector pKK223–3 (Amersham Pharmacia Biotech, Piscataway, NJ). *E. coli* strains were routinely grown at 37°C in LBK medium (47) with appropriate antibiotics at the following concentrations: 100 μg/ml ampicillin, 25 μg/ml kanamycin, 25 μg/ml chrolamphenicol, and 300 μg/ml erythromycin. For growth of some transformants, 10 mM glucose was added to support a sufficient growth yield. For cloning in pMW118, S-gal/LB agar (Sigma–Aldrich, St. Louis, MO) plates were used to select positive clones. GSH-reduced form, ATP bis-Tris salt dihydrate, β-NAD, and β-NADH di-Tris salt were purchased from Sigma–Aldrich. Sodium cyanide was obtained from Fisher Scientific Co. (Pittsburgh, PA).

Cloning of Genes Encoding the Two-Component CPA2s Transporters and Their Individual Components. Cloning of pKK223–3-*yhaU* and pKK223–3-*yhaTU* was described elsewhere (12). For the constructs produced expressly for this study, PCR was carried out on chromosomal DNA by using AccuPrime *Pfx* DNA Polymerase (Invitrogen, Carlsbad, CA) or Vent DNA Polymerase (New England BioLabs, Ipswich, MA) according to the instructions. In all instances, the structural genes were cloned together with their native ribosome-binding sites behind the *tac* promoter in pKK223–3 constructs and behind the *lac* promoter in pMW118 constructs. Basal levels of expression of the cloned genes, without addition of inducer, were used for all experiments. The sequences of all constructs were verified by sequence analyses performed by Hokkaido System Sciences (Hokkaido, Japan), by using an ABI-100 model 377 sequencer, or by the DNA Sequencing Core Facility at Mount Sinai School of Medicine, by using an Applied Biosystems (Foster City, CA) 373A DNA sequencer. Details of the constructions and primers used are provided in *SI Methods*.

Preparation of Membrane Vesicles and Antiport Assays. Everted membrane vesicles were prepared by the method described by Rosen (48). Fluorescence assays of $K^+(Rb^+, Na^+, Li^+)/H^+$ antiport activity by using AO as a probe of the ΔpH were conducted in everted membrane vesicles at room temperature (47) using spectrofluorophotometer RF-5301PC (Shimadzu, Kyoto, Japan). The excitation and emission wavelengths were 420 nm and 500 nm, respectively, with a 10-nm slit unless otherwise noted. The membrane protein concentration was assayed by the Folin reagent method (49), using BSA as a standard. The antiport assay buffer, to which 100 μg of everted membrane vesicle protein was added, contained 10 mM Tris·HCl, 140 mM Cl, 5 mM $MgCl_2$ and 1 μM AO. The pH was adjusted to 8.5 except where indicated. To start the assay, Tris-D-lactate (pH 8.0) was added as an electron donor to a final concentration of 2 mM from a more concentrated buffered solution. After the initial fluorescence quenching leveled off, reflecting the establishment of a steady-state, respiration-generated ΔpH, test cations were added as their chloride salts at concentrations indicated for particular experiments. The resulting dequenching of fluorescence that reflects antiport activity was calculated from the value obtained 1 min after salt addition. For some experiments in which GSH was added and in those experiments in which the effects of NADH and NAD^+ on antiport activity were assessed, 1 mM Tris-ATP was used to energize the vesicles instead of electron donors to prevent consumption of added NADH. In this protocol, the vesicles were preincubated with 100 mM sodium cyanide for 10 min at room temperature before the assay. After dilution of these vesicles into the assay mixture, the final $[Na^+]$ in the assay was 0.5 mM. The assays were conducted at pH 7.5, and AO fluorescence was monitored at 580 nm instead of 500 nm. Use of 580 nm avoided interference from NADH while making only a minimal change in AO fluorescence. All fluorescence assays were conducted in duplicate on at least two independent vesicle preparations.

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