KefF, the Regulatory Subunit of the Potassium Efflux System KefC, Shows Quinone Oxidoreductase Activity††

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Electrophilic compounds are often highly toxic, because they readily react with nucleophiles found both in the bases of DNA and within functionally important side chains of proteins, especially cysteine. Bacteria produce electrophiles themselves as metabolic by-products when they are unable to regulate carbon influx (39). In addition, pathogens may become exposed to electrophiles from exogenous sources (e.g., during entry into macrophages [10]). After entering the cell, electrophiles either react spontaneously with glutathione (GSH) or are conjugated to the nucleophile by glutathione S-transferase (40), which is the first step in detoxification. The glutathione adducts (GSX) can then be further detoxified (14, 27, 28). Despite active detoxification, potentially lethal direct reaction with DNA and other important macromolecules may occur, and ancillary damage may arise from depletion of the GSH pool (E. Ozynamak, C. Almeida, A. Moura, S. Miller, and I. R. Booth, unpublished data). Bacterial survival increases significantly if the cytosol is acidified during electrophile exposure (11, 13), possibly due to the reduction in nucleophile reactivity caused by protonation or partial protonation at lower pH values. Potassium efflux via KefC is accompanied by \( \text{H}^+ \) and \( \text{Na}^+ \) influx and hence causes acidification of the cytoplasm. Kef is activated by GSH and inactivated by GSH, binding to its cytosolic regulatory \( \text{K}^+ \) transport and nucleotide binding (KTN) domain. Kef systems are found only in Gram-negative bacteria and have been most extensively studied in \textit{Escherichia coli}, which has two homologues, KefB and KefC, showing differential sensitivity to electrophiles (12). Recently, we showed that GSH and GSX occupy overlapping binding pockets in KefC but that the additional steric bulk of GSX causes a significant conformational change, which results in activation of KefC (35). In addition, the KTN domain has a nucleotide binding site on a Rossmann fold (36), and it has been suggested that KefC is inactivated when NADH is bound at this site (16).

In many bacteria, including \textit{E. coli}, regulation of Kef is still more complicated, as soluble KefB/KefC-binding proteins have been discovered (KefG and KefF, respectively) that are required to associate with KefB/KefC to allow full activation of these systems (29). An X-ray crystal structure of the KefC KTN domain in complex with KefF indicates that binding of the KefF dimer to KefC holds the KTN domains at a specific angle that is believed to be favorable for KefC activity (36). Surprisingly, the sequences of KefG/KefF show homology to quinone reductases, such as the human quinone reductase 1 (QR1) and QR2. Comparison of the X-ray crystal structures of KefF and human QR1 demonstrates that the sequence similarity to QR1 and QR2 is also reflected in the fold of the proteins (see Fig. S1 in the supplemental material). Furthermore, the flavin mononucleotide cofactor, FMN, required for oxidoreductase catalytic reaction, is bound to KefF in the crystal structure. Immediately adjacent to the bound FMN, there is a hydrophobic cleft suitable for substrate binding (Fig. 1). Within this cleft, electron density is present in the X-ray crystal structure, but the bound compound could not be identified (36). Thus, we sought to establish whether KefF has enzyme
activity in addition to its role as an activator of KefC and whether these two functions are directly connected.

We demonstrate here that KefF is an effective oxidoreductase for a wide range of electron acceptors, but not for formerly known electrophiles that activate the KeF systems, like, for example, methylglyoxal (MG) and N-ethyl maleimide (NEM). The enzymatic activity of KefF is not directly required for activation of KefC. Furthermore, electrophilic quinones, which readily form adducts with GSH, were established as potent activators of KefC and are also good substrates for the enzymatic activity of KefF.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are described in Table 1. The expression construct pTrcYabFH6, used for most experiments, carries a C-terminal His6 tag. The gene was cloned via an NcoI site at the N-terminus. The expression construct pTrcYabFH6, used for most experiments, carries a C-terminal His6 tag. The gene was cloned via an NcoI site at the N-terminus. The resulting DNA was purified using a Qiagen I-quick Kit, treated with DpnI, and transformed by electroporation into BW25113 pKD46. The transformed cells were recovered on LK medium (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter KCl) containing kanamycin (25 μg/ml) and then tested for growth on LK medium containing kanamycin or ampicillin (25 μg/ml).

Colonies that retained only kanamycin resistance (thus judged to no longer carry the pK46 plasmid) were verified for creation of kefF::kan knockout by PCR using primers designed for regions outside the kefF locus. The resultant strain, BW25113 ΔyabF::kan, was transduced to kefGB::apr using E. coli strain DY330 ΔkefGB::apr (E. Ozyamak and I. R. Booth, unpublished data) as a donor and a standard P1 phage protocol to create strain MJF654 (ΔyabF::kan/kefGB::apr). MJF654 colonies were selected by resistance to 60 μg/ml apramycin (apr) and 25 μg/ml kanamycin (kan).

E. coli MJF654 cells were cultured in K115 minimal medium at 37°C to an optical density of 0.5 at 650 nm (OD650) of ~0.8 to 1 (9). Minimal medium consists of 46 mM KH2PO4, 23 mM KH2PO4, and 8 mM (NH4)2SO4 supplemented with separately autoclaved 0.4 mM MgSO4, 6 μM (NH4)2SO4, 1 mg/liter thiamine hydrochloride, and 2 g/liter glucose. The cell culture was harvested by filtration, and the cells were washed with K0 buffer before they were suspended in potassium-free K+ buffer. For the K+ buffer, the potassium salts of the K+ medium were replaced by sodium salts and the supplements were omitted. The K+ buffer was prepared by adding 10 mM KCl to K0 buffer. Equal volumes of cells were then poured into two insulated stirring vessels maintained at 37°C, and 0.5 mM NEM was added to one vessel. Samples were taken from each vessel at defined time intervals for measurement of the retained cellular potassium content by flame photometry. The logarithmic values of the intracellular potassium concentrations were plotted against the time after addition of NEM, and the slope was determined by linear regression. Potassium efflux triggered by quinones was measured with an ion-selective electrode as described previously (35) using the strains MJF274, MJF276, Frag56, and MJF654. Cells were grown and washed as described above. They were suspended in 5 ml potassium-free K+ buffer and added to 30 ml of K+ buffer in a single stirred vessel held at 37°C with equilibrated electrodes inserted. After 5 min, either quinones or NEM (0.5 mM final concentration) was added, and the potassium content of the medium was recorded continuously. Rate constants were obtained, using the program Origin 8.0 (Originlab) (35), by fitting exponentials to the change in potassium concentration after addition of an activator.

Protein expression and purification. KefF constructs were transformed into the E. coli strain MJ362 for expression. The culture was grown in 0.5 liter terrific broth (TB) at 37°C. For the TB medium, 12 g tryptone, 24 g yeast extract, 10 g NaCl, 5 g/liter yeast extract, 5 g/liter KCl, and 1 g/liter MgSO4 were added. The K10 buffer was prepared by adding 10 mM KCl to K0 buffer. Equal volumes of cells were then poured into two insulated stirring vessels maintained at 37°C, and 0.5 mM NEM was added to one vessel. Samples were taken from each vessel at defined time intervals for measurement of the retained cellular potassium content by flame photometry. The logarithmic values of the intracellular potassium concentrations were plotted against the time after addition of NEM, and the slope was determined by linear regression. Potassium efflux triggered by quinones was measured with an ion-selective electrode as described previously (35) using the strains MJF274, MJF276, Frag56, and MJF654. Cells were grown and washed as described above. They were suspended in 5 ml potassium-free K+ buffer and added to 30 ml of K+ buffer in a single stirred vessel held at 37°C with equilibrated electrodes inserted. After 5 min, either quinones or NEM (0.5 mM final concentration) was added, and the potassium content of the medium was recorded continuously. Rate constants were obtained, using the program Origin 8.0 (Originlab) (35), by fitting exponentials to the change in potassium concentration after addition of an activator.

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and 4 ml glycerol were dissolved in 900 ml deionized water and autoclaved. One hundred milliliters of separately sterilized phosphate buffer (0.17 M K$_2$HPO$_4$ and 0.72 M K$_2$HPO$_4$) was added after cooling. When the culture reached an OD$_{650}$ of 0.8 to 1, expression was induced with 1.1 mM IPTG (isopropyl-$\beta$-D-thiogalactopyranoside) for 3 h at 25°C. Cells were harvested by 15 min of centrifugation at 4,000 $\times$ g, suspended in 20 ml lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol), and stored at $-80^\circ$C. Phenylmethylsulfonyl fluoride (PMSF) (1 mM), 10 mM benzamidine, and 10 $\mu$M FMN were added to the cell suspension before lysis in a French press (SLM Aminco) at a pressure of 18,000 lb/in$^2$. Bacterial membranes were removed by ultracentrifugation at 100,000 g for 1 h. The supernatant was filtered before binding to 2 ml Ni-nitriol triaetate acide (NTA) affinity matrix (Sigma) packed in a column. After washing the column with 30 ml washing buffer (50 mM imidazole, 50 mM HEPES, 150 mM NaCl, and 10% glycerol), KefF was eluted with 5 ml elution buffer (same as washing buffer but with 300 mM imidazole). Purified protein was analyzed by SDS-PAGE using a precast 4 to 12% NuPage Novex Bis-Tris gradient gel, and sharp bands observed for other proteins on similar SDS-PAGE loading concentrations, it runs as a diffuse band rather than the sharp bands for free FMN of 12.5 mM. The protein concentration was determined using the Lowry method (25) with bovine serum albumin (BSA) as a standard, and the FMN concentration was determined using the fluorescence of the flavin. For the FMN determination, purified KefF samples and free FMN as calibration samples were diluted in buffer (50 mM MES [morpholinolinosulfonic acid]-NaOH, pH 6.0, and 50 mM NaCl) to appropriate dilutions. The samples and calibration samples were incubated for 10 min at 100°C wrapped in tin foil. After cooling, they were centrifuged at 13,000 $\times$ g for 10 min at 4°C. Fluorescence was measured with an FLS920 spectrometer (Edinburgh Instruments) at an excitation wavelength of 450 nm and an emission wavelength of 530 nm. Concentrations of KefF and FMN were confirmed by UV/visible-light (Vis) absorption. Extinction coefficients for the FMN bound to KefF were determined by comparison of absorption spectra of native KefF before and after incubation with 0.2% SDS for 10 min in the dark at room temperature (RT) to release the FMN. An extinction coefficient for free FMN of 12.5 mM$^{-1}$ cm$^{-1}$ at 446 nm ($\varepsilon_{446}$) was assumed (26).

**Enzyme kinetics.** Enzyme kinetics were measured using a Cary50 UV/Vis spectrometer (Varian) with a temperature-controlled Pelletier cell holder and stirred cells (25°C) in 50 mM HEPES, pH 7.5, with 150 mM NaCl and 3 $\mu$M FMN. A constant concentration of 0.75 mM NADH was used, and its oxidation was detected at 340 nm using an extinction coefficient ($\varepsilon_{340}$) of 6.22 mM$^{-1}$ cm$^{-1}$, while acceptor substrates were used at a final concentration of 0.1 mM, except oxygen, which was air-saturated buffer (approximately 0.25 mM at 25°C). A stock solution of 20 mM benzoquinone was prepared in assay buffer, and activities were corrected for the spontaneous reaction of this acceptor. For other quinones, stocks were made in ethanol, keeping the final concentration of ethanol in the assay buffer 0.5%. The enzymatic reduction of the substrate thiazolylblue tetrazolium bromide (MTT) was measured at different concentrations of MTT and NADH/NADPH by the increase in the absorbance at 610 nm due to the formation of blue formazan using an extinction coefficient ($\varepsilon_{610}$) of 11.3 mM$^{-1}$ cm$^{-1}$.

Specific activities were fitted to the Michaelis-Menten equation using Origin 8.0 (OriginLab), and mean values with standard deviations for 3 independent measurements are reported.

**RESULTS**

**Purification of KefF.** A C-terminally His-tagged construct was used to express and purify KefF in a two-step protocol. During washing of the Ni$^{2+}$ immobilized-metal affinity chromatography (IMAC) column, the bed color changed to yellow due to the presumed oxidation of the FMN cofactor (this was not observed for the mutant G107S described below). After elution from the Ni$^{2+}$ column, KefF was further purified and characterized by size exclusion chromatography (see Fig. S4 in the supplemental material). KefF elutes as a homogeneous peak at an elution volume corresponding to a dimeric complex, as expected from the crystal structure and homologous quinone reductases (15, 23, 35, 36). For a soluble protein, KefF shows unusual behavior on SDS gels as, even at moderate loading concentrations, it runs as a diffuse band rather than the sharp bands observed for other proteins on similar SDS-PAGE gels (Fig. 2A). Attempts to obtain sharper bands by modifying the denaturation before loading and by addition of urea to the loading buffer were unsuccessful.

UV/Vis spectra of purified KefF showed the typical absorption peaks of oxidized flavins, with absorption maxima at 379 and 457 nm (in comparison to 373 and 446 nm for free FMN) and extinction coefficients as follows: $\varepsilon_{379}$ 8.1 mM$^{-1}$ cm$^{-1}$ and $\varepsilon_{457}$ 10.0 mM$^{-1}$ cm$^{-1}$ (Fig. 2B). After purification, the flavin occupancy was never 100% and varied between 40 and 70% (Table 2). Others have made similar observations for proteins that bind FMN (34). Attempts to reconstitute FMN to

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**TABLE 2. Apparent steady-state parameters and FMN contents of KefF**

<table>
<thead>
<tr>
<th>KefF</th>
<th>FMN/KefF ratio (mol/mol)</th>
<th>$k_{on}^{app}$ (NADH) (s$^{-1}$)</th>
<th>$K_m^{app}$ (NADH) (mM)</th>
<th>$k_{cat}^{app}$ (MTT) (s$^{-1}$)</th>
<th>$K_m^{app}$ (MTT) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (NADH)</td>
<td>0.4 ± 0.1</td>
<td>1.9 ± 0.6</td>
<td>0.15 ± 0.04</td>
<td>2.2 ± 0.8</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>WT (NADPH)*</td>
<td>0.4 ± 0.1</td>
<td>1.7 ± 0.5</td>
<td>0.07 ± 0.06</td>
<td>2.7 ± 1.9</td>
<td>0.7 ± 0.6</td>
</tr>
<tr>
<td>G107S</td>
<td>not detectable</td>
<td>0.04 ± 0.03</td>
<td>1.1 ± 0.1</td>
<td>0.015 ± 0.005</td>
<td>0.13 ± 0.06</td>
</tr>
<tr>
<td>H112W</td>
<td>0.61 ± 0.05</td>
<td>0.28 ± 0.04</td>
<td>4.75 ± 2.5</td>
<td>0.06 ± 0.03</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>F149W</td>
<td>0.7 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.05 ± 0.02</td>
<td>0.3 ± 0.1</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>C147S</td>
<td>0.5 ± 0.1</td>
<td>2.1 ± 0.2</td>
<td>0.06 ± 0.03</td>
<td>2.7 ± 0.6</td>
<td>0.43 ± 0.06</td>
</tr>
<tr>
<td>C151S</td>
<td>0.53 ± 0.07</td>
<td>2.6 ± 0.7</td>
<td>0.07 ± 0.03</td>
<td>3.2 ± 0.7</td>
<td>0.33 ± 0.06</td>
</tr>
<tr>
<td>C147S/C151S</td>
<td>0.42 ± 0.07</td>
<td>2.0 ± 0.7</td>
<td>0.05 ± 0.03</td>
<td>2.9 ± 0.9</td>
<td>0.53 ± 0.06</td>
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</tbody>
</table>

*a The values in this row are for the reaction of NADPH instead of NADH.*
and MG could not directly serve as electron acceptors in a detoxification reaction catalyzed by KefF (see Fig. S2C in the supplemental material).

**Enzymatic activity of KefF is not required for activation of KefC.** We have established that KefF shows redox enzymatic activity with a wide range of substrates. This posed the question whether there is a linkage between this enzymatic activity and the role of KefF as a modulator of potassium efflux through KefC. To address this question, we introduced mutations in the active site of KefF designed to inhibit the enzymatic redox activity. To avoid nonspecific effects on the regulation of KefC, for example, by misfolding of the KefF protein, we used the X-ray crystal structure to choose residues that when mutated were unlikely to modify the structural integrity of KefF. Three mutants, G107S, H112W, and F149W, were constructed. The first mutant was envisaged to disrupt binding of FMN to KefF, since the side chain of serine extends to a region where the ribose backbone of FMN is observed in the crystal structure. The other two mutations were intended to block the substrate-binding pocket with the more bulky tryptophan side chain. Upon purification, all of these mutated KefF constructs were obtained in yields similar to those of the wild type, and similar size exclusion profiles of the dimeric complex were observed (see Fig. S4 in the supplemental material). As expected, KefF G107S had no FMN bound to it, and accordingly, no yellow coloration was seen during purification. All three mutations caused a significant reduction of redox enzymatic activity (Table 2). These constructs were then transformed into a strain lacking KefF and tested for their abilities to activate potassium efflux via KefC. All three constructs were able to activate potassium efflux, despite their lack of enzymatic activity (Fig. 3). This observation showed clearly that the enzymatic activity was not required for activation of potassium efflux.

On inspection of the crystal structure of KefF, we observed two cysteine residues, C147 and C151, on the protein surface close to the active site. The activity of the quinolinate synthase

<table>
<thead>
<tr>
<th>Electron acceptor</th>
<th>Sp act (μmol min⁻¹ mg⁻¹)</th>
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<tbody>
<tr>
<td>Benzoquinone</td>
<td>125 ± 29</td>
</tr>
<tr>
<td>Methylbenzoquinone</td>
<td>146 ± 22</td>
</tr>
<tr>
<td>Duroquinone</td>
<td>110 ± 30</td>
</tr>
<tr>
<td>2,3-Dimethoxy-5-methyl-p-benzoquinone (Qₐ)</td>
<td>109 ± 30</td>
</tr>
<tr>
<td>Menadione</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>DCIP</td>
<td>131 ± 35</td>
</tr>
<tr>
<td>Ferricyanide</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>FMN</td>
<td>0.2 ± 0.02</td>
</tr>
<tr>
<td>Oxygen</td>
<td>0.09 ± 0.02</td>
</tr>
</tbody>
</table>

**KefF shows oxidoreductase activity.** As KefF has sequence and structural similarities to quinone reductases and retains the FMN cofactor, we evaluated its ability to act as a redox enzyme. First, we tested whether the FMN could be reduced with the physiological reductants NADH and NADPH. It was observed that both were able to reduce FMN bound to KefF, as judged from absorption spectra. The FMN reverted to its oxidized state under aerobic conditions, but not under anaerobic conditions, demonstrating that the reduced flavin cofactor in KefF showed some reactivity to oxygen (see Fig. S2A in the supplemental material).

Several electron acceptors for the oxidative half-reaction were tested in enzymatic steady-state assays. Many substrates were reduced with a high specific activity, while the turnover with oxygen was slow (Table 3). Quinones, including benzoquinone, menadione, and duroquinone, were readily reduced by KefF. For duroquinone, an apparent $k_{cat}$ of $99 \pm 27 \text{ s}^{-1}$ and $K_m$ of $14 \pm 2 \text{ μM}$ were determined (at a constant concentration of 0.75 mM NADH), which are comparable to other quinone reductases (24, 34). The 1-electron acceptors 2,6-dichloroindophenol (DCIP) and ferricyanide are good substrates for KefF (Table 3). MTT could be directly used as an electron acceptor (Table 2), while FMN, if used as a substrate, showed only low activity. On the other hand, no activity toward the nitro compound nitrofurantoin and the azo dyes Congo red and Orange II was detected. NADH and NADPH were comparable as electron donors for KefF in steady-state experiments with MTT as an acceptor (Table 2). Measurement of the enzyme rates with different substrate concentrations, for both the electron donor and acceptor, resulted in a group of parallel lines in the Lineweaver-Burk plot, indicating a Ping-Pong Bi-Bi mechanism, in which one substrate reacts with the enzyme and the product is released before a second substrate reacts with the modified enzyme (see Fig. S3 in the supplemental material). Although stable reconstitution with FMN could not be achieved, as described above, functional reconstitution was seen by a significant increase in activity if 3 μM FMN was present in the assay buffer (see Fig. S2B in the supplemental material). Similarly, the azoreductase AzoR was reported to require free FMN in the assay buffer for enzymatic activity (24). No enzymatic activity was found for typical electrophiles that activate potassium efflux through the Kef systems. NEM and MG could not directly serve as electron acceptors in a

![FIG. 3. Comparison of enzymatic activity of KefF with potassium efflux of KefFC. Relative $k_{cat}$ values of purified WT KefF and mutants with the substrates NADH and MTT (open circles) are compared with the relative rate constants for potassium efflux of the plasmid-free and transformed strain MJF654 triggered by NEM (closed circles). The data were normalized with WT KefF (NADH) values. For WT KefF, the enzymatic activity with NADPH is also shown. The error bars indicate standard deviations.](http://jb.asm.org/Downloaded from on March 8, 2021 by guest)
(NadA) from *E. coli* is regulated by the redox state of a surface-exposed cysteine pair (37). Similarly, surfaced-exposed cysteine residues in KefF could potentially sense oxidative stress of the cell by forming a disulfide bridge or reacting directly with electrophiles, as their surface location will likely lead to high reactivity. Therefore, we introduced the C147S and C51S mutations and measured the enzymatic activities of the resultant proteins and their abilities to activate potassium efflux. The single mutants and the double mutant displayed no significant difference from the wild type in NADH-MTT oxidoreductase activity and potassium efflux, giving no indication of a regulatory function for these cysteine residues (Fig. 3 and Table 2). Furthermore, no change in oxidoreductase activity could be seen when wild-type KefF was preincubated with NEM and MG prior to determination of the enzymatic activity (measured with NADH and MTT). Thus, modification of the cysteine residues is unlikely to modulate the behavior of KefF.

**Activation of potassium efflux by electrophilic quinones.**

Known activators of the Kef system, such as NEM and menadione, which belong to this group of GSH-reactive quinones, elicit potassium efflux via KefC (Fig. 4B and C). When KefFC was overexpressed, the rate constant for efflux was enhanced relative to the rate observed with just the chromosomal copy of the *kefFC* genes (Fig. 4, inset). Benzoquinone, in particular, gave a level of activation similar to that of NEM, judged by the rate constants (Fig. 4, inset). In contrast, no increase in K+/H1001 efflux was observed for quinones that undergo conjugate addition reactions less readily, such as duroquinone. It should be noted that each of the three quinones used in our experiments triggered some loss of K+/H1001 via a mechanism that was independent of KefB and KefC (Fig. 4B to D).

We utilized an isogenic glutathione-deficient strain (Frag56 *gshA* [Table 1]) to determine whether activation of KefC by electrophilic quinones was GSH dependent. When GSH was present in the growth medium, Frag56 became GSH replete, and potassium efflux was seen after the addition of benzoquinone. In contrast, in the absence of GSH, only a small effect occurred, likely due to the above-mentioned background efflux mechanism (Fig. 5A). This difference showed that GSH was required for quinone-elicited efflux, as expected for KefC activation. Quinone-activated KefC activity was diminished in the absence of KefF, but the KefF mutants G107S and H112W could restore full activity (equivalent to wild-type KefF) in the KefF-null strain when benzoquinone was used as an electrophile (Fig. 5B).

**DISCUSSION**

Soluble quinone reductases often have a broad substrate specificity for the oxidative half-reaction. Therefore, these enzymes have often been named according to their different acceptors, for example, azoreductase, nitroreductase, chromate reductase, ferric reductase, or flavin reductase. These additions at the different ortho positions can take place if the quinone is reoxidized to the quinone (22). Benzoquinone and menadione, in particular, gave a level of activation similar to that of NEM, judged by the rate constants (Fig. 4, inset). In contrast, no increase in K+ efflux was observed for quinones that undergo conjugate addition reactions less readily, such as duroquinone. It should be noted that each of the three quinones used in our experiments triggered some loss of K+ via a mechanism that was independent of KefB and KefC (Fig. 4B to D).

We utilized an isogenic glutathione-deficient strain (Frag56 *gshA* [Table 1]) to determine whether activation of KefC by electrophilic quinones was GSH dependent. When GSH was present in the growth medium, Frag56 became GSH replete, and potassium efflux was seen after the addition of benzoquinone. In contrast, in the absence of GSH, only a small effect occurred, likely due to the above-mentioned background efflux mechanism (Fig. 5A). This difference showed that GSH was required for quinone-elicited efflux, as expected for KefC activation. Quinone-activated KefC activity was diminished in the absence of KefF, but the KefF mutants G107S and H112W could restore full activity (equivalent to wild-type KefF) in the KefF-null strain when benzoquinone was used as an electrophile (Fig. 5B).
enzymes have similar structures, possessing a flavin cofactor at the active site and a common flavodoxin fold, but often display low sequence similarity (7). In *E. coli*, several of these soluble NADH/NADPH-dependent oxidoreductases have been described: the tryptophan repressor-binding protein WrbA (34), the azoreductase AzoR (32), nitroreductases (41), and the so-called modulator of drug activity MdaB (17). With several similar oxidoreductases present with overlapping substrate selectivities, it is difficult to reveal the physiological function for each of them. In a comparison with structures in the Protein Data Bank (PDB) using the program DALI (18, 19), KefF shows much higher similarity to the human quinone reductases QR1 and QR2 than to the other oxidoreductases in *E. coli*. Human QR1 showed the highest similarity, with a Z score of 20.3 and a root mean square (RMS) of 2.2 Å for 167 of 272 residues (PDB code, 1QBG) (18, 38). Like QR1, KefF can accept NADH and NADPH as electron donors; it has a size similar to that of QR2 but is shorter than QR1. QR1 provides protection against oxidative stress caused by quinones, while the function of QR2 is unknown.

Comparison of the crystal structure of KefF with those of human QR1 or QR2 revealed that both the substrate-binding pocket and the flavin in KefF are much more accessible: residues 48 to 80 shield the active site in the human QRs and are missing in KefF (see Fig. S1 in the supplemental material). This difference might enable KefF to accept bigger substrates, such as MTT. The binding pocket of KefF is almost exclusively lined by aromatic residues (W67, Y68, Y88, H112, F113, and Y134) and FMN (Fig. 1). Both subunits provide residues to the active site. In QR1, the reduction of the flavin is facilitated by the provision of a proton from Y155 to O5 of the flavin adenine dinucleotide (FAD) and further charge distribution to H161 (23). The residue homologous to Y155 in KefF is F113, which is not able to provide a proton to the flavin. This function could be fulfilled by H112, which is in closer proximity to the flavin than H161 in human QRs. Mutation of H112 to tryptophan caused loss of catalytic activity in KefF, which could arise either because it simply blocks the binding site or because H112 is involved in catalysis. A charge on H112 might be stabilized by interaction with Y68 or Y134. The charge relay mechanism of human QR1 is conserved in some QRs, including *E. coli* MdaB (Y119) and human QR2 (Y155), but variations are seen for other QRs, for example, WrbA. Sequence alignments show that for *E. coli* KefG, which partners KefB, a tyrosine is conserved in this position; the H112 of KefF is not conserved in KefG. H112, which is at the end of the binding pocket, is also well positioned to form hydrogen bonds with the substrate in a manner similar to that of H161 in human QR1 and QR2.

The binding pocket of KefF is more hydrophilic and possesses more residues capable of forming H bonds with the substrates than the human QRs. Y68 and Y134 in KefF are equivalent to F106 and F178 in human QRs (Fig. 1). Y88 of KefF is conserved in human QR1 (Y126), while a phenylalanine is found in QR2 (F126). Y134 is positioned parallel to the aromatic system of the flavin, leaving space where an aromatic substrate could fit to form a π stack, as seen in QR1 with the nicotinamide ring of NADPH (23) and in QR2 with menadione (15) and the inhibitor resveratrol (3). A phenylalanine in this position is also conserved in MdaB and AzoR from *E. coli* (1, 20). In *E. coli* WrbA, W67 fulfills the function, and a stacked structure with benzoquinone has been resolved (2, 5).

Given the structural similarities between the binding pocket of KefF and other oxidoreductases, it is perhaps no surprise that KefF is a competent enzyme, and it seems unlikely that its redox activity is an evolutionary relic. Interestingly, in a screen of environmental samples for genes with alcohol oxidoreductase activity, a gene with 90% amino acid sequence identity to *E. coli* KefF was identified (21). Activity measurements in extracts showed oxidation activity toward 1,2-ethanediol with NAD as a cosubstrate. This gene was not found together with the gene for KefC in the DNA fragment isolated, as is usually the case for KefF (and, similarly, for KefG with KefB). If this gene is used as a query in a BLAST search, the highest similarity (97% amino acid identity) is found for a KefF homologue from *Citrobacter* sp. strain 30-2. The *Citrobacter* KefF, however, is clustered with a *kefC* gene.

We wondered if the redox enzymatic activity of KefF influences the known function of KefF as the activator of KefC. Three mutants that showed no enzymatic activity, G107S, H112W, and F149W, were constructed, but all were capable of activating KefC. These mutations most likely did not disturb the structural integrity of KefF, since the mutant KefF proteins accumulated to similar levels in cells, and these proteins can activate KefC. Therefore, enzymatic activity is not required for KefC activation. Another possible link between enzymatic activity and KefC activation could be the fact that KefF reduces electrophiles and in this way detoxifies them. The Kef complex would then combine short-term rescue from the effects of electrophiles and their long-term detoxification. However, typical electrophiles that activate KefC, for example, NEM, were not reduced by KefF. In the case of this electrophile, an NEM reductase (NemA) that is homologous to the “old yellow enzyme” found in yeast has been described in *E. coli* (31). Previously, we established that the NEM adduct of GSH, N-ethylsulfinimido-S-glutathione, is also rapidly broken down by *E. coli* to release N-ethylmaleamic acid and free GSH (28). Similarly, there are multiple pathways for detoxifying MG (27, 30). Thus, *E. coli* has multiple mechanisms for dealing with the toxic effects of both quinone and nonquinone electrophiles.

We demonstrate here that electrophilic quinones, which form adducts with GSH, activate KefC and are also good substrates for KefF (Fig. 4 and Table 3). These compounds are ubiquitous, and it seems likely that bacteria have developed defense mechanisms against them. Through redox cycling of quinones, reactive oxygen species, including *H<sub>2</sub>O<sub>2</sub>*, superoxide, and hydroxyl radicals, can be formed, which causes stress for the cell. In addition, electrophilic quinones react with nucleophilic compounds, which in particular results in thiol-specific stress. Recently, it has been suggested that quinones are the native substrates for the azoreductase AzoR from *E. coli* (24). Our results indicate that the function of the enzymatic activity of KefF is to reduce the redox toxicity of electrophilic quinones in parallel with acting as
triggers for the KefC efflux system. The two threats caused by these quinones, redox reactivity and electrophilicity, however, are interlinked, since (i) GSH not only forms adducts with them, but can also be oxidized in an nonadditive redox reaction, and (ii) the enzymatic reduction of the quinones removes their ability to undergo conjugate addition reactions.

The regulation of KefC is based on three regulatory binding sites on the KTN domain: a nucleotide binding site in the Rossman fold, overlapping binding sites for GSH and GSQ, and the protein-protein interface with KefF. Our new findings, presented here, indicate a further dimension to this pattern, namely, a role for KefF in activation of KefC by electrophilic quinones, summarized in Fig. 6. These quinones react with GSH, forming adducts (GSQ) that are strong activators for KefC, while GSH itself inhibits KefC. GSH can also reduce the quinones directly, resulting in the oxidation of the GSH pool. Alternatively, the quinones are reduced by NADH, a process that is catalyzed by KefF. KefF is required for full activation of KefC but does not trigger potassium efflux itself. Rather, it may increase the activity of KefC in two interdependent ways: first, by modulating the potassium efflux itself. Rather, it may increase the activity of KefC and, hence, bacterial K⁺ transport loci in Escherichia coli K-12. J. Bacteriol. 196:639–644.


