Energetics of Cd\textsuperscript{2+} efflux system in cadmium-resistant \textit{Staphylococcus aureus} 17810R

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Energetics of \textsuperscript{109}Cd efflux in resting cells of cadmium-resistant \textit{Staphylococcus aureus} 17810R was assayed in 1 or 100 mM potassium/sodium phosphate buffer, pH 7 (PB). Experiments with the use of inhibitors and ionophores showed that Cd\textsuperscript{2+} extrusion in this organism required ATP and either a pH gradient (ΔpH) in 1 mM PB or membrane potential (Δψ) in 100 mM PB. The role of high phosphate ion concentration in Δψ-dependent Cd\textsuperscript{2+} efflux is discussed.

According to Tynecka \textit{et al.} \cite{1}, plasmid-mediated cadmium resistance in \textit{Staphylococcus aureus} is due to the presence of the \textit{cad}A-coded Cd\textsuperscript{2+} efflux system. This system extrudes Cd\textsuperscript{2+} from the cytoplasm immediately after its entry via the Mn\textsuperscript{2+} porter down the membrane potential (Δψ), which prevents accumulation of Cd\textsuperscript{2+} and its toxic effects \cite{1–3}. Nigericin, an ionophore which selectively collapses the pH gradient (ΔpH), caused enhancement of 109\textsuperscript{Cd} accumulation by growing cells of cadmium-resistant \textit{S. aureus} 17810R which oxidized exogenous amino acids \cite{1}. On the basis of this observation, Tynecka \textit{et al.} \cite{1} suggested that the Cd\textsuperscript{2+} efflux system operated as an electroneutral Cd\textsuperscript{2+}/2H\textsuperscript{+} antipporter down the ΔpH generated by the respiratory chain.

Silver and coworkers \cite{4, 5} suggested, on the basis of the predicted amino-acid sequence of CadA protein, that Cd\textsuperscript{2+} efflux system is the Cd\textsuperscript{2+}-ATPase, belonging to the P-type cation-translocating ATPases. Recently, Tsai & Linet \cite{6} demonstrated, in membranes of \textit{Bacillus subtilis} in which \textit{cad}A determinant was subcloned and expressed, that the CadA protein formed a phosphorylated enzyme intermediate characteristic of the P-type ATPases. Tsai \textit{et al.} \cite{7}, using everted membrane vesicles of the same organism, showed that Cd\textsuperscript{2+} efflux was energized solely by ATP. However, the experiments by these authors \cite{7} with the use of inhibitors and ionophores suggested that Cd\textsuperscript{2+} extrusion could require the energy of ΔpH in addition to ATP and that Cd\textsuperscript{2+}-ATPase had unique properties.

In the present work we studied energization of Cd\textsuperscript{2+} efflux system in resting cells of cadmium-resistant \textit{S. aureus} 17810R. In some experiments, a cadmium-sensitive derivative strain \textit{S. aureus} 17810S which lacks the Cd\textsuperscript{2+} efflux system, was used as a control organism.

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\textsuperscript{1}Abbreviations: CCCP, carbonyl cyanide m-chlorophenyl hydrazone; DCCD, dicyclohexylcarbodiimide; HQNO, 2-heptyl-4-hydroxyquinoline N-oxide; PB, potassium/sodium phosphate buffer, pH 7; Δψ, electrochemical proton gradient; Δψ, membrane potential; ΔpH, pH gradient.
MATERIALS AND METHODS

*Staphylococcus aureus* 17810R, carrying a penicillinase plasmid pIII7810 with the cadA gene, and its plasmidless, cadmium-sensitive variant strain 17810S were described previously [2]. Experiments were performed on resting, aerobically grown, early-exponential phase cells of both strains, which were obtained according to [2, 8]. In most of the experiments, cells were suspended in 1 or 100 mM potassium/sodium phosphate buffer, pH 7 (further called PB), at a density of 0.2 mg dry wt. ml⁻¹. ¹⁰⁹Cd (carrier-free) uptake at its 10 μM concentration was assayed as described previously [2]. Steady-state ¹⁰⁹Cd efflux was studied according to [1]. Oxygen uptake was determined manometrically [1]. ATP content was measured by the luminescence method [9]. The experiments shown in each Figure were done in triplicates and representative data are presented.

RESULTS AND DISCUSSION

Resting cells of *S. aureus* suspended in buffers in the absence of exogenous energy donors, utilize endogenous energy reserves, such as the amino-acid pool or ATP for membrane energization [8, 10] As shown in Fig. 1A, resting cells of cadmium-resistant *S. aureus* 17810R, similarly as growing cells of this organism [1, 2], incubated in 100 mM PB did not accumulate ¹⁰⁹Cd. This was due to the activity of the plasmid-coded Cd²⁺ efflux system [1]. Under similar conditions, the resting cells of cadmium-sensitive control strain 17810S accumulated ¹⁰⁹Cd via the high-affinity Mn²⁺ porter down the membrane potential (Δψ), as indicated by inhibition of Cd²⁺ accumulation with Mn²⁺ or with valinomycin + K⁺ (Fig. 1A).

In contrast to growing cells of strain 17810R [1], in resting cells of this organism nigericin did not cause enhancement of Cd²⁺ accumulation in 100 mM PB (Fig. 1A). This suggests that, in the absence of pH gradient (ΔpH) abolished by nigericin, Δψ generated by endogenous respiration (Fig. 1A, inset) could energize Cd²⁺ efflux.

A decrease of PB concentration from 100 to 1 mM caused some net ¹⁰⁹Cd accumulation by strain 17810R, which is insensitive to either Mn²⁺ or valinomycin + K⁺ (Fig. 1B); this accumulation could occur via the low affinity Cd²⁺ uptake system without involvement of the Mn²⁺ porter [2]. DCCD, a known inhibitor of the ATP synthetase complex, stimulated Cd²⁺ accumulation via the Mn²⁺ porter (Fig. 1B). Under these conditions, the HQNO-sensitive respiratory Δψ energized Cd²⁺ entry via the Mn²⁺ porter (Fig. 1B), while ΔpH did not support Cd²⁺ efflux, since Cd²⁺ acted as an inhibitor of endogenous respiration (Fig. 1B, inset) and ΔpH formation. In the absence of DCCD, inhibition of respiration by Cd²⁺, caused a reversal of the direction of the ATP synthetase

Fig. 1. ¹⁰⁹Cd uptake by resting cells of *S. aureus* 17810R and 17810S. (A) Cells of both strains were suspended in 100 mM PB. Strain 17810R: ○, control cells; ●, cells pretreated with 0.5 μM nigericin. Strain 17810S: □, control cells; cells pretreated with: Δ, 1 μM valinomycin + 50 mM KCl; ■, 100 μM MnCl₂. (B) Cells of strain 17810R were suspended in 1 mM PB. ○, control cells; cells pretreated with: □, 100 μM DCCD; ●, 0.5 μM nigericin; △, 1 μM valinomycin + 100 mM KCl; ■, 100 μM MnCl₂; △, DCCD- or nigericin-pretreated cells + 1 μM valinomycin + 100 mM KCl; ◇, DCCD- or nigericin-pretreated cells + 100 μM MnCl₂; V, DCCD-pretreated cells + 100 μM HQNO. Cells were pretreated with inhibitors and ionophores for 10 min at 37°C before addition of ¹⁰⁹Cd. Insets: the effect of 10 μM Cd²⁺ on endogenous respiration of strain 17810R in 100 (A) or 1 mM PB (B). ○, control cells; ●, cells treated with CdCl₂.
activity into the hydrolysis [11, 12]. The \( \Delta \psi \) generated via hydrolysis of endogenous ATP promoted \( Cd^{2+} \) influx via the \( Mn^{2+} \) porter, while \( \Delta pH \), not sensitive to \( Cd^{2+} \), energized \( Cd^{2+} \) exit. Thus, by collapsing \( \Delta pH \), nigericin restored \( Cd^{2+} \) accumulation via the \( Mn^{2+} \) porter in strain 17810R (Fig. 1B). Our data are in agreement with those obtained by Tsai et al. [7] in everted membrane vesicles of \( B. subtilis \) containing the \( cadA \) determinant. These authors demonstrated that in the presence of ATP as a sole energy source, \( Cd^{2+} \) efflux was inhibited by either DCCD or nigericin [7].

An increase of PB concentration from 1 to 100 mM at steady-state in the \( Cd^{2+} \)-preloaded, nigericin-pretreated cells of strain 17810R, caused a partial extrusion of \( Cd^{2+} \) (Fig. 2A). This extrusion was insensitive to CCCP (Fig. 2A) and probably represented an energy-independent loss of \( Cd^{2+} \) bound to the low affinity \( Cd^{2+} \) uptake system.

In 1 mM PB, \( Cd^{2+} \) inhibited endogenous respiration in strain 17810R, (Fig. 1B, inset) while nigericin deprived the cells of ATP (Fig. 2C, inset). Therefore L-lactate, the cadmium-insensitive respiratory substrate [13], was used as an exogenous energy donor. Addition of L-lactate to the \( Cd^{2+} \)-preloaded, nigericin-pretreated cells, restored respiration (Fig. 2B) and also caused some transient ATP resynthesis down \( \Delta \psi \), sensitive to valinomycin + \( K^+ \) or to DCCD (Fig. 2C). However, L-lactate did not cause energy-dependent \( Cd^{2+} \) extrusion (Fig. 2A). This indicates that in the nigericin-pretreated cells the energy of \( \Delta \psi \) generated by L-lactate oxidation could not energize \( Cd^{2+} \) efflux. Only simultaneous addition of L-lactate and 100 mM PB at steady-state resulted in energy-dependent \( Cd^{2+} \) extrusion, sensitive to valinomycin + \( K^+ \) or to CCCP (Fig. 2A). These data suggest an important role of high PB concentration in the presence of L-lactate in \( \Delta \psi \) driven \( Cd^{2+} \) efflux in the nigericin-pretreated cells of strain 17810R.

![Diagram](image_url)

Fig. 2. (A) Steady-state \(^{105}\)Cd efflux from the nigericin-pretreated, \( Cd^{2+} \)-preloaded cells of \( S. aureus \) 17810R.

- Cells suspended in 1 mM PB and pretreated for 10 min at 37°C with 0.5 \( \mu M \) nigericin and for 40 min with 10 \( \mu M \)
- 10\(^{10}\)Cd. At the time indicated by the arrow, cell suspensions were supplemented with: - 10 mM L-lactate; - 100 mM PB; - 100 mM PB + 5 \( \mu M \) CCCP; - 10 mM L-lactate + 100 mM PB; - 10 mM L-lactate + 100 mM PB and 1 \( \mu M \) valinomycin + 50 \( mM \) KCl or 5 \( \mu M \) CCCP; - 10 mM L-lactate + 100 mM PB and 100 \( \mu M \) DCCD.

(B) L-Lactate oxidation by the nigericin-pretreated, \( Cd^{2+} \)-preloaded cells of \( S. aureus \) 17810R.

- Endogenous respiration in cells suspended in 1 mM PB and pretreated with nigericin and \( Cd^{2+} \). At the time indicated by the arrow, cell suspensions were supplemented with: - 10 mM L-lactate; - 100 mM PB; - 100 mM PB + 1 \( \mu M \) valinomycin + 50 \( mM \) KCl or 5 \( \mu M \) CCCP; - 100 mM PB and 100 \( \mu M \) DCCD.

(C) ATP resynthesis in the nigericin-pretreated, \( Cd^{2+} \)-preloaded cells of \( S. aureus \) 17810R.

- Endogenous ATP level in cells suspended in 1 mM PB and pretreated with nigericin and \( Cd^{2+} \). At the time indicated by the arrow, cell suspensions were supplemented with: - 10 mM L-lactate; - 100 mM PB; - 100 mM PB + 1 \( \mu M \) valinomycin + 50 \( mM \) KCl or 5 \( \mu M \) CCCP; - 100 mM PB and 100 \( \mu M \) DCCD.

Oxygen consumption by the cells was measured manometrically.

- Time (min)
Fig. 3. Steady-state $^{109}$Cd efflux from the nigericin-pretreated, Cd$^{2+}$-preloaded cells of S. aureus 17810R in various media.

O, Cells suspended in 1 mM PB and pretreated for 10 min at 37° C with 0.5 mM nigericin and for 40 min with 10 μM Cd. (A) Cell suspensions were supplemented with: ●, 100 mM KCl, 100 mM NaCl, 100 mM Tris/HCl buffer, pH 7, 100 mM Mops/NaOH buffer, pH 7 + 10 mM L-lactate; or with ○, the same media + 10 mM L-lactate + 5 μM CCCP. (B) Cell suspensions were supplemented with: ■, 100 mM triethanolamine-phosphate buffer, pH 7; ▲, 100 mM triethanolamine-phosphate buffer, pH 7 + 10 mM L-lactate; ▲, 100 mM triethanolamine-phosphate buffer, pH 7 + 10 mM L-lactate and 1 μM valinomycin + 50 mM KCl. The efflux was initiated at the time indicated by the arrow by addition of L-lactate.

Figure 3A shows that neither K$^+$ nor Na$^+$, present in PB, played any role in the L-lactate-induced Cd$^{2+}$ efflux from the nigericin-pretreated cells of strain 17810R. Similarly, other 100 mM buffers, pH 7, Tris/HCl or Mops/NaOH, did not cause energy-dependent Cd$^{2+}$ extrusion in the presence of L-lactate (Fig. 3A). The above data point to an important role of phosphate ions in Cd$^{2+}$ efflux. This was confirmed by the energy-dependent Cd$^{2+}$ extrusion caused by 100 mM triethanolamine-phosphate buffer supplemented with L-lactate (Fig. 3B).

When the cells of strain 17810R, devoid of endogenous energy reserves by starvation and not pretreated with nigericin, were energized by L-lactate in 1 mM PB, they accumulated Cd$^{2+}$ only via the Mn$^{2+}$ porter (Fig. 4A) at the same rate as the control strain 17810S (Fig. 4B). This indicates that in 1 mM PB neither ΔΨ nor ΔpH generated by L-lactate oxidation could energize Cd$^{2+}$ efflux in strain 17810R, although CCCP-sensitive ATP synthesis was driven by the total electrochemical proton gradient — ΔpH$^+$ (Fig. 4A, inset). Only in 100 mM PB, the ΔΨ generated by L-lactate oxidation, but not ΔpH, energized Cd$^{2+}$ efflux. This was evidenced by the lack of enhancement of 109 Cd accumulation with nigericin (Fig. 4A). Our data indicate that, the ΔpH generated by L-lactate oxidation can not energize Cd$^{2+}$ extrusion, while ΔΨ can do it but only in combination with 100 mM PB.

The question arises as to what is the role of the high phosphate ion concentration in the ΔΨ-dependent Cd$^{2+}$ efflux in strain 17810R? It should be stressed, that according to our polarographic studies, 100 mM PB complexed Cd$^{2+}$ only in about 15% (not shown). We suggest that phosphate ions may provide extra protons during formation of cadmium phosphate in the cytoplasm. These protons pumped out via the

Fig. 4. $^{109}$Cd uptake by starved cells of S. aureus 17810R (A) and 17810S (B) oxidizing L-lactate.

Cells of both strains were deprived of endogenous energy reserves in 100 mM PB, containing 5 mM MgCl$_2$ at 37°C for 3 h on a shaker. Starved cells were suspended in 1 or 100 mM PB and preincubated with 10 mM L-lactate for 20 min at 37°C. In 1 mM PB: ●, control cells; cells pretreated with: ●, 1 μM valinomycin + 100 mM KCl; ○, 100 μM MnCl$_2$. In 100 mM PB: ■, control cells; ○, cells pretreated with 0.5 mM nigericin. Inset: ATP synthesis in starved cells of S. aureus 17810R oxidizing L-lactate. ATP content was estimated in starved cells suspended in 1 ○ or 100 mM PB (●) after addition of L-lactate. ● Cells pretreated with 5 μM CCCP in 1 or 100 mM PB before addition of the substrate.
respiratory chain may return through the \( \text{Cd}^{2+} \) efflux system down the \( \Delta \psi \) generated by \( \text{L-lactate} \) oxidation. Our suggestion is in agreement with a model of Nicholls & Akerman [14] explaining the role of “phosphate” protons in \( \text{Ca}^{2+} \) efflux in mammalian mitochondria.

As shown in Fig. 2A, DCCD, which stopped ATP resynthesis (Fig. 2C) but not \( \text{L-lactate} \) oxidation (Fig. 2B), inhibited the energy-dependent \( \text{Cd}^{2+} \) efflux from the nigercin-pretreated cells of strain 17810R oxidizing \( \text{L-lactate} \) in 100 mM PB. This suggests that, besides \( \Delta \psi \) and phosphate ions, ATP was also needed for \( \text{Cd}^{2+} \) efflux in strain 17810R. The ATP requirement for \( \text{Cd}^{2+} \) extrusion is in agreement with data by other authors [4–7].

To summarize, the activity of the \( \text{Cd}^{2+} \) efflux system in \( \text{S. aureus} \) strain 17810R depends not only on ATP required for phosphorylation of CadA protein but also on either \( \Delta \psi \) or \( \Delta \text{pH} \) needed for proton circulation. Our suggestion is in accord with the data by Serrano [15] that some cation translocating P-type ATPases require for their activity both ATP and the proton current.

REFERENCES