SHORT COMMUNICATION

A Dimeric Complex of Ruthenium: A New Inhibitor of Respiration-driven Calcium Transport in *Escherichia coli* K12

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Succinate-driven calcium uptake by everted membrane vesicles from *Escherichia coli* was sensitive to 2 mM-KCN and a dimeric, mixed valence complex of ruthenium, $\text{Ru}_2(\text{NH}_3)_6\text{Br}_5(\text{H}_2\text{O})$ (100 μM), but relatively insensitive to 100 μM -Ruthenium Red. The sensitivity of uptake to KCN and the slight inhibition caused by Ruthenium Red could be attributed to the different potencies of these compounds as respiratory inhibitors. Respiration was completely insensitive to the dimeric ruthenium complex, however, suggesting that this compound is an inhibitor of respiration-driven calcium transport.

INTRODUCTION

Bacteria generally maintain a low intracellular concentration of calcium, excreting the element by a highly specific, active transport system (Silver, 1977). Disruption of *Escherichia coli* in a French press yields membranes with an 'inside-out' orientation, which accumulate calcium in an energy-dependent fashion (Tsuchiya & Rosen, 1975). Neither La^{3+} nor Ruthenium Red (1 or 10 μM) inhibit $^{45}\text{Ca}^{2+}$ accumulation by everted membrane vesicles (Tsuchiya & Rosen, 1975), although these materials specifically and competitively inhibit calcium accumulation by mitochondria (Moore, 1971; Silver, 1977).

During an extensive survey of the interactions of ruthenium compounds with micro-organisms (Gibson, 1981), one compound proved to be a potent inhibitor of $^{45}\text{Ca}^{2+}$ accumulation, suggesting that it may be a useful tool in studies of the mechanism and functions of calcium extrusion. This effect is the subject of the present communication.

METHODS

Organism and growth conditions. *Escherichia coli* K12, strain A1002, was grown on a defined medium that contained (l^{-1}): glycerol, 5 g; K_2HPO_4 , 4 g; KH_2PO_4 , 1 g; NH_4Cl , 1 g; K_2SO_4 , 2.6 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 g; isoleucine, valine and methionine, 1 g each; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2 g; trace elements (Poole *et al.*, 1979), 10 ml. Cultures were grown in 200 ml batches of medium in 1 l Erlenmeyer flasks, shaken at 200 r.p.m. after inoculation with 2.5 ml of a 12 to 14 h starter culture.

Preparation of everted membrane vesicles. Cells from 1.4 l culture were harvested after 5 h growth, when A_{420} (1 cm path length; Pye-Unicam SP1700 spectrophotometer) was 1 to 1.2, by centrifugation at 23 000 g for 5 min. The wet weight of cells, after washing the pellets once with a buffer (I) that contained 100 mM-potassium phosphate and 10 mM-disodium EDTA (pH 6.6), was 1.5 to 1.9 g (l culture) $^{-1}$. Disruption of the organism in a French press at 27.6 MPa and the isolation of vesicles by differential centrifugation was exactly as described by Rosen & Tsuchiya (1979). The yield was approximately 2 to 3 mg protein (l culture) $^{-1}$. Vesicles were suspended in

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a buffer that contained 10 mM-Tris/HCl, 140 mM-KCl, 0.5 mM-dithiothreitol and 10% (v/v) glycerol (pH 7.2; buffer II) to a protein concentration of 2 to 4 mg ml⁻¹.

For measurements of oxygen uptake in the open system (see below) vesicles were prepared in high yields [10 to 11 mg protein (1 culture)⁻¹] by sonication (Poole & Haddock, 1975) in buffer I, followed by the differential centrifugation procedure of Rosen & Tsuchiya (1979). Such vesicles are generally considered to have the same 'inside-out' orientation as those prepared in the French press (Coakley *et al.*, 1977).

Calcium transport assays. These were carried out in a buffer (III) that contained 10 mM-Tris/HCl, 10 mM-K₂HPO₄ and 140 mM-KCl (pH 8.0), with 20 mM-sodium succinate as energy source, as described by Rosen & Tsuchiya (1979). The assay mixture was stirred at 25 °C. Uptake was initiated by adding CaCl₂ [0.76 to 1.14 mCi ⁴⁵Ca (mmol Ca)⁻¹ (1 mCi = 3.7 × 10⁷ becquerels)] to a final concentration of 0.5 mM. Samples (200 µl) were taken at intervals and filtered on the centres of nitrocellulose filters (0.45 µm pore size; 25 mm diam.). They were dried at 37 °C overnight prior to counting in a Packard Tri-Carb 460 CD liquid scintillation system (lower channel: 0; upper channel: 250; 10 min counts). The scintillation fluid contained 2,5-diphenyloxazole in toluene (3.93 g l⁻¹).

Oxygen uptake. The short term effects of various concentrations of putative inhibitors were studied using a conventional O₂ electrode system (Rank Bros, Bottisham, Cambridge). For prolonged measurements, not limited by the onset of anoxia, the system was used in the open mode (Poole, 1977) with a constant rate of stirring provided by an IKA-Combimag RET magnetic stirrer (Sartorius Instruments Ltd). In both modes, the volume was 2 ml and the temperature was 26 °C.

Chemicals. The dimeric, mixed-valence complex of ruthenium, of composition Ru₂(NH₃)₆Br₅(H₂O) was prepared by the method of Bottomley & Tong (1971). The precise formulation of the complex is not clear. For the corresponding chloride, these authors suggest a 1:1 electrolyte [Ru₂(NH₃)₆Cl₄]Cl·H₂O, while other workers (Mercer & Gray, 1972) propose [Ru₂(NH₃)₆Cl₃]Cl₂·H₂O. The distinction depends on the interpretation of conductivity data and on analysis of ionic halide by ion-exchange techniques. Our experiments (D. J. O'Reardon, M. N. Hughes and R. K. Poole, unpublished results) support the latter formulation, which will probably involve triply chloride-bridged ruthenium(II) and ruthenium(III). After considerable effort, crystals have been prepared and the structure should be established in due course.

Ruthenium Red (practical grade; Sigma) also contains inert salts. Concentrations given were calculated according to the percentage of dye quoted for each batch by the manufacturers; the quoted concentration lay between 20 and 45%. Optical densities (533 nm) of such solutions were in good agreement (± approx 5%) with the values calculated using the extinction coefficients given by either Fletcher *et al.* (1961) or Reed & Bygrave (1974) for the purified dye.

All inhibitors were used as freshly prepared aqueous solutions. DL-Dithiothreitol was from Sigma; all other reagents were from BDH or Fisons and of the highest purity available.

RESULTS AND DISCUSSION

In the absence of added ATP, calcium accumulation by everted membrane vesicles is dependent on the availability of an oxidizable substrate such as succinate (Fig. 1*a*). Uptake was sensitive to the respiratory inhibitor, KCN, and the uncoupler carbonylcyanide *m*-chlorophenylhydrazine (5 µM) as reported by Tsuchiya & Rosen (1975). The dimeric ruthenium complex (100 µM) inhibited the maximum level of ⁴⁵Ca²⁺ accumulated by >70% (Fig. 1*b*). Ruthenium Red, however, inhibited it by <30% (Fig. 1*c*), even at 10-fold the concentrations used by Tsuchiya & Rosen (1975). The low residual uptake in the presence of KCN, absence of succinate or presence of the dimeric ruthenium complex may be due to any energy-independent facilitated transport of calcium (Silver *et al.*, 1975).

Since any inhibitor of respiration could be expected to decrease calcium uptake under these conditions, it was important to determine the effects on respiration of the compounds used. Conventional polarographic measurements showed that short-term exposure of vesicles (prepared using a French press; final concentration 0.17 mg protein ml⁻¹) to either Ruthenium Red (up to 190 µM) or the dimeric ruthenium compound (up to 240 µM) was without effect on rates of succinate oxidation (results not shown). The effect of KCN (2 mM) was not immediate (Pudek & Bragg, 1974), but approx. 90% inhibition was achieved within 14 min of its addition. The open O₂ electrode was used to study the possibility of inhibition by the ruthenium compounds over the longer periods shown in Fig. 1. Addition of succinate to a suspension of vesicles (prepared by sonication) resulted in a rapid decline in the dissolved O₂ tension to reach a new, lower steady state (Fig. 2*a*). The gradual increase in dissolved O₂ over the next 30 min is most

Fig. 1. Respiration-driven calcium accumulation and its inhibition in everted membrane vesicles from *E. coli*. In each of (a) to (c) $^{45}\text{Ca}^{2+}$ was added at zero time and its uptake (O) in the presence of 20 mM-succinate and absence of inhibitors is shown. In (a), the effects of omitting succinate (●) or adding 2 mM-KCN (□) are shown. The effects of including the dimeric mixed valence ruthenium compound (100 μM) (■) or Ruthenium Red (100 μM) (Δ) are shown in (b) and (c), respectively. The final protein concentration was 0.18 mg ml $^{-1}$.

Fig. 2. Polarographic determination of respiration rates of everted membrane vesicles using the open O_2 electrode. The liquid volume was 2 ml and the O_2 transfer rate (K) was 0.25 min $^{-1}$. The gas phase was air, the temperature 26 °C and the protein concentration 0.18 mg ml $^{-1}$. Addition of aqueous solutions of inhibitors were made as indicated by the arrows. The inflection in each curve within the first 5 min is the time of adding 20 mM-succinate.

likely due to a small loss of liquid from the vessel by evaporation and the consequent increase in the rate of O_2 transfer from the gaseous to liquid phases. Subsequent addition of 2 mM-KCN caused the dissolved O_2 tension to increase to a final steady state that reflected approx. 90% inhibition of respiration. In a similar experiment (Fig. 2b), 100 μM -Ruthenium Red caused an 11% increase in the steady state O_2 concentration (equivalent to 11% inhibition of respiration), which could account for the results in Fig. 1(c). The divalent mixed-valence compound of ruthenium (100 μM) caused a small decrease (which was too low to quantify) in the respiration rate (Fig. 2c). The protein concentration in these experiments was identical to that used in the Ca^{2+} transport assays.

The two ruthenium compounds have a reciprocal effect on the activity of the Mg^{2+} -activated ATPase in *E. coli*: Ruthenium Red inhibits the ATPase with I_{50} values in the range 26 to 75 μM (Scherr & Günther, 1978; Scott *et al.*, 1980; Gibson, 1981), whereas the dimeric mixed valence

compound gave only 21% inhibition at 690 μM (Gibson, 1981). These results, and those in Fig. 1, agree with the finding (Brey & Rosen, 1979; Tsuchiya & Takeda, 1979) that, unlike calcium transport in *Streptococcus faecalis* (Kobayashi *et al.*, 1978), the *E. coli* system is not linked obligatorily to ATP hydrolysis by the ATPase, but is an electrogenic proton/calcium antiport driven by uncoupler-sensitive proton circulation, generated by ATP or respiration. The stoichiometry of the antiport has not been firmly established.

The only other compound reported to inhibit Ca^{2+} uptake by *E. coli* vesicles is 1 mM-S-adenosylmethionine (Mato, 1979). The mechanism of action of neither of these inhibitors is known; either could be explained by an inhibition of Ca^{2+} transport *per se* or by making vesicles more permeable to Ca^{2+} .

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