Inhibition of Mg, Ca-ATPase from *E. coli* by Ruthenium Red

Franz Scherr and Theodor Günther

Institut für Molekularbiologie und Biochemie, Freie Universität Berlin

Z. Naturforsch. **33 c**, 61–64 (1978); received November 3, 1977/ January 10, 1978

E. coli, ATPase, Ruthenium Red, Inhibition

The membrane-bound, solubilized, and trypsin-treated forms of Mg, Ca-ATPase from *E. coli* are inhibited by ruthenium red [RR]. The inhibition is noncompetitive and is reduced at higher substrate concentrations.

n-Butanol-extracted ATPase is not inhibited by ruthenium red and is not activated by KCl.

In mitochondria ruthenium red (RR) inhibits energy-dependent Ca^{2+} uptake, and energy-independent Ca^{2+} binding [1–6] and promotes Ca^{2+} release [3, 6]. A Ca^{2+}-binding protein which is specifically inhibited by RR was isolated from mitochondria [7].

Moreover, the Ca^{2+}-dependent ATPase activities of erythrocytes [8], sarcoplasmic reticulum [9] and sarcolemma [10], which are probably involved in Ca^{2+}-transport [11, 12], are inhibited by RR. Since the ATPase of *E. coli* is also activated by Ca^{2+}, we tested the effect of RR on it.

Materials and Methods

ATPase preparations

E. coli strain B 163 was cultured as already described [13]. Membrane-bound ATPase was prepared according to Evans [14]. For preparation of solubilized ATPase, the membrane vesicles were washed three times in 30 mM Tris HCl 1 mM EDTA, pH 7.5 and sedimented at 30 000 × *g*. The pellet was washed with 3 mM Tris-HCl pH 7.5 and centrifuged at 30 000 × *g*. The supernatant fluid contained the solubilized ATPase.

n-Butanol-extracted ATPase was prepared from membrane-bound ATPase and checked by polyacrylamide gel electrophoresis according to Salton and Schor [15].

Pyridine-extracted ATPase was prepared from solubilized ATPase according to Nelson *et al.* [16].

For preparation of trypsin-treated ATPase, 0.3 ml solubilized ATPase [100 µg protein] was incubated with 0.1 ml 200 mM Tris HCl, pH 9, and 0.2 ml trypsin [50 µg, specific activity 33 U/mg] for 5 min at 37 °C. After addition of 0.1 ml H_2O, 0.1 ml 300 mM KCl or RR respectively, incubation was continued for 5 min and then the ATPase reaction was started by addition of substrate.

For treatment of ATPase with neuraminidase, 0.3 ml membrane-bound ATPase [0.9 mg protein] was incubated with 100 µg neuraminidase [from *Clostridium perfringens*, specific activity 0.6 U/mg] and 50 µl 0.1 M sodium acetate buffer, pH 5.0, for 30 min at 37 °C. After addition of 0.9 ml 25 mM Tris HCl, pH 9, 0.1 ml was used for testing ATPase activity.

Determination of ATPase activity

The ATPase was assayed at 37 °C in a shaking water bath in 1 ml samples with Tris HCl buffer as indicated in the legends, 0.2 mM ATP adjusted to pH with Tris, and 0.04 mM $CaCl_2$ or $MgCl_2$. The protein content of the samples amounted to 0.1 mg. After 10 min preincubation, substrate was added and incubation was continued for 20 min. Under these conditions the ATPase activity was linear with respect to time.

The reaction was stopped with 0.5 ml 15% cold TCA. After centrifugation double determinations of the inorganic phosphate with 0.2 ml or 0.5 ml of the supernatant were made according to Anner and Moosmayer [17]. The protein content was determined according to Lowry *et al.* [18].

Chemicals

Trypsin, neuraminidase, ATP and Tris were obtained from Boehringer, Mannheim, Germany.

RR and the other reagents, which were of reagent grade, were purchased from E. Merck, Darmstadt, Germany.

Commercial RR contains RV and RB. In some experiments purified RR, RV and RB were used.

Requests for reprints should be sent to Professor Dr. T. Günther, Institut für Molekularbiologie und Biochemie, Freie Universität Berlin, Arnimallee 22, D-1000 Berlin 33.

Abbreviations: RR, ruthenium red; RB, ruthenium brown; RV, ruthenium violet.

RR and RV were purified from commercial RR according to Luft [19]. RB was prepared from RR according to Fletcher *et al.* [20].

Results

As Fig. 1 shows, the *E. coli* ATPase is inhibited by RR. The inhibition is not specific for Ca^{2+}, as it also occurs in the presence of Mg^{2+}. The KCl-activated Mg, Ca-ATPase is also inhibited by RR.

To determine the type inhibition, $1/v$ was plotted against $1/[S]$ (Fig. 2). It can be seen that both V and K_m are changed, and that RR is thus a "mixed-type" inhibitor [21]. The same type of inhibition was found with purified RR [not shown]. Thus the inhibition is especially pronounced at lower substrate concentrations.

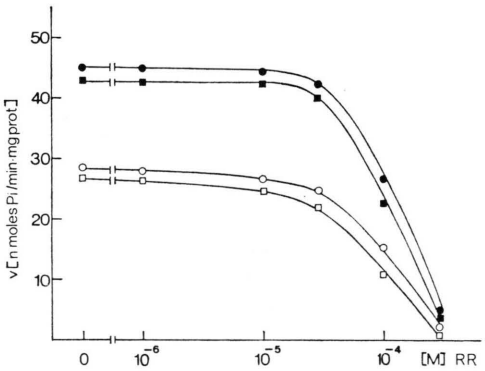

Fig. 1. Inhibition of ATPase from *E. coli* by ruthenium red. Membrane-bound ATPase was incubate for 20 min at 37 °C in 20 mM Tris HCl buffer, pH 9.0. Commercial RR was used. [ATP] = 0.2 mM; □, ■: 0.04 mM $CaCl_2$; ○, ●: 0.04 mM $MgCl_2$; full symbols in presence of 30 mM KCl. Mean of 3 experiments.

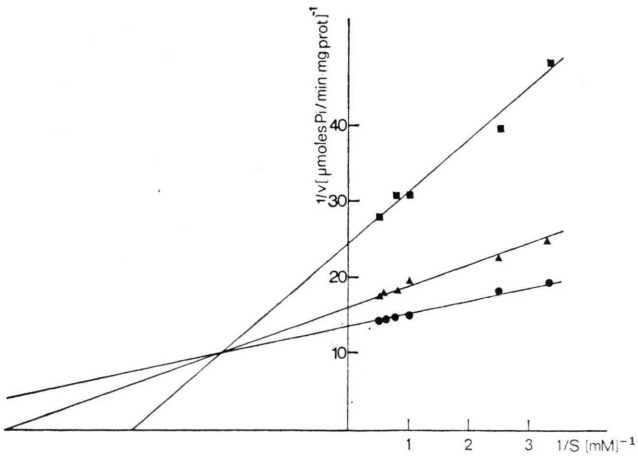

Fig. 2. Plot of $1/v$ *vs* $1/[S]$ at various ruthenium red [RR] concentrations. ●: without RR; ▲: 3×10^{-5} M RR; ■: 1×10^{-4} M RR. [Commercial RR] Membrane-bound ATPase was incubated for 20 min at 37 °C in 30 mM Tris HCl buffer, pH 8.5, in presence of 30 mM KCl. Ca ATP in the ratio 1 : 5 was used as substrate.

Ca^{2+} transport and binding are inhibited by RR, again noncompetitively [1, 4, 5]. Like the *E. coli* ATPase, the sarcolemma ATPase is less inhibited at higher substrate concentrations [10]. However, an increase in the Ca^{2+} concentration did not decrease the inhibition of the *E. coli* ATPase (not shown). The same was observed with sarcolemma ATPase [10].

Commercial RR contains RV and RB [19]. These impurities also inhibit the *E. coli* ATPase [Fig. 3], although less strongly than RR. There are further impurities in commercial RR, *e. g.* several ruthenium ammines, chloride and nitrosylruthenium complexes [19, 20], probably more than 9 different substances [22]. The ruthenium ammines, however, are inactive [22]. According to Luft [19], commercial RR contains only about 15% RR. Therefore, the difference in half maximal inhibition between commercial RR [Fig. 1] and purified RR, RB and RV [Fig. 3] may result from inactive impurities.

In order to characterize the RR-binding groups, the *E. coli* ATPase was treated with neuraminidase.

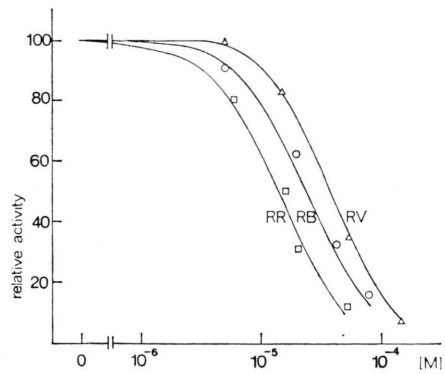

Fig. 3. Inhibition of ATPase from *E. coli* by purified ruthenium red [RR], ruthenium violet [RV] and ruthenium brown [RB]. Membrane-bound ATPase was incubated for 20 min at 37 °C in 20 mM Tris HCl buffer pH 9.0 in presence of 30 mM KCl. [ATP] = 0.2 mM, $[CaCl_2] = 0.04$ mM. Mean of 2 experiments.

It was still inhibited by RR after this treatment. The binding of Ca^{2+} to sarcolemma ATPase was also not significantly affected by the treatment with neuraminidase [23].

The *E. coli* ATPase consists of 5 different subunits, (α, β, γ, δ, ε, for review see [24]), which

have different functions [24]. In order to characterize the point of attack of RR, ATPase preparations with various subunit compositions were tested. Solubilized ATPase was inhibited in the same way by RR as membrane-bound ATPase. We found the same behaviour with solubilized ATPase whose ε subunit had been destroyed by treatment with trypsin [not shown].

ATPase prepared by extraction with *n*-butanol, which preserves the α and β subunits [15, 25], was no longer inhibited by RR [Table I]. Interestingly

Table I. Effect of ruthenium red [RR] and KCl on the activity of *n*-butanol-extracted ATPase from *E. coli*. Conditions: 20 mM Tris HCl buffer, pH 9.0, [ATP] = 0.2 mM, [$CaCl_2$] = 0.04 mM, 20 min incubation at 37 °C. Commercial RR was used.

[RR] [mM]	Control [nmol/min · mg prot]	30 mM KCl
0	18.8	18.8
0.1	19.0	18.8
0.3	18.6	19.1

enough, this ATPase preparation was also no longer capable of activation by KCl. (Membrane-bound, solubilized and trypsin-treated ATPase is activated by KCl [26].)

The RR inhibition therefore appears to be connected with the γ or δ subunit.

Further differentiation along these lines was not possible because after preparation with pyridine, which produces a complex of α, β and γ subunits, the residual ATPase activity was too low for kinetic measurements. Similarly, treatment of mitochondrial ATPase with pyridine destroyed all measurable activity [27].

Discussion

RR acts as a "mixed type" inhibitor [Fig. 2], giving a combination of partially competitive [type I b] and non-competitive inhibition [21]. In earlier experiments with ATPase from *Bac. megaterium*, no inhibition by RR was found [28]. However, in these experiments a maximum of 1×10^{-5} M RR was added, at an ATP concentration of 8 mM. As shown here with the *E. coli* ATPase, the inhibition of bacterial ATPase only becomes noticeable at higher RR and lower substrate concentrations. A quantitative comparison is also encumbered by the fact that various commercial RR preparations differ in purity and content of RR, RB and RV [19], which cause varying degrees of inhibition.

According to Luft [19], RR reacts with polyanions with higher charge densities, *i. e.* glycosaminoglycans, glycoproteins, phospholipids, polyglutamic acid or polyaspartic acid. Glycosaminoglycans do not occur in *E. coli*. Glycoproteins containing neuraminic acid must probably also be excluded, since RR continues to inhibit after neuraminidase treatment, unless neuraminidase-insensitive sialic acid residues are involved in the RR binding. A participation of phospholipids is also improbable, because the binding of Ca^{2+} to sarcolemma and sarcoplasmic reticulum was also inhibited by RR after the lipids had been extracted from the membrane [9, 10]. One could therefore assume, as did Madeira and Antunes-Madeira [10], that the high content in aspartic and glutamic residues in bacterial ATPase [28 – 30] is responsible for the binding of RR. However, *n*-butanol-treated ATPase, which has the same high content in glutamic and aspartic residues as shockwash ATPase [29], is not inhibited by RR.

The results with trypsin-treated or *n*-butanol-extracted ATPase suggest that the binding site for RR is located on the γ or δ subunit. Since the δ subunit is only responsible for the binding of the ATPase complex to the membrane [24], the RR inhibition could occur through the γ subunit.

The inhibition of the *E. coli* Mg, Ca-ATPase by RR suggests that this enzyme, like the Ca-ATPases of erythrocytes and sarcoplasmic reticulum, may be involved in the transport of Ca^{2+} [and possibly Mg^{2+}] into *E. coli* cells. This is supported by the facts that the ATP-dependent component of the active Ca^{2+} transport into vesicles from *E. coli* [31] and *A. vinelandii* [32] is inhibited by DCCD, a specific inhibitor of the membrane-bound bacterial ATPase, and that the ATP-dependent flux of Mg^{2+} in mitochondria is also inhibited by RR [33].

[1] C. L. Moore, Biochem. Biophys. Res. Comm. **42**, 298 – 305 (1971).
[2] F. D. Vasington, P. Gazzotti, R. Tiozzo, and E. Carafoli, Biochim. Biophys. Acta **256**, 43 – 54 (1973).
[3] C. S. Rossi, F. D. Vasington, and E. Carafoli, Biochem. Biophys. Res. Comm. **50**, 846 – 852 (1973).
[4] G. R. Ash and F. L. Bygrave, FEBS Letters **78**, 166 – 168 (1977).
[5] K. C. Reed and F. L. Bygrave, Biochem. J. **140**, 143 – 155 (1974).
[6] L. A. Sordahl, Arch. Biochem. Biophys. **167**, 104 – 115 (1974).

[7] G. Sottocasa, G. Sandri, E. Panfili, B. Bernard, P. Gazzotti, F. D. Vasington, and E. Carafoli, Biochem. Biophys. Res. Comm. **47,** 808–813 (1972).
[8] E. L. Watson, F. F. Vincenzi, and P. W. Davis, Biochim. Biophys. Acta **249,** 606–610 (1971).
[9] M. G. P. Vale and A. P. Carvalho, Biochim. Biophys. Acta **325,** 29–37 (1973).
[10] V. M. C. Madeira and M. C. Antunes-Madeira, J. Membr. Biol. **17,** 41–50 (1974).
[11] A. E. Shamoo, T. R. Thompson, K. P. Campbell, T. L. Scott, and D. A. Goldstein, J. Biol. Chem. **250,** 8289–8291 (1975).
[12] J. T. Buckley, Biochem. J. **142,** 521–526 (1974).
[13] T. Günther and F. Dorn, Z. Naturforsch. **21 b,** 1076–1081 (1966).
[14] D. J. Evans, J. Bacteriol. **100,** 914–922 (1969).
[15] M. R. J. Salton and M. T. Schor, Biochim. Biophys. Acta **345,** 74–82 (1974).
[16] N. Nelson, D. W. Deters, H. Nelson, and E. Racker, J. Biol. Chem. **248,** 2049–2055 (1973).
[17] B. Anner and M. Moosmayer, Analyt. Biochem. **65,** 305–309 (1975).
[18] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. **193,** 265–275 (1951).
[19] J. H. Luft, Anat. Rec. **171,** 347–368 (1971).
[20] J. M. Fletcher, B. F. Greenfield, C. J. Hardy, D. Scargill, and J. L. Woodhead, J. Chem. Soc. **1961,** 2000–2006.
[21] M. Dixon and E. C. Webb, Enzymes, 315 pp., Longmans, London 1953.
[22] K. C. Reed and F. L. Bygrave, FEBS Letters **46,** 109–114 (1974).
[23] C. J. Limas, Arch. Biochem. Biophys. **179,** 302–309 (1977).
[24] B. A. Haddock and C. W. Collins, Bacteriol. Rev. **41,** 47–99 (1977).
[25] M. R. J. Salton and M. T. Schor, Biochem. Biophys. Res. Comm. **49,** 350–357 (1972).
[26] J. Ahlers and T. Günther, Arch. Biochem. Biophys. **171,** 163–169 (1975).
[27] J. B. Smith and P. C. Sternweis, Biochem. Biophys. Res. Comm. **62,** 764–771 (1975).
[28] R. Mirsky and V. Barlow, Biochim. Biophys. Acta **291,** 480–488 (1973).
[29] M. T. Schor, M. C. Heincz, M. R. J. Salton, and F. Zaboretzky, Microbios **10 A,** 145–150 (1974).
[30] H. P. Schnebli, A. E. Vatter, and A. Abrams, J. Biol. Chem. **245,** 1122–1127 (1970).
[31] B. P. Rosen and J. S. McClees, Proc. Nat. Acad. Sci. U.S. **71,** 5042–5046 (1974).
[32] P. Bhattachryya and E. M. Barnes, J. Biol. Chem. **251,** 5614–5619 (1976).
[33] E. Kun, Biochemistry **15,** 2328–2336 (1976).