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Inhibition of Mitochondrial Calcium Ion Transport by an Oxo-Bridged Dinuclear Ruthenium Ammine Complex†

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ABSTRACT: Ruthenium red is a well-known and effective inhibitor of the mitochondrial Ca^{2+} uniporter; however, Reed and Bygrave [(1974) *FEBS Lett. 46*, 109–114] tentatively attributed this inhibition to a colorless impurity present in commercial samples of ruthenium red (RR). This component has now been isolated and a derivative, $(\mu\text{-O})[(HCO_2)(NH_3)_4Ru]_2Cl_3$, structurally characterized. The active species in solution appears to be the symmetrical oxo-bridged ion, $[X(NH_3)_4Ru\text{–}O\text{–}Ru(NH_3)_4X]^{3+}$, where X = Cl^- or OH^-. Its absorption spectrum shows a maximum at 360 nm. The dinuclear ruthenium ammine complex inhibits Ca^{2+}-stimulated respiration of rat liver mitochondria with an I_{50} of 3.5 pmol/mg of protein compared to the value of 60 pmol of RR/mg of protein. The inhibition by the dinuclear compound is noncompetitive with Ca^{2+}. Respiration-linked swelling of mitochondria induced by Cd^{2+} also responds similarly to both the dinuclear complex and RR. A close correlation was observed between binding to mitochondria as monitored with ^{103}Ru-labeled dinuclear complex and inhibition of Ca^{2+} transport. A Scatchard plot yielded estimates of maximum specific binding and dissociation constant of 7.5 pmol/mg of protein and 1.3 nM, respectively. The inhibitor has the characteristics of a satisfactory affinity ligand for purification of the uniporter.

Ruthenium red is a cytological stain widely used for acidic mucopolysaccharides and selectively staining mitochondria for both visible and electron microscopy (Clarke, 1980). It has the structure $[(NH_3)_5Ru^{III}\text{–}O\text{–}(NH_3)_4Ru^{IV}\text{–}O\text{–}Ru^{III}(NH_3)_5]^{6+}$ and specifically inhibits respiration-driven uptake of Ca^{2+} by mitochondria (Lehninger et al., 1967; Moore, 1971; Vasington et al., 1972; Reed & Bygrave, 1974a) as well as the Ca^{2+} release mechanism of the sarcoplasmic reticulum (Antoniu et al., 1985; Chiesi et al., 1988). Reed and Bygrave (1974b) noted that commercial preparations of ruthenium red which are often less than 20% pure, produced stronger inhibition than purified preparations and used thin-layer chromatography to partially separate an active component that absorbed strongly around 355 nm. Experiments on similarly purified radiolabeled ruthenium red preparations showed that these nearly colorless fractions had a higher inhibitory activity per atom of ruthenium than ruthenium red itself (Reed & Bygrave, 1974b). In this paper, we report the synthesis and isolation of the inhibitor $(\mu\text{-O})[(HCO_2)(NH_3)_4Ru]_2Cl_3$, obtained by the reaction of the active ion (presumbly $[X(NH_3)_4Ru\text{–}O\text{–}Ru(NH_3)_4X]^{3+}$, where X = Cl^- or OH^-) with formate.

Experimental Procedures

Ruthenium red was obtained from Eastman Kodak Co., ruthenium chloride from AESAR, CM-cellulose from Whatman and Bio-Gel P-6 from Bio-Rad. $^{103}RuCl_3$ was supplied by NEN Research Products (Du Pont). Arsenazo[1] III supplied by Aldrich Chemical Co. was purified by passage through a column of Chelex 100 (Bio-Rad). The absorption spectra were measured in the Perkin-Elmer 557 double-beam, dual-wavelength spectrophotometer.

Rat liver mitochondria were prepared according to Johnson and Lardy (1967). Oxygen consumption was measured po-

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[1] Abbreviations: Arsenazo, 2,2′-[1,8-dihydroxy-3,6-disulfo-2,7-naphthylenebis(azo)]bis(benzenearsonic acid); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ru360, the dinuclear ammine complex; Tris, tris(hydroxymethyl)aminomethane; RR, ruthenium red.

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larographically (Estabrook, 1967) and mitochondrial swelling by the apparent absorbance change at 546 nm (Rasheed et al., 1984; Chavez et al., 1985). For the kinetic experiments, Ca^{2+} uptake in mitochondria was also measured with the use of Arsenazo III as the indicator with the wavelength pair λ 685–675 nm (Scarpa, 1979). Analysis for Ru was carried out by atomic absorption on an Instrumentation Laboratory 557 spectrometer (Clarke, 1978). Elemental analyses for C, H, N, and Cl were done at the Robertson Laboratory, Madison, NJ.

RESULTS

Synthesis and Analysis. The synthesis of the inhibitory dinuclear ruthenium ammine complex was based on the method for the preparation of ruthenium red (Fletcher et al., 1961). The product was purified by ion-exchange chromatography instead of crystallization.

$RuCl_3 \cdot 3H_2O$ (250 mg) was dissolved in 1.5 mL of 6 N HCl and 0.2 mL of absolute ethanol was added. The solution was placed in a water bath at 90 °C in order to reduce any Ru(IV) normally present in commercial Ru(III). After 1 h, 0.2 mL of concentrated aqueous ammonia was added, which immediately produced a heavy, black, granular precipitate. It was collected by centrifugation, resuspended in 20 mL of 12 N NH_4OH, maintained at 90 °C for 20 min, and then held overnight at 40 °C. The portion of the precipitate that remained was removed by centrifugation, and the supernatant, which contained about 40% Ru360 and 10% ruthenium red was dissolved in 0.2 M ammonium formate adjusted to pH 5.5, and the solution was loaded on a 2 × 12 cm carboxymethylcellulose (CM-52-cellulose) column previously equilibrated with 0.2 M ammonium formate. The column was eluted with a linear ammonium formate gradient from 0.2 to 0.6 M. Yellowish fractions (λ_{max} = 360 nm) eluted with about 0.4 M ammonium formate were combined and lyophilized to dryness. The residue was dissolved in 5 mL of distilled water, and 20 mL of absolute ethanol was added to precipitate Ru360, leaving the residual ammonium formate in solution. The precipitate was similarly redissolved and precipitated twice more. The final precipitate (17 mg), presumably the formate salt, was dried at room temperature and stored in the refrigerator for the experiments on Ca^{2+} transport. The supernatants contained about 4 mg of Ru360.

The formate counterion was exchanged for chloride by passing a solution through a DEAE-cellulose chloride column and elution with distilled water. The compound was lyophilized to dryness. Elemental analyses gave the following results. Anal. Calcd for $[H_{26}C_3N_3O_5Ru_2]Cl_3$: H, 4.76; C, 4.36; N, 20.34; Cl, 19.31; Ru, 36.70. Found: H, 5.10; C, 4.98; N, 20.16; Cl, 19.89; Ru, 37.5.

A reference sample of ruthenium red was prepared by dissolving the commercial product in 0.1 M ammonium formate and chromatography on CM-52-cellulose as described above. The fractions eluted with 0.6 M ammonium formate and absorbing at 532 nm were collected, combined, and lyophilized. Excess ammonium formate was extracted by 80% ethanol washing.

The spectra of purified ruthenium red and Ru360 shown in Figure 1 reveal no detectable cross-contamination. The molar absorbance of Ru360 at 360 nm was 2.6×10^4 M^{-1} cm^{-1}. The weak band centered at 360 nm shown by ruthenium red remains constant relative to the 532-nm peak even after repeated CM-52-cellulose chromatography. Consistent with the tripositive versus the hexapositive charge for ruthenium red, Ru360 eluted from CM-cellulose with 0.4 M ammonium acetate as opposed to 0.6 M for ruthenium red.

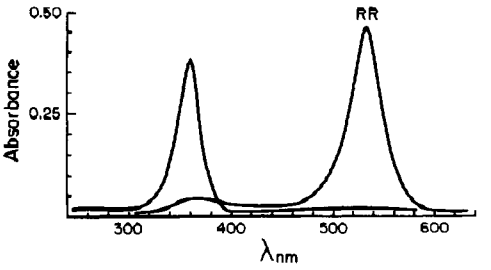

FIGURE 1: Absorption spectra of ruthenium red and the dinuclear ruthenium ammine complex. Ruthenium red was purified by chromatography on CM-cellulose, and the dinuclear compound was synthesized from $RuCl_3$ (see text). The spectra were determined in 0.1 M ammonium acetate, pH 5.4. The peaks were at 533 and 360 nm.

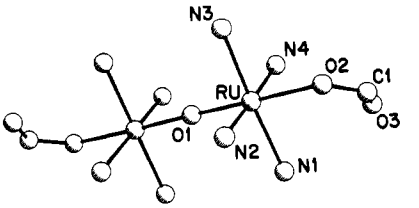

FIGURE 2: A PLUTO drawing of the structure of $(\mu\text{-O})[(HCO_2)(NH_3)_4Ru]_2^{3+}$. The bridging Ru–O bond is quite short (1.8233(4) Å), while the $Ru-O_{formate}$ bond length (2.034(3) Å) is typical for a Ru–O single bond. The equatorial ammine–ruthenium bond lengths, which average 2.11 Å, are well within the expected range for a Ru–N single-bond distance.

The radiolabeled Ru360 was synthesized as described above with 2.5 mg of $RuCl_3 \cdot 3H_2O$ combined with 1 mCi of $^{103}RuCl_3$. All additions were proportionately reduced. The CM-52 column dimension was 0.5 × 5 cm. The yield was 20 nmol of Ru360 with a specific activity of 100 cpm/pmol determined by scintillation counting in a Beckman 5500 γ counter.

For X-ray analysis, crystals were obtained by dissolving 30 mg of the dinuclear ruthenium ammine complex and allowing ethanol to diffuse into the solvent by placing the test tube in a beaker containing 10 mL of ethanol and covering the assembly tightly with parafilm. After 2 days, about 5 mg of irregular hexagonal dark-red crystals were collected by decanting the supernatant from the tube.

The crystal structure $(\mu\text{-O})[(HCO_2)(NH_3)_4Ru]_2Cl_3$ or (μ-oxo)bis(*trans*-formatotetraammineruthenium) trichloride revealed a nearly linear molecule containing two octahedral rutheniums linked by an oxygen bridge. The ends of the molecule are capped with formates (Figure 2). The placement of the μ-O on a crystallographic mirror plane dictates that the two metal ions are identical. The quartet of amines around each Ru are eclipsed with respect to one another.

Inhibition of Ca^{2+} Transport. The specificity of ruthenium red for the Ca^{2+} uniporter is demonstrated by the observation that even at the high levels of about 20 nmol/mg of protein, it had no effect on the stimulation of state 4 respiration by ADP (Moore, 1971; Vasington et al., 1972). The respiratory stimulation by added Ca^{2+}, however, was totally blocked. Figure 3 shows the relative inhibitory effects of the dinuclear ruthenium ammine complex and ruthenium red on Ca^{2+}-stimulated respiration. The 50% inhibition levels are 0.0035 and 0.05 nmol/mg of protein respectively. The value for ruthenium red is close to that reported by Reed and Bygrave (1974a), which was 0.07 nmol/mg of protein.

The transport of Cd^{2+} into mitochondria also is inhibited by ruthenium red (Rasheed et al., 1984; Chavez et al., 1985). Cd^{2+} transport is measured by the mitochondrial swelling it produces in acetate medium. In the swelling assay, there is

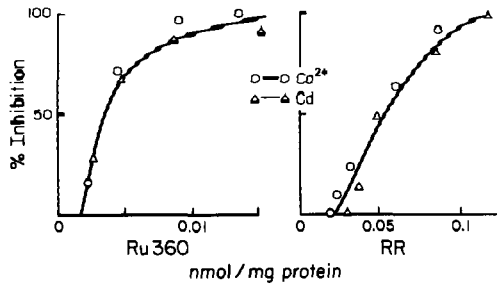

FIGURE 3: Inhibition of Ca^{2+} and Cd^{2+} transport by Ru360 and ruthenium red. Ca^{2+} transport was measured by the stimulation of state 4 respiration of rat liver mitochondria (3 mg) in 2 mL of 0.25 M sucrose/5 mM Tris–HEPES, pH 7.4/5 mM Tris–succinate at 30 °C. The Cd^{2+} transport was measured by the swelling of mitochondria on the addition of 3 μM Cd^{2+} to 1.5 mg of rat liver mitochondria in 1 mL of 0.25 M sucrose/2 mM Tris–HEPES/2 mM succinate at 25 °C. The swelling was monitored at 546 nm.

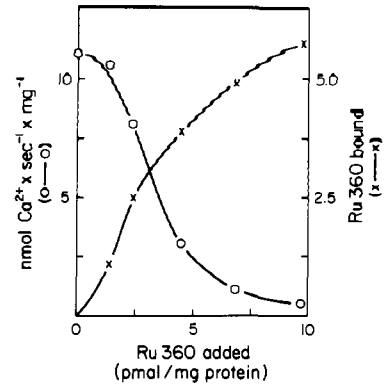

FIGURE 4: Relationship of the inhibition of Ca^{2+} transport by the dinuclear ruthenium ammine complex to its binding to mitochondria. Mitochondria (20 mg of protein) were suspended 4 mL of 0.25 M sucrose/5 mM Tris–HEPES and the indicated amount of ^{103}Ru-labeled dinuclear complex (specific activity 25 cpm/pmol Ru360). After 10 min at 4 °C, mitochondria were collected by centrifugation and washed once with 10 mL of the same medium. A 4-mg aliquot of mitochondria was counted. It may be noted that additional washing did not yield detectable radioactivity in the supernatant. Ca^{2+} transport was measured as in Figure 3.

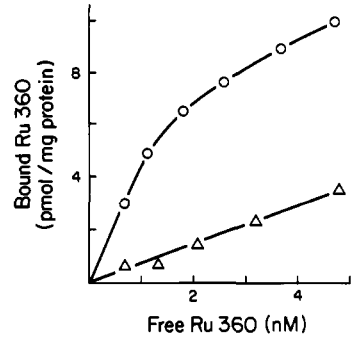

FIGURE 5: Equilibrium binding of dinuclear ruthenium ammine complex to intact mitochondria. Mitochondria (4 mg of protein) were incubated in 5 mL of 0.25 M sucrose/10 mM HEPES, pH 7.4/2.5 mM $CaCl_2$ and the indicated concentration of ^{103}Ru-labeled inhibitor at about 0 °C. After 10 min, the samples were centrifuged for 10 min at 20000 rpm and the pellets were washed with 10 mL of medium to remove free inhibitor. To determine nonspecific binding, 10 μM unlabeled dinuclear compound was added to the mitochondria that had been exposed to labeled inhibitor for 10 min. After another 10 min, the samples were centrifuged and washed as above. Total binding to mitochondria minus nonspecific binding represents specific binding, as in receptor type assays (Borsotto et al., 1984a,b).

generally a lag of several seconds before onset of the osmotic response. The duration of the lag proved to be a more reliable measure of the rate of Cd^{2+} uptake than the relative rate of swelling expressed as the inverse of the time taken to reach maximal swelling (Rasheed et al., 1984). Both the dinuclear complex and ruthenium red inhibited Cd^{2+}-dependent swelling, and the inhibition curves determined as above were almost identical with those for inhibition of Ca^{2+}-stimulated respiration (Figure 3). It also appears that the inhibition curves intersect the abscissa around 0.001 and 0.002 nmol for the dinuclear complex and ruthenium red, respectively, or the curves are sigmoidal. Reed and Bygrave (1974a,b) have reported similar results with the latter compound. Affolter and Carafoli (1981) observed that the activity response when Ca^{2+} concentration was increased was sigmoidal, indicating that $\Delta\mu H^+$ formation may be rate limiting rather than Ca^{2+} uniporter concentration.

The Ca^{2+}-stimulated respiration induced by saturating levels of Ca^{2+} is a suitable assay for Ca^{2+} transport activity but does not provide the sensitivity needed for analysis of the inhibition kinetics. The Ca^{2+} indicator Arsenazo III, has been found to be useful for such measurements (Scarpa, 1979). The double-reciprocal plots of the initial rates of Ca^{2+} uptake with and without added dinuclear ruthenium ammine complex (10 pmol/mg of protein) showed noncompetitive inhibition (data not shown). The K_m for Ca^{2+} was 28 μM, and the V_{max} without inhibitor was 10 nmol·mg^{-1}·s^{-1}. Similar kinetics were observed in earlier determinations with ruthenium red (Vasington et al., 1972; Reed & Bygrave, 1974a).

The relationship between binding of the dinuclear ruthenium ammine complex to mitochondrial protein and inhibition of Ca^{2+} uptake has been determined by use of the radiolabeled compound (Figure 4). In the 2.5–5 pmol/mg range, the activity decreased with the increase in inhibitor concentration. At concentrations of the inhibitor greater than 7.5 nM, the binding was not saturated, although the inhibition appeared to approach a maximal value, presumably because of significant nonspecific binding, as in the case of ruthenium red (Reed & Bygrave, 1974b). Figure 5 illustrates equilibrium binding to whole mitochondria in the absence and in the presence of a large excess of unlabeled dinuclear ruthenium complex. Similar to the binding of [^{3}H]nitrindipine to muscle T-tubule membrane (Borsotto et al., 1984a,b), the nonspecific binding of Ru360 that persisted in the presence of unlabeled inhibitor increased linearly with the concentration of free inhibitor whereas specific binding, defined as the difference between total and nonspecific binding, appeared to be a saturable function. On account of the high nonspecific binding, it was difficult to measure specific binding at concentrations higher than those shown in Figure 5. Nevertheless, a Scatchard plot (not shown) yielded a dissociation constant of 1.3 nM and the maximum specific binding was 7.5 pmol/mg of protein, which are consistent with the inhibition data shown in Figure 3.

Discussion

Structure. The occurrence of the capping formate ions as shown in Figure 2, which probably substituted onto the ion during the chromatographic purification, implies that the end positions of the dimeric core are readily substitutable by good inorganic ligands. Consequently, the active component in Ru360 is very likely $[X(NH_3)_4Ru–O–Ru(NH_3)_4X]^{3+}$, where X = Cl^- or OH^-. The tripositive charge of the complex may cause it to ion-pair with anionic carboxylate sites on the surface of proteins. Such outer-sphere binding also would be facilitated by hydrogen bonding via the ammine or apical ligand protons. The ready substitution of the apical sites should provide a facile means of linking the $[Ru–O–Ru]^{5+}$ core to a variety of ligands or immobilizing it on a chromatographic substrate. Moreover,

it is possible that the dinuclear complex covalently binds at the apical positions to carboxylate residues near the surface of the Ca^{2+} uniporter.

The short μ-O-Ru bond length indicates significant π bonding, which probably involves p orbitals on the oxygen and d_π (d_{xz} and d_{yz}) orbitals on the metal ions, which have lobes of symmetry directed down the internuclear z axis. Since the charge distributed over the two ruthenium ions is odd (7+), the metal ions should be considered as Ru(3.5) with the odd electron delocalized through the π bonding network between the two metals.

In collaboration with J. J. Kang and N. Ikemoto, the effects of the dinuclear ruthenium ammine complex and ruthenium red on Ca^{2+} release by the heavy fraction of sarcoplasmic reticulum of rabbit skeletal muscle were compared. The Ca^{2+} release was induced by the addition of 2 mM caffeine, and the time course was monitored by stopped-flow spectrophotometry with Arsenazo III as the indicator (Ikemoto et al., 1988). Concentrations of the dinuclear complex up to 5 μM did not show detectable inhibition while 1 μM ruthenium red produced complete inhibition. The data point to interesting differences in the specificity of the di- and trinuclear ruthenium ammine complexes that may be explored in other Ca^{2+} transport systems, e.g., voltage-dependent Ca^{2+} channels in cisplatin-sensitive and cisplatin-resistant leukemia cells (Vassilev et al., 1987), neurotransmitter release mechanisms (Taipale et al., 1989; Wieraszko, 1986), and Ca^{2+} sequestration in the endoplasmic reticulum (Hurley, 1988).

These studies provide a new ruthenium ammine complex of potential usefulness as an affinity ligand for the purification of the Ca^{2+} uniporter.

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