The Interaction of Rhodium(II) Carboxylates with Enzymes

R. A. Howard, T. G. Spring, and J. L. Bear

Departments of Chemistry [J. L. B.] and Biophysical Sciences [R. A. H., T. G. S.], University of Houston, Houston, Texas 77004

SUMMARY

The effect of rhodium(II) acetate, propionate, and methoxyacetate on the activity of 17 enzymes was evaluated. The enzymes were preincubated with the rhodium(II) complexes in order to detect irreversible inhibition. All enzymes that have essential sulfhydryl groups in or near their active site were found to be reversibly inhibited. Those enzymes without essential sulfhydryl groups were not affected. In each case, the rate of inactivation closely paralleled the observed toxicity and antitumor activity of rhodium(II) carboxylates; that is, rhodium(II) propionate > rhodium(II) acetate > rhodium(II) methoxyacetate. In addition, those enzymes that have been demonstrated to be most sensitive to established sulfhydryl inhibitors, such as glyceraldehyde-3-phosphate dehydrogenase, were also most sensitive to rhodium(II) carboxylate inactivation. Proton magnetic resonance measurements made during the titration of rhodium(II) acetate with cysteine showed that breakdown of the carboxylate cage occurred as a result of reaction with this sulfhydryl-containing amino acid.

INTRODUCTION

A number of rhodium(II) carboxylates (Chart 1) have been shown to exhibit carcinostatic activity against Ehrlich ascites and L1210 ascites tumors in mice (2, 5, 10). Both the antitumor effect and toxicity of these neutral rhodium(II) cage complexes are shown to be related to their hydrophobicity (partition coefficient). The chemical mechanism by which these compounds exert their biological effect is not known, but several investigations have been made with regard to the reactions of rhodium(II) carboxylates with biological molecules. It has been demonstrated that ligands can bind to rhodium(II) carboxylates (Chart 1, L) at the 2 axial positions through such donor atoms as nitrogen, sulfur, oxygen, and phosphorus (14, 19). Ligands with unprotonated amino groups, such as adenine nucleotides and phosphorolytic acid, were also most sensitive to rhodium(II) carboxylate inactivation. Proton magnetic resonance measurements made during the titration of rhodium(II) acetate with cysteine showed that breakdown of the carboxylate cage occurred as a result of reaction with this sulfhydryl-containing amino acid.

Also inhibited in vivo, one possible explanation for the toxic and antitumor effects of rhodium(II) carboxylates is the interruption of DNA replication. It was unclear, however, whether the effect on the polymerase activities was due to direct polymerase inhibition (either reversible or irreversible) or to interactions with the substrates and/or templates. Subsequent studies on the interaction of rhodium(II) carboxylates with cysteine revealed that this amino acid uniquely causes the breakdown of the rhodium(II) carboxylate cage structure. In order to determine whether this reaction is important in explaining the biological effects of rhodium(II) carboxylates, we have tested a number of different enzymes to ascertain whether those enzymes that possess free —SH groups would behave differently after exposure to a rhodium(II) carboxylate than those that do not possess free —SH groups. This paper reports the results of this investigation.

MATERIALS AND METHODS

Rhodium(II) acetate was purchased from Matthey Bishop, Inc., Malvern Pa. The other rhodium(II) carboxylates were synthesized by exchange with the free acid, as previously described (15). All rhodium(II) carboxylates were recrystallized from acetone or an acetone-water mixture before use. Enzymes and substrates were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of the highest purity available from Aldrich Chemical Co., Milwaukee, WI. The enzymes studied and the references for the assay procedures used appear in Table 1.

Enzymes were preincubated in the absence of substrate for fixed periods of time with the rhodium(II) carboxylate under investigation and then were assayed for activity. This allowed us to measure the rate of irreversible enzyme inactivation in the absence of substrate protection. The protocol involved the dilution of 50 μl of an undialyzed enzyme concentrate into 1 ml of rhodium(II) carboxylate in 0.05 M sodium phosphate buffer (pH 7.5). Concurrently, 50 μl of the enzyme concentrate were also diluted into 1 ml of buffer alone, for a control. At specified time intervals, an appropriate volume of the preincubated enzyme was removed, and enzyme activity was measured. The rate of inactivation was then calculated as (experimental activity/control activity) versus time.

The interaction of rhodium(II) acetate with cysteine (free base) was performed in 0.05 M sodium phosphate buffer (pH 7.5). The NMR scans were run on a Varian T-60 NMR spectrometer, with trimethylsilylamine as an external standard.

1 Recipient of Grant GM-20479 from the NIH.
2 Recipient of Grant CA-13817 from the NIH. To whom requests for reprints should be addressed, at the Department of Chemistry, University of Houston, Houston, Texas 77004.
4 Received May 21, 1976; accepted August 23, 1976.

4 The abbreviations used are: NMR, nuclear magnetic resonance; PCMB, paracloromercuribenzoate; LDH, lactate dehydrogenase; GPDH, glyceraldehyde-3-phosphate dehydrogenase.
RESULTS AND DISCUSSION

The reaction of cysteine with rhodium(II) carboxylates is dramatically different from the reaction involving any of the other amino acids. Instead of reversible axial binding, characteristic or other ligands, cysteine causes the breakdown of the carboxylate cage structure. This fact is demonstrated by proton NMR measurements made during the titration of rhodium(II) acetate with cysteine in buffer at pH 7.5. Adding cysteine to a solution of rhodium(II) acetate caused the radio frequency absorption of the methyl protons of the acetates to shift upfield from the position of rhodium(II)-bound acetates (2.12 δ) to that of free acetate ions (2.27 δ). Integration of the 2 types of acetate methyl protons indicates the reaction to be complete when 4 cysteines have been added per Rh₂(OOCCH₃)₄ (Chart 2).

The previous studies on enzyme inhibition by rhodium(II) carboxylates have included the rhodium(II) compound directly in the assay, leaving unresolved the question of whether the observed inhibition is reversible or irreversible. In this study, the enzymes were instead preincubated with either rhodium(II) propionate, rhodium(II) acetate, or rhodium(II) methoxyacetate and then were rapidly diluted into the assay mixture for measurement of activity. This procedure allowed the measurement of irreversible inhibition as a function of time. On the basis of the previously described reaction of rhodium(II) carboxylates with cysteine, we predicted that only those enzymes having essential —SH groups, required for or related to enzymatic activity, would be irreversibly inhibited. The results show this prediction to be valid (Table 2). Although binding of rhodium(II) carboxylates to proteins by axial bonding was previously demonstrated, this interaction did not result in inhibition (reversible or irreversible) of non-SH-bearing enzymes (Tables 1 and 2). Thus, for example, although pancreatic RNase (with no free —SH groups) was shown by equilibrium dialysis to bind rhodium(II) acetate (3), no inhibition of activity toward 2',3'-CMP is seen. Even the level of rhodium(II) carboxylate introduced into the assay with the enzyme from the preincubation mixture (10⁻⁶ M) was not inhibitory during the assay.

Only 2 of the enzymes tested which were known to have —SH groups were not inactivated by rhodium(II) carboxylates, namely, enolase and phosphoglycerate mutase (Table 2). There is, however, no evidence that the —SH groups on enolase are essential for activity (26). It should be noted that enolase did show a relatively slow rate of inactivation [t½ = 22 min in 1 mM rhodium(II) propionate] when Mg²⁺ ion, an essential cofactor, was omitted in the preincubation experiment. This slow irreversible inhibition could have been caused by the exposure of an —SH group on the enzyme during subunit dissociation or unfolding of the protein. Although rabbit muscle phosphoglycerate mutase has 4 cysteine groups which can be titrated with PCMB (18), there is no evidence that the cysteines are present in the active site. Rhodium(II) carboxylates apparently do not react with these —SH groups, or else the complexing of a rhodium at these sites does not alter activity.

Enzymes that have been shown to have —SH groups at or near their active site were all inhibited irreversibly by preincubation with rhodium(II) carboxylates (Table 2). In each case, the rate of inactivation was rhodium(II) propionate >

---

Table 1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>EC No.</th>
<th>Sigma Cat. no.</th>
<th>Assay ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoglycerate mutase</td>
<td>Rabbit muscle</td>
<td>2.7.5.3</td>
<td>P8252</td>
<td>9</td>
</tr>
<tr>
<td>GPDH</td>
<td>Rabbit muscle</td>
<td>1.2.1.12</td>
<td>G5126</td>
<td>24</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>Rabbit muscle</td>
<td>2.7.1.40</td>
<td>P1506</td>
<td>3</td>
</tr>
<tr>
<td>LDH</td>
<td>Rabbit muscle</td>
<td>1.1.1.27</td>
<td>L1254</td>
<td>20</td>
</tr>
<tr>
<td>Enolase</td>
<td>Rabbit muscle</td>
<td>4.2.1.11</td>
<td>e</td>
<td>25</td>
</tr>
<tr>
<td>Chymotrypsin*</td>
<td>Bovine</td>
<td>3.4.4.5</td>
<td>C1384</td>
<td>11</td>
</tr>
<tr>
<td>Lysozyme^</td>
<td>Chicken</td>
<td>3.2.1.17</td>
<td>L6876</td>
<td>22</td>
</tr>
<tr>
<td>Alkaline phosphatase*</td>
<td>Escherichia coli</td>
<td>3.1.3.1</td>
<td>P4252</td>
<td>7</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>Yeast</td>
<td>1.1.1.49</td>
<td>G7878</td>
<td>27</td>
</tr>
<tr>
<td>Glycerolphosphate dehydrogenase</td>
<td>Rabbit muscle</td>
<td>1.1.1.8</td>
<td>G6880</td>
<td>21</td>
</tr>
<tr>
<td>Glucose oxidase*</td>
<td>Aspergillus niger</td>
<td>1.1.3.4</td>
<td>G6500</td>
<td>8</td>
</tr>
<tr>
<td>Peroxidase*</td>
<td>Horseradish</td>
<td>1.11.1.7</td>
<td>P8125</td>
<td>17</td>
</tr>
<tr>
<td>Trypsin*</td>
<td>Bovine</td>
<td>3.4.4.4</td>
<td>T8003</td>
<td>13</td>
</tr>
<tr>
<td>Glutamic-oxaloacetic transaminase^</td>
<td>Porcine heart</td>
<td>2.6.1.1</td>
<td>G2751</td>
<td>1</td>
</tr>
<tr>
<td>Malic dehydrogenase</td>
<td>Bovine</td>
<td>1.1.1.37</td>
<td>M9004</td>
<td>23</td>
</tr>
<tr>
<td>Aldolase</td>
<td>Rabbit muscle</td>
<td>4.1.2.13</td>
<td>A5511</td>
<td>12</td>
</tr>
<tr>
<td>RNase A^</td>
<td>Bovine</td>
<td>2.7.7.16</td>
<td>R4875</td>
<td>4</td>
</tr>
</tbody>
</table>

* Purified by the method of Winstead and Wold (12).
^ Denotes those enzymes that lack free —SH groups.

---

DECEMBER 1976 4403
Results of enzyme inactivation studies

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Inactivated by Rh₂(OOCR)₄</th>
<th>t₁/₂ (min) in 10⁻⁴ M rhodium (II) propionate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoglycerate mutase</td>
<td>No</td>
<td>Not affected</td>
</tr>
<tr>
<td>GPDH</td>
<td>Yes</td>
<td>6</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>Yes</td>
<td>200</td>
</tr>
<tr>
<td>LDH</td>
<td>Yes</td>
<td>17</td>
</tr>
<tr>
<td>Enolase</td>
<td>No</td>
<td>Not affected</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>Yes</td>
<td>8</td>
</tr>
<tr>
<td>Glycerol phosphate dehydrogenase</td>
<td>Yes</td>
<td>21</td>
</tr>
<tr>
<td>Malic dehydrogenase</td>
<td>Yes</td>
<td>16</td>
</tr>
<tr>
<td>Aldolase</td>
<td>Yes</td>
<td>200</td>
</tr>
</tbody>
</table>

a Those enzymes that lack —SH groups (Table 1) were all unaffected by rhodium(II) carboxylates.

b t₁/₂, the time required for enzyme activity to decrease to 50% of control activity.

c Inactivation seen when Mg²⁺ was not present in preincubation.

R. A. Howard et al.

Prevented and full activity would return when dithiothreitol was added to the assay. The results of this experiment showed that enzyme inhibition due to PCMB was reversible but that PCMB-blocked —SH groups apparently still reacted with rhodium(II) carboxylates, although much more slowly (t₁/₂ = 4 min for PCMB blocked; t₁/₂ < 15 sec for free enzyme in 1 mM rhodium(II) propionate).

For any enzyme that has an essential sulfhydryl group in its active site we expected to see some substrate protection against inactivation, since the —SH groups would be less accessible to the rhodium(II) complex in substrate-protected enzymes. With GPDH, both the substrate, glyceraldehyde 3-phosphate, and coenzyme, NAD, slowed but did not prevent the inactivation by 1 mM rhodium(II) propionate (t₁/₂ < 15 sec for free enzyme, t₁/₂ = 1 min with 5 × 10⁻⁴ M glyceraldehyde 3-phosphate present, and t₁/₂ = 2.5 min with 2.5 × 10⁻⁴ M NAD present). Our present hypothesis is that enzyme inhibition is due to an initial interaction between one or more —SH groups and a rhodium(II) carboxylate, followed by cage breakdown and the tight binding of a rhodium (probably in its more kinetically inert +3 oxidation state) to the enzyme active site. Possible involvement of other active components has not been ruled out.

Relationship of Enzyme Data to Antitumor Activity. The reaction of rhodium(II) carboxylates with —SH enzymes closely parallels the toxicity and antitumor activity that these compounds display. In fact, a close numerical correlation is observed between the concentration of a particular rhodium(II) carboxylate which will cause inactivation of an enzyme to 5% activity (relative to control) in a specified time interval and the dose of a rhodium(II) carboxylate that yields maximum survival rates in mice (approximately the dose that is lethal in 10% of the cases) (Chart 4). The correlation of the antitumor activity with —SH reactivity is much better than the correlation with the binding strength of amino acids.

rhodium(II) acetate > rhodium(II) methoxyacetate. The inactivation of LDH as a function of time by each of the 3 rhodium(II) carboxylates at a concentration of 10⁻⁴ M (Chart 3) demonstrates the relative potency of the rhodium(II) carboxylates tested. In addition, those enzymes that have been demonstrated to be very sensitive to established —SH inhibitors, such as GPDH, are most sensitive to rhodium(II) carboxylate inhibition.

Once a sensitive enzyme has been exposed to a rhodium(II) carboxylate, activity is not readily restored. Incubation of LDH that had been deactivated with rhodium(II) propionate with a 10-fold excess of dithiothreitol, cysteine, EDTA, or a dithiothreitol and EDTA mixture did not result in recovery of any activity after 6 weeks of exposure. The effect, therefore, appears to be irreversible using normal chemical techniques.

To prove further the requirement of an —SH group for deactivation of an enzyme by a rhodium(II) carboxylate, the —SH groups of LDH were first blocked with the use of PCMB, the effect of which can be reversed by dithiothreitol. After reaction with PCMB, the enzyme was exposed to rhodium(II) propionate in the hope that irreversible inactivation of the enzyme by the rhodium(II) complex would be prevented and full activity would return when dithiothreitol was added to the assay. The results of this experiment showed that enzyme inhibition due to PCMB was reversible but that PCMB-blocked —SH groups apparently still reacted with rhodium(II) carboxylates, although much more slowly (t₁/₂ = 4 min for PCMB blocked; t₁/₂ < 15 sec for free enzyme in 1 mM rhodium(II) propionate).

For any enzyme that has an essential sulfhydryl group in its active site we expected to see some substrate protection against inactivation, since the —SH groups would be less accessible to the rhodium(II) complex in substrate-protected enzymes. With GPDH, both the substrate, glyceraldehyde 3-phosphate, and coenzyme, NAD, slowed but did not prevent the inactivation by 1 mM rhodium(II) propionate (t₁/₂ < 15 sec for free enzyme, t₁/₂ = 1 min with 5 × 10⁻⁴ M glyceraldehyde 3-phosphate present, and t₁/₂ = 2.5 min with 2.5 × 10⁻⁴ M NAD present). Our present hypothesis is that enzyme inhibition is due to an initial interaction between one or more —SH groups and a rhodium(II) carboxylate, followed by cage breakdown and the tight binding of a rhodium (probably in its more kinetically inert +3 oxidation state) to the enzyme active site. Possible involvement of other active components has not been ruled out.

Relationship of Enzyme Data to Antitumor Activity. The reaction of rhodium(II) carboxylates with —SH enzymes closely parallels the toxicity and antitumor activity that these compounds display. In fact, a close numerical correlation is observed between the concentration of a particular rhodium(II) carboxylate which will cause inactivation of an enzyme to 5% activity (relative to control) in a specified time interval and the dose of a rhodium(II) carboxylate that yields maximum survival rates in mice (approximately the dose that is lethal in 10% of the cases) (Chart 4). The correlation of the antitumor activity with —SH reactivity is much better than the correlation with the binding strength of amino acids.

To prove further the requirement of an —SH group for deactivation of an enzyme by a rhodium(II) carboxylate, the —SH groups of LDH were first blocked with the use of PCMB, the effect of which can be reversed by dithiothreitol. After reaction with PCMB, the enzyme was exposed to rhodium(II) propionate in the hope that irreversible inactivation of the enzyme by the rhodium(II) complex would be prevented and full activity would return when dithiothreitol was added to the assay. The results of this experiment showed that enzyme inhibition due to PCMB was reversible but that PCMB-blocked —SH groups apparently still reacted with rhodium(II) carboxylates, although much more slowly (t₁/₂ = 4 min for PCMB blocked; t₁/₂ < 15 sec for free enzyme in 1 mM rhodium(II) propionate).

For any enzyme that has an essential sulfhydryl group in its active site we expected to see some substrate protection against inactivation, since the —SH groups would be less accessible to the rhodium(II) complex in substrate-protected enzymes. With GPDH, both the substrate, glyceraldehyde 3-phosphate, and coenzyme, NAD, slowed but did not prevent the inactivation by 1 mM rhodium(II) propionate (t₁/₂ < 15 sec for free enzyme, t₁/₂ = 1 min with 5 × 10⁻⁴ M glyceraldehyde 3-phosphate present, and t₁/₂ = 2.5 min with 2.5 × 10⁻⁴ M NAD present). Our present hypothesis is that enzyme inhibition is due to an initial interaction between one or more —SH groups and a rhodium(II) carboxylate, followed by cage breakdown and the tight binding of a rhodium (probably in its more kinetically inert +3 oxidation state) to the enzyme active site. Possible involvement of other active components has not been ruled out.

Relationship of Enzyme Data to Antitumor Activity. The reaction of rhodium(II) carboxylates with —SH enzymes closely parallels the toxicity and antitumor activity that these compounds display. In fact, a close numerical correlation is observed between the concentration of a particular rhodium(II) carboxylate which will cause inactivation of an enzyme to 5% activity (relative to control) in a specified time interval and the dose of a rhodium(II) carboxylate that yields maximum survival rates in mice (approximately the dose that is lethal in 10% of the cases) (Chart 4). The correlation of the antitumor activity with —SH reactivity is much better than the correlation with the binding strength of amino acids.
This is related to the toxic effects of rhodium on tumor and the interaction of the same enzymes in vivo as in vitro, and whether rhodium compounds cause appreciable inactivation of cytochrome C. The latter data were obtained as described elsewhere (4): O, rhodium(II) methoxycaceta; ■, rhodium(II) acetate; ●, rhodium(III) propionate.

Chart 4. Correlation between concentration of rhodium(II) carboxylate required to inactivate LDH 50% in 1 min (y axis) and the therapeutic dose of rhodium(II) carboxylate which results in maximum survival rate of mice carrying Ehrlich ascites tumor cells (x axis). The latter data were obtained as described elsewhere (4): O, rhodium(II) methoxycaceta; ■, rhodium(II) acetate; ●, rhodium(III) propionate.

group ligands (19). On the basis of this strong correlation, we propose that the antitumor activity of rhodium(II) carboxylates is due to the reaction of enzymes or proteins containing —SH groups. In addition, Eck et al. (6) observed that only a small fraction of the rhodium in a dose of rhodium(II) acetate administered to mice appeared in the urine, whereas most of the acetate portion of the molecule was metabolized to CO2 within 2 hr. Noble metal chlorides, on the other hand, are known to be readily excreted in the urine (16). This evidence further supports the supposition that the rhodium is bound to tissue and/or free proteins which serve to prevent excretion. It may also be possible that rhodium(II) carboxylates exert their effect by binding to cellular membrane proteins, many of which are known to contain —SH groups. The differential effects of the rhodium(II) carboxylates might then be due to their differential ability to dissolve in the lipid portion of the membrane where reaction with —SH groups could occur.

In conclusion, the in vitro studies strongly indicate that the interaction between the rhodium(II) carboxylates and molecules of biological importance containing sulphydryl groups is related to the observed antitumor activity. However, whether rhodium compounds cause appreciable inactivation of the same enzymes in vivo as in vitro, and whether this is related to the toxic effects of rhodium on tumor and normal cells, remains to be determined.

REFERENCES

The Interaction of Rhodium(II) Carboxylates with Enzymes

R. A. Howard, T. G. Spring and J. L. Bear


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/36/12/4402

E-mail alerts Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.