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INHIBITION OF HYDROXYPROLINE SYNTHESIS BY PALLADIUM IONS

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Summary

Palladium ions, administered as $PdSO_4$, markedly affect the incorporation of L-[3,4-$^3H_2$]proline into non-dialyzable fractions in 10-day chick embryo cartilage explants with a 55—65% reduction in the concentration range 0.06—0.6 mM. Under these conditions the synthesis of [^{3}H]hydroxyproline was nearly completely inhibited. Experiments with prolyl hydroxylase (EC 1.14.11.2) indicated a strong irreversible inhibition of the enzyme with a competition between Fe^{2+} and Pd^{2+}. The K_i for the inhibition was 0.02 mM. Pd^{2+}-treated enzyme remained inactive after extensive dialysis. These studies suggest that Pd^{2+} may inhibit collagen synthesis by replacing Fe^{2+} in the active site of prolyl hydroxylase and forming strong complexes with the enzyme. These studies also point to a potential mechanism of Pd^{2+} toxicity.

Introduction

The structural protein collagen is synthesized in a series of biochemical events in the first of which specific proline and lysine residues in the initially synthesized polypeptide undergo hydroxylation to hydroxyproline and hydroxylysine (see ref. 1 for review). The hydroxylases for proline (EC 1.14.11.2) as well as lysine require Fe^{2+} for their activity and chelating agents inhibit the hydroxylation step both in tissues [2—5] and in vitro reactions of prolyl hydroxylase [5]. Hydroxylation in chelator-inhibited systems can usually be restored by the addition of excess Fe^{2+} [3—5]. We report here the unexpected

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observation that Pd^{2+} ions irreversibly inhibit prolyl hydroxylase both in a tissue actively engaged in collagen synthesis in vitro and in reactions of the purified enzyme. The inhibition of prolyl hydroxylase was caused by competition between Pd^{2+} and Fe^{2+} for the same site on the enzyme. Pd^{2+} also suppressed the overall incorporation of radioactive proline into non-dialyzable fractions in the tissue incorporation studies to a smaller extent than the inhibition of hydroxyproline synthesis.

Materials and Methods

Materials

L-[3,4-$^3H_2$]proline, 4.8 Ci/mM, was obtained from New England Nuclear. Palladium sulfate was a product of Research Organic/Inorganic Chemicals. Embryonated chicken eggs incubated for varying periods in a commercial hatchery were obtained and kept in a humidified incubator at 39° C until ready to use.

Explants and incubation procedures

10-day old chick tibiae are largely cartilaginous and a major part of the protein output in these tissues is collagen. These tissues were used in all experiments. All tissue incubations were made with tibiae explanted by microdissection under Krebs medium [6]. One tibia from each pair was used in the control and the counter member was used in the test incubation. In most experiments, two tibiae were used in each flask. All additives were dissolved in the incubation medium and the tissues were preincubated for 10 min under the experimental conditions before the addition of the radioactive precursor. Incubations were carried out in a shaker water bath maintained at 37° C and terminated by homogenizing the tissues in chilled, distilled water. The homogenates were dialyzed overnight against water in the cold to remove unbound radioactivity and hydrolyzed in 6 M HCl at 110° C for 20 h. The hydrolyzates were prepared for assays by evaporating them in vacuo and the residues were dissolved in distilled water.

Assays

Radioactivity was determined in a liquid scintillation counter and observed cpm corrected to dpm after quench correction. Radioactive hydroxyproline was assayed by the procedure described by Juva and Prockop [7]. Total ninhydrin-reactive material in the hydrolyzates was determined as an index of protein content, using leucine standards. Although throughout the very large number of similar experiments in our laboratory, little difference has been noted in the incorporation of radioactive precursors between the two members of each pair of tibiae, the radioactive incorporation has been related to the total tissue protein content in these studies and is expressed as a specific activity or hydroxyproline radioactivity incorporated per μmol leucine equivalent.

Collagenase reaction

The incorporation of radioactive proline into the collagen sequence was

confirmed in some of these studies using a virtually protease-free collagenase preparation (Advance Biofactures, New York), using minor modifications of a procedure described before [9]. In determining the distribution of radioactivity between dialyzable and non-dialyzable materials, corrections were made to include the dialyzable radioactivity present inside the dialysis bag.

Prolyl hydroxylase reaction

The enzyme was prepared by minor modifications of a published method [10] and the preparations used here were enriched in enzyme close to 400-fold. The enzyme activity was assayed by the method described by Hutton et al. [11] using L-[3,4-$^3H_2$]proline-labeled unhydroxylated collagen ("Protocollagen"), prepared by incubating cartilage explants with radioactive proline in the presence of 1.0 mM α,α'-bipyridyl. The hydroxylation reaction was stopped by adding trichloroacetic acid to a final concentration of 5 per cent. ^{3}HHO, formed in stoichiometric amounts during [^{3}H]prolyl hydroxylation was collected by vacuum distillation. The radioactivity of the ^{3}HHO was used as a measure of hydroxylation.

Results

Experiments with intact cartilage explants

Initial experiments using several Pd^{2+} salts established that Pd^{2+} rather than the counter-ion was responsible for the biological effects of these compounds. Preliminary experiments also indicated that Pd^{2+} in a 10-fold concentration range, 0.06—0.6 mM, reduced the overall incorporation of proline by 45—55

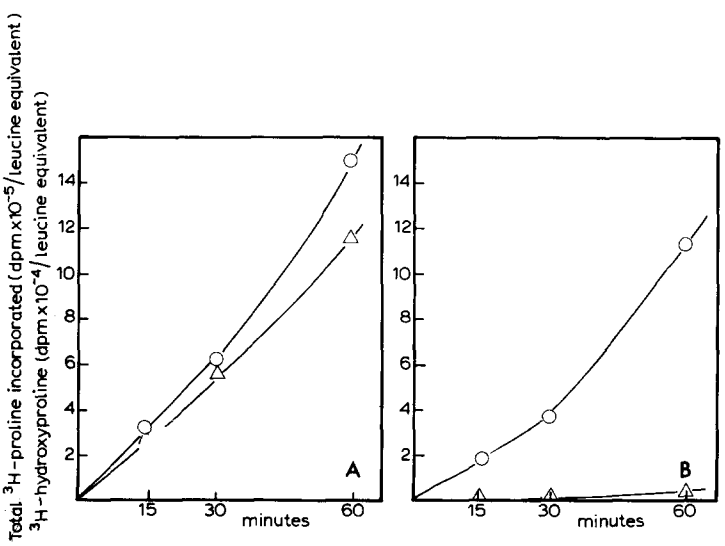


Fig. 1. Incorporation of [^{3}H]proline and synthesis of [^{3}H]hydroxyproline in the presence of Pd^{2+}. Sets of two cross-matched tibiae were incubated in the control medium (A) and in medium containing 0.37 mM $PdSO_4$ (B) in the presence of 5 μCi [^{3}H]proline for varying lengths of time; the incubation and assay procedures have been described in the text. Total ^{3}H-incorporation, ○———○; [^{3}H]hydroxyproline synthesized, △———△.

TABLE I

INCORPORATION OF ^{3}H-PROLINE INTO COLLAGENASE-SUSCEPTIBLE PROTEIN IN THE PRESENCE OF Pd^{2+}

Tissues were incubated for 1 h with 5 μCi [^{3}H] proline in the presence of the indicated concentration of $PdSO_4$ and the dialyzed homogenates of the labeled tissues were analyzed for ^{3}H-incorporation into collagen and non-collagenous proteins as described in the text.

Additions	Percent of total ^{3}H-radioactivity inside dialysis bag (non-collagenous protein)	Percent of total ^{3}H-radioactivity outside dialysis bag ("collagen-sequence")
None	47	53
+0.012 mM $PdSO_4$	47	53
+0.062 mM $PdSO_4$	62	38
+0.620 mM $PdSO_4$	38	62

percent. Under the same conditions, however, the synthesis of [^{3}H] hydroxyproline was inhibited 90 percent or more.

When the tissues were incubated with [^{3}H] proline in the presence of 0.37 mM Pd^{2+}, the radioactive precursor continued to be incorporated at lowered rates, for 1 h and longer (Fig. 1). Very little [^{3}H] hydroxyproline was synthesized in the tissues exposed to Pd^{2+}.

Hydroxyproline in collagen is synthesized by the enzymatic hydroxylation of proline already incorporated into polypeptides with the collagen sequence [1]. The reduced synthesis of hydroxyproline may be explained either by a specific inhibition of collagenous polypeptide synthesis or by the inhibition of prolyl hydroxylase in the collagen synthesizing cells. This question was examined by assaying the incorporation of [^{3}H] proline into the collagen sequence by using a highly purified Clostridial collagenase preparation (Table I). Control experiments using tissues labeled with [^{14}C] tryptophan and [^{3}H] proline indicated that most of the [^{3}H] hydroxyproline and less than 5 percent of the

TABLE II

IRREVERSIBILITY OF Pd^{2+} INHIBITION OF INTRACELLULAR PROLINE HYDROXYLATION IN THE PRESENCE OF Fe^{2+}

The tissues were incubated for 2 h with 5 μCi [^{3}H] proline and the indicated additives. Incubation was terminated by homogenizing the tissues and the tissues were processed and analyzed as described in the text.

Additions	Total ^{3}H-incorporation (dpm/leucine equiv. · 10^{-5})	Total [^{3}H]-hydroxyproline (dpm/leucine equiv. · 10^{-4})
None	3.57	2.44
+Pd^{2+}, 0.2 mM	2.64	0.18
+Fe^{2+}, 0.2 mM	4.96	4.13
+Pd^{2+}, 0.2 mM +Fe^{2+}, 0.2 mM	1.64	0.24
+Fe^{2+}, 0.4 MM	4.55	3.62
+Pd^{2+}, 0.2 mM + +Fe^{2+}, 0.4 mM	1.65	0.26

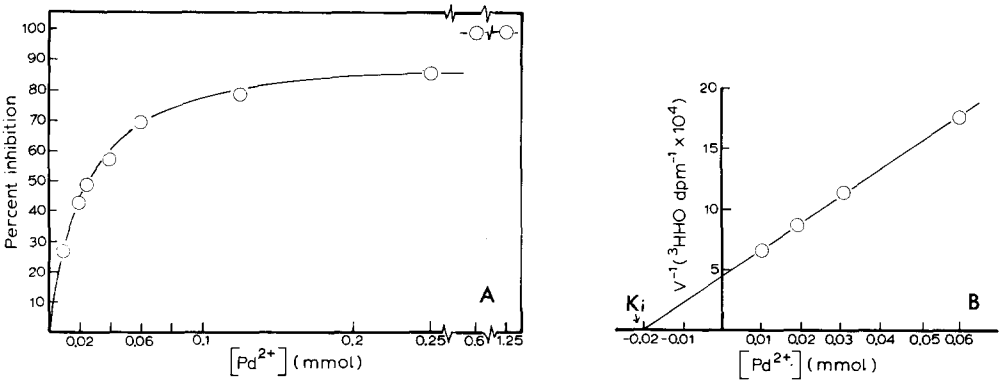


Fig. 2. Concentration dependence of inhibition of prolyl hydroxylase by Pd^{2+}. The enzyme reaction was carried out as described in the text and in the legend to Table III, in the presence of varying concentration of $PdSO_4$.

[^{14}C]tryptophan radioactivity was rendered dialyzable under our experimental conditions. As seen in Table I, there was no difference in the proportion of [^{3}H]proline-derived radioactivity in collagenase-susceptible material in control or Pd^{2+}-treated tissues. These data ruled out the unlikely possibility of a specific inhibition of collagenous polypeptide synthesis by Pd^{2+} and indicated that Pd^{2+} may be inhibitory to prolyl hydroxylase.

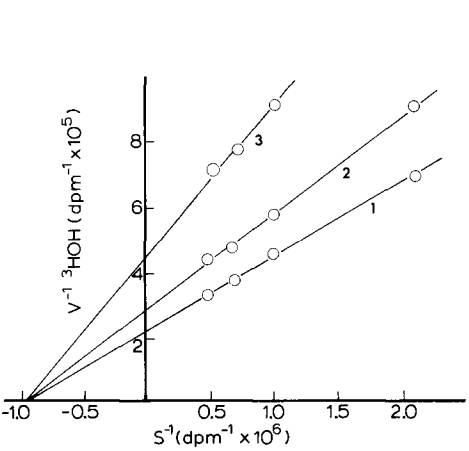
Although the above studies, and experiments with purified prolyl hydroxylase discussed later in this paper, suggested that the inhibition of hydroxyproline synthesis was caused by the inhibition of prolyl hydroxylase, by a mechanism involving competition between Fe^{2+} and Pd^{2+}, attempts to reverse this inhibition by excess Fe^{2+} were not successful (Table II), suggesting an irreversible change in the proline hydroxylating system.

Experiments with prolyl hydroxylase

Tissue studies indicated that Pd^{2+} may be inhibitory to prolyl hydroxylase. This question was examined by including Pd^{2+} in the prolyl hydroxylase reaction system. The prolyl hydroxylase preparation used in these studies was purified approx. 400-fold. A strong concentration-dependent inhibition of the reaction was observed, with a nearly maximal inhibition at Pd^{2+} concentrations of 0.6 mM and above (Fig. 2a). The apparent K_i for this inhibition was 0.02 mM Pd^{2+} (Fig. 2b).

The inhibition of prolyl hydroxylase was non-competitive with respect to substrate binding (Fig. 3) indicating that the interaction of Pd^{2+} with the enzyme occurred at a site different from the one involved in substrate binding. However, when double reciprocal plots were constructed at varying concentrations of Fe^{2+} at constant substrate concentration, a clear-cut competition between Fe^{2+} and Pd^{2+} was observed (Fig. 4). These data suggest that Pd^{2+} may be competing with Fe^{2+} for the same site, on the enzyme.

The competition between Fe^{2+} and Pd^{2+} suggested the possibility of reversing the inhibition of prolyl hydroxylase by Pd^{2+}, by increasing the Fe^{2+}-concentration in the reaction system. As in the case of intact tissues, however, there was no reversal of inhibition even in the presence of a 40-fold excess of Fe^{2+}



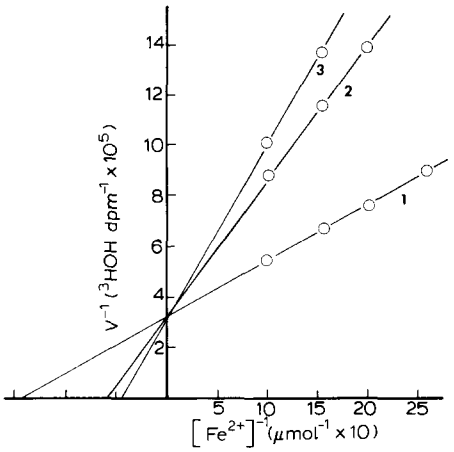


Fig. 3. Inhibition of prolyl hydroxylase by Pd^{2+} is non-competitive with respect to substrate binding. The reaction was carried out as described in the legend to Table III with varying concentrations of substrate, (1) in the absence of Pd^{2+}, (2) in the presence of 0.125 mM $PdSO_4$ and (3) in the presence of 0.625 mM $PdSO_4$.

Fig. 4. Competition between Pd^{2+} and Fe^{2+} during the inhibition of prolyl hydroxylase by Pd^{2+}. The reaction was carried out as described in Table III, in the presence of varying concentrations of Fe^{2+}, and (1) no inhibitor, (2) in the presence of 0.125 mM Pd^{2+}, and (3) in the presence of 0.625 mM Pd^{2+}.

over Pd^{2+} (Table III). When Pd^{2+} was added at a concentration of 0.6 mM, the formation of hydroxyproline was completely inhibited; however, as in the experiment described in Table II, no reversal was obtained at equivalent Fe^{2+} concentrations. We examined the possibility that the irreversibility of the inhibition by Pd^{2+} was due to a strong complexation between prolyl hydroxylase and the cation. The enzyme was pre-incubated with 0.2 mM Pd^{2+} followed by extensive dialysis to remove as much of the metal as possible. As seen in Table IV, this preparation exibited very little activity when compared to a control preparation which was not exposed to Pd^{2+} but was dialyzed under identical conditions. These data suggest that the complex of prolyl hydroxylase with Pd^{2+} is not dissociated under these conditions and support the observations in Tables II and III which suggest that the inhibition is essentially irreversible.

Discsussion

Palladium metal in various forms has been used in catalysis for a long time but very little information is available on its biological effects. Available data [12,13] suggest variable toxicity of palladium compounds which depended on the mode of administration. Our in vitro studies indicated that ionic Pd^{2+} may be toxic and may interfere with connective tissue metabolism, by lowering over-all protein synthesis and inhibiting the hydroxylation step in collagen synthesis. Since prolyl and lysyl hydroxylase reactions are very similar, it can be presumed that Pd^{2+} would inhibit the formation of hydroxylysine as well. Hydroxyproline contributes stability to the collagen triple helix [14—16] by contributing hydrogen bonds [17,18] and the inhibition of proline hydroxylation would have severe effects on tissue physiology and function, if a sufficient quantity reaches the target system.

TABLE III

IRREVERSIBILITY OF Pd^{2+} INHIBITION OF PROLYL HYDROXYLASE

Prolyl hydroxylase assays were carried out as described in the text. The reaction mixture contained [3,4-$^3H_2$]proline-labeled unhydroxylated collagen ($8.9 \cdot 10^5$ dpm), α-ketoglutarate, 0.5 mM, ascorbate, 0.5 mM, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.1 mM and enzyme, 0.2 mg in a final volume of 2.0 ml in Tris · HCl, 0.1 M, pH 7.6. $PdSO_4$ and excess ferrous ammonium sulfate were added as indicated in the Table. The reaction was terminated after 10 min by adding trichloroacetic acid to a final concentration of 5 percent and the analysis completed as described in the text.

Additions	Enzyme activity (^{3}HHO formed, dpm · 10^{-4}) *
Complete system	4.95
+Fe^{2+}, 0.2 mM	5.12
+Fe^{2+}, 0.4 mM	4.64
+Pd^{2+}, 0.025 mM	2.23
+Pd^{2+}, 0.025 mM + Fe^{2+}, 0.2 mM	2.36
+Pd^{2+}, 0.025 mM + Fe^{2+}, 0.4 mM	2.40

* ^{3}HHO: The prolyl hydroxylase assay is based on the release of one ^{3}H atom from each 3,4($^3H_2$)-prolyl residue hydroxylated and we have used the term ^{3}HHO to signify this stoichiometry in measuring the radioactivity of the water in the reaction medium.

Although the present studies do not provide information on the stoichiometry and the nature of Pd^{2+}-binding to prolyl hydroxylase, the clear cut competition between Pd^{2+} and Fe^{2+} suggests a mechanism involving complex formation between Pd^{2+} and the enzyme, in which Pd^{2+} replaces Fe^{2+} in the catalytic site. Fe^{2+} may be associated with a hydrophobic portion of the enzyme [19—22], but the mode of binding of Fe^{2+} to the enzyme has not been described. Preliminary studies in our laboratory have shown that other transition metals, such as Hg^{2+}, Cd^{2+}, Cu^{2+}, and Pt^{4+} which have a high affinity for -SH groups, inhibit prolyl hydroxylase in the same manner as Pd^{2+}. Pd^{2+} has been shown to form stable complexes with -SH groups and has been used as an assay for these

TABLE IV

PROLYL HYDROXYLASE · Pd^{2+} COMPLEX REMAINS INACTIVE AFTER EXTENSIVE DIALYSIS

The enzyme (1.0 mg) was incubated with 0.2 mM $PdCl_2$ in a final volume of 2.0 ml in the cold room for 15 min, after which it was dialyzed for 24 h against three 0.5 l changes and finally, against 1.0 l of a buffer, containing 0.1 M NaCl, 0.1 M glycine, 0.01 M Tris · HCl, pH 7.0. A control preparation was dialyzed under identical conditions. The reaction was carried out as described in Table III except that the enzyme concentration was 0.06 mg/ml in this experiment.

Additions	Enzyme activity (^{3}HHO formed, dpm · 10^{-3})
Untreated, dialyzed enzyme	
+0.1 mM Fe^{2+}	9.72
+0.2 mM Fe^{2+}	9.08
+0.4 mM Fe^{2+}	8.60
Pd^{2+}-treated, dialyzed enzyme	
+0.1 mM Fe^{2+}	0.09
+0.2 mM Fe^{2+}	0.45
+0.4 mM Fe^{2+}	0.33

groups [23]. The competition between Fe^{2+} and Pd^{2+} suggests that Fe^{2+} may be bound to the enzyme by -SH groups. Binding of Fe^{2+} to other non-heme iron enzymes has been shown to involve -SH groups [24,25].

The complex between prolyl hydroxylase and Pd^{2+} is apparently much more stable than the enzyme · Fe^{2+} complex, since the addition of Fe^{2+} in larger excess did not cause significant reversal of the inhibition either in the intracellular or in in vitro reaction. This virtual irreversibility was supported by the failure to recover enzyme activity even after extensive dialysis. These studies do not rule out the possibility that Pd^{2+} may also bind to -SH groups at sites other than the catalytic center of the enzyme.

Fe^{2+} has been shown to be an integral part of the active site of several other enzymes which catalyze the incorporation of oxygen into a variety of substrates [26—29]. The active site Fe^{2+} plays a role in the reductive fixation of oxygen in prolyl hydroxylase [30] and other similar enzymatic reactions [31]. Replacement of the active site Fe^{2+} with Pd^{2+} may inhibit either the binding or the transfer of oxygen, but it does not appear to interfere with the binding of the peptidyl proline substrate as seen from the non-competitive inhibition with respect to the substrate. These data also suggest a topological separation of the peptidyl substrate-binding site and the active site of the enzyme, a conclusion supported by the minimum chain-length requirement for peptidyl proline substrates [32] and the sequential asymmetry in the hydroxylation of proline observed in small repeating polypeptide substrates [33]. The binding of the substrate apparently does not involve the metal cofactor.

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