Inhibition of prolyl 4-hydroxylase by hydroxyanthraquinones

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Prolyl 4-hydroxylase (EC 1.14.11.2) is an essential enzyme in the post-translational modification of collagen. Inhibitors of this enzyme are of potential interest for the treatment of diseases involving excessive deposition of collagen. We have found that anthraquinones with at least two hydroxy groups *ortho* to each other are potent inhibitors of this enzyme. Kinetic studies revealed that 2,7,8-trihydroxyanthraquinone (THA) competitively inhibited the co-substrate, 2-oxoglutarate, but was non-competitive with regard to ascorbate and was tentatively considered to be uncompetitive with regard to protocollagen. The inhibition by THA was greatly enhanced in the absence of added Fe^{2+} and was partially reversed by the addition of concentrations of Fe^{2+} in excess of the optimum for the enzymic reaction. Binding studies indicated that THA is an effective chelating agent for Fe^{2+} . Several non-quinoidal compounds bearing the catechol moiety also inhibited the enzyme. The results suggest that THA inhibited prolyl 4-hydroxylase by binding to the enzyme at the site for 2-oxoglutarate possibly involving the Fe^{2+} atom, rather than by complexing with Fe^{2+} in free solution. The inhibition of prolyl 4-hydroxylase by THA exhibited strong positive co-operativity and may involve three distinct but non-independent binding sites.

INTRODUCTION

A critical step in the post-translational modification of collagen pro- α -chains is the hydroxylation of certain prolyl residues (Prockop *et al.*, 1979a). Two enzymes are involved, prolyl 3-hydroxylase (EC 1.14.11.7) and prolyl 4-hydroxylase (EC 1.14.11.2). The physiological role of the latter enzyme seems well established, since collagen must have about 90 4-hydroxyproline residues per chain in order to form the characteristic triple helix, which is stable at 37 °C (Prockop *et al.*, 1979a). Prolyl 4-hydroxylase has been purified to homogeneity from several sources and is now well-characterized (Prockop *et al.*, 1979a). The avian enzyme is a tetramer consisting of two different monomers, with M_r of approx. 64000 and 60000, and requires the co-factors Fe^{2+} ions, molecular oxygen, 2-oxoglutarate and ascorbate. Kivirikko and his colleagues studied the enzymic mechanism in detail (Myllyla *et al.*, 1977; Tuderman *et al.*, 1977) and concluded that it involves the sequential binding of Fe^{2+} , 2-oxoglutarate, O_2 and the polypeptide substrate. The Fe^{2+} atom is apparently in thermodynamic equilibrium with the enzyme and is believed to form part of its active centre (Majamaa *et al.*, 1984).

The importance of prolyl-4-hydroxylase to the correct processing and subsequent secretion of collagen (Prockop *et al.*, 1979a) suggests that it could be a useful target for a new generation of drugs to treat diseases involving excessive deposition of collagen and connective tissue. These include fibrosis of the lungs and liver, arteriosclerosis, scleroderma, keloid disease (Prockop *et al.*, 1979b), the proliferative pannus of rheumatoid arthritis (Boyd, 1970) and possibly muscular dystrophy (Fujii & Murota, 1983). Among the known inhibitors of prolyl 4-hydroxylase are $\alpha\alpha'$ -bipyridine, which is an effective chelator of Fe^{2+} , a number of pyridine mono- and di-carboxylic acids (Majamaa *et al.*, 1984, 1985) and an antitumour antibiotic, P-1894B (Ishimaru *et al.*, 1982),

otherwise known as vineomycin A1 (Ohta *et al.*, 1982). Unfortunately none of these compounds seems likely to be useful as an effective antifibrotic agent *in vivo*. During a search for novel inhibitors of prolyl 4-hydroxylase, we discovered that certain polyhydroxylated anthraquinones inhibit purified preparations of this enzyme from embryonic chicks and neonatal rats and also depress collagen deposition in models of fibrosis *in vivo* (T. J. Franklin & W. P. Morris, unpublished work). In the present paper we describe the effects of polyhydroxy-anthraquinones and related compounds on embryonic-chick prolyl 4-hydroxylase purified to homogeneity.

MATERIALS AND METHODS

Materials

Prolyl 4-hydroxylase was purified from the legs of 17-day chick embryos by the method of Kedersha & Berg (1981). When examined by SDS/polyacrylamide-gel electrophoresis, the enzyme preparation consisted solely of two proteins of M_r 60000 and 64000, considered to be the subunits of prolyl 4-hydroxylase. [^3H]Proline-labelled protocollagen was prepared by incubating 17-day embryonic-chick calvaria with $\alpha\alpha'$ -bipyridine and L-[3,4- ^3H]proline (Amersham International) by the method of Peterkofsky & DiBlasio (1975). The specific radioactivity of the purified protocollagen was approx. 100 kBq/ng. 2-Oxoglutarate was purchased from C. F. Boehringer & Soehne G.m.b.H., Mannheim, Germany. Ascorbic acid (sodium salt), DL-dithiothreitol, catalase (twice crystallized from bovine liver), bovine serum albumin (essentially fatty acid-free) and Tris were all purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K. THA (2,7,8-trihydroxyanthraquinone) and compounds A, C, D, E and F were all synthesized in the laboratories of Imperial Chemical Industries PLC by our colleagues Mr. R. I. Dowell and Mrs. E. M. Hadley. Compound B was

purchased from Fluka and compound G from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Assay of prolyl 4-hydroxylase

Most assays were carried out by measuring the release of ^3H , as $^3\text{H}_2\text{O}$, from $[^3\text{H}]$ prolyl-protocollagen, which accompanies the hydroxylation of proline at the 4-position. The reaction was carried out in a final volume of 0.5 ml, containing 5–10 ng of prolyl 4-hydroxylase, 40 μg of $[^3\text{H}]$ prolyl-protocollagen, 2-oxoglutarate (100 μM), ascorbate (1 mM), dithiothreitol (100 μM), catalase (0.1 mg/ml), bovine serum albumin (2 mg/ml) and 50 μM -ferrous ammonium sulphate, all dissolved in 50 mM-Tris/HCl buffer, pH 7.6. The mixture was incubated at 37 °C and the reaction stopped after 20 min by adding trichloroacetic acid to a final concentration of 5% (w/v) together with $(\text{NH}_4)_2\text{SO}_4$ (50% satn.) and bovine serum albumin (10 mg). The precipitated protein was removed by centrifugation at 11000 g for 8 min, and a sample (1 ml) of the supernatant was mixed with 10 ml of PCS scintillation fluid (Amersham International) for the determination of released ^3H by a liquid-scintillation counter. The release of $^3\text{H}_2\text{O}$ remained linear with time over 20 min. Initial velocities were determined by extrapolation from curves plotted with points at 2.5 min intervals. Inhibitors were usually dissolved in dimethyl sulphoxide and added to the reaction mixture to give a final concentration of solvent of 1% (v/v), which had a negligible effect on enzyme activity. The data points were fitted to the best hyperbolae representing the reaction velocity versus substrate concentration by using a computer program ('Hypfit'). The hyperbolae were then transformed into the Lineweaver–Burk plots given in the Results section.

RESULTS

Inhibition of prolyl 4-hydroxylase by THA, and its reversal by Fe^{2+}

The ability of THA to inhibit purified chick prolyl 4-hydroxylase was discovered during a broad search for inhibitors of this enzyme. Fig. 1 indicates that a concentration of 47 μM inhibited the enzyme by 50% (IC_{50}).

The existence of the catechol and the adjacent OH–CO groupings in THA suggested that the compound might complex with Fe^{2+} . The effect of varying the concentrations of Fe^{2+} in the reaction mixture was therefore tested. Although prolyl 4-hydroxylase requires added Fe^{2+} for optimal activity, it will function in the absence of added Fe^{2+} , presumably because sufficient Fe^{2+} is retained by the enzyme during purification. Fig. 1 shows that the inhibitory activity of THA was markedly increased when Fe^{2+} was omitted from the reaction mixture, giving an IC_{50} of 4.6 μM . Fig. 2 presents a Lineweaver–Burk plot of a more detailed experiment with various concentrations of Fe^{2+} and THA. There was no evidence for a simple competitive relationship between Fe^{2+} and THA.

Kinetics of inhibition of prolyl 4-hydroxylase by THA versus 2-oxoglutarate, protocollagen and ascorbate

The kinetic data obtained with THA and 2-oxoglutarate are shown in Fig. 3 and demonstrate competitive antagonism between 2-oxoglutarate and the anthraquin-

Fig. 1. Inhibition of prolyl 4-hydroxylase by THA in the presence or absence of added Fe^{2+}

Prolyl 4-hydroxylase was incubated according to the reaction conditions in the text in the presence (●) or absence (○) of added 0.05 mM- Fe^{2+} . The reaction was stopped after 20 min and the release of $^3\text{H}_2\text{O}$ measured.

Fig. 2. Lineweaver–Burk plot of the inhibition of prolyl 4-hydroxylase by THA with respect to Fe^{2+}

Initial velocities were determined with various concentrations of Fe^{2+} in the presence of 0 μM - (□), 0.25 μM - (●), 1.0 μM - (■) and 2.5 μM - (△) THA.

one derivative. A plot of the intercepts of $1/[\text{S}]$ of Fig. 3 against the concentration of THA was non-linear (not shown) and indicated a positively co-operative interaction between THA and prolyl 4-hydroxylase, i.e. the inhibitor constant, K_i , was inversely proportional to the concentration of inhibitor. Fig. 4 indicates a non-competitive

Fig. 3. Inhibition of prolyl 4-hydroxylase by THA with respect to 2-oxoglutarate

Initial velocities were determined with various concentrations of 2-oxoglutarate in the presence of 0 μM - (□), 19.5 μM - (●), 39.1 μM - (■) and 48.8 μM - (Δ) THA.

Fig. 5. Inhibition of prolyl 4-hydroxylase by THA with respect to protocollagen

Initial velocities were determined with various concentrations of protocollagen in the presence of 0 μM - (□), 19.5 μM - (●), 29.3 μM - (■) and 39.1 μM - (Δ) THA.

Fig. 4. Inhibition of prolyl 4-hydroxylase by THA with respect to ascorbate

Initial velocities were determined with various concentrations of ascorbate in the presence of 0 μM - (□), 19.5 μM - (●), 35.2 μM - (■) and 46.9 μM - (Δ) THA.

relationship between THA and ascorbate. Mathematical modelling of the plot of the $1/[S]$ intercept versus the concentration of THA (not shown) suggested that the shape of the curve was consistent with the interaction of THA with three distinct binding sites on the enzyme. The K_i for THA was calculated to be 40.3 μM . Kinetic studies with protocollagen were technically difficult because of the high blank (i.e. without enzyme) values that were observed with high concentrations of protocollagen. However, from the data shown in Fig. 5 an uncompetitive relationship may be tentatively inferred between THA and protocollagen. Analysis of the plot of the $1/[S]$ intercept versus the concentration of THA (not shown) again suggested that THA binds to three separate sites on prolyl 4-hydroxylase.

Structural requirements for inhibition of prolyl 4-hydroxylase

Table 1 shows that a catechol group was important for inhibition of prolyl 4-hydroxylase. Inhibitory activity fell markedly in the monohydroxy anthraquinone (C). The presence or position of a third hydroxy group, as in THA, had little effect on the inhibitory potency of these compounds (A and B). Because of the redox nature of the catalytic cycle of prolyl 4-hydroxylase we considered the possibility that the quinoidal function of the anthraquinones might be involved in their inhibitory action, although anthraquinone (D) itself was inactive at 481 μM . The hydroxylated xanthenes, E and F, were approximately equally as active as THA. This suggests that the redox nature of the anthraquinones is probably not a requirement for the inhibition of prolyl 4-hydroxylase, although it is possible that the modes of action of the catechol-bearing anthraquinones and xanthenes are different. 1,2-Dihydroxynaphthalene (G) was also an effective inhibitor of the enzyme, but we have not yet investigated the nature of its inhibitory action.

We thought it important to establish the potential effects of some representative compounds on the concentration of free Fe^{2+} in solution, in view of the stimulatory action of this metal ion on the activity of isolated prolyl 4-hydroxylase. Our colleagues Dr. J. L. Longridge and Mr. R. G. Button used a potentiometric method to determine the stability constants for mono- and bi-dentate complexes between Fe^{2+} and some of the inhibitors. The concentrations of the free ligands, Fe^{2+} and the complexes in incubation buffer at pH 7.4 were then computed by using the COMICS program of Perrin & Sayce (1967). Table 1 shows that the monohydroxyanthraquinone, C, which was a poor inhibitor of prolyl 4-hydroxylase, was somewhat more effective in complexing Fe^{2+} than was THA. In contrast, the hydroxyxanthone, E, and dihydroxynaphthalene, which were effective inhibitors of the enzyme, had minimal effects on the concentration of free Fe^{2+} at pH 7.4.

Table 1. Inhibitors of prolyl 4-hydroxylase and their complexation with Fe²⁺

The concentrations of free Fe²⁺ and the monodentate (Fe²⁺L) and bidentate (Fe²⁺L₂) complexes in the presence of 50 μM total Fe²⁺ and 50 μM ligand (L) were calculated for each compound as described in the text.

Compound	Concn. for 50% inhibition (μM)	Concn. of free Fe ²⁺ and complexed species (μM)		
		[Fe ²⁺]	[Fe ²⁺ L]	[Fe ²⁺ L ₂]
THA	47	25	6.7	18.0
A	51	—	—	—
B	79	24.0	15.0	12.0
C	> 1 mM	13	23	13
D	> 1 mM	—	—	—
E	74	—	—	—
F	41	49.0	0.42	0.74
G	< 16	50.0	0.2	0.0001

DISCUSSION

Polyhydroxyanthraquinones occur widely in nature and have a long history in medicine and as dyestuffs. We have found that such compounds, provided that they possess a pair of neighbouring hydroxy groups, are effective inhibitors of prolyl 4-hydroxylase. Other compounds with dihydroxy functions, such as the xanthenes E and F and 1,2-dihydroxynaphthalene, also inhibited the enzyme. The ability of added Fe²⁺ partially to reverse the inhibition of prolyl 4-hydroxylase by THA suggested that an inactive complex might be formed between THA and Fe²⁺ ions in solution. Potentiometric studies confirmed that THA is indeed an effective chelating agent for Fe²⁺, according to the equilibria:

However, the monohydroxyanthraquinone, C, shown in Table 1 is also an effective chelator, but it is virtually inactive as an inhibitor of prolyl 4-hydroxylase, whereas compounds such as 1,2-dihydroxynaphthalene and the xanthone, F, which are less effective in lowering the concentration of free Fe²⁺, are all good inhibitors of prolyl 4-hydroxylase. It might appear, therefore that the chelating property of THA has little to do with its enzyme-inhibiting activity. However, the interaction of Fe²⁺ with hydroxyanthraquinones is complex. The metal probably binds to the catechol function in addition to the

hydroxy-carbonyl system, although the affinity of the latter function for Fe²⁺ at pH 7.5 is certainly much higher. These considerations may be important in the light of studies by Majamaa *et al.* (1984). They found that a series of pyridinecarboxylic acids, capable of forming weak bidentate or terdentate complexes with Fe²⁺, are effective inhibitors of chick prolyl 4-hydroxylase by competitive antagonism with 2-oxoglutarate. Majamaa *et al.* (1984) suggested that 2-oxoglutarate binds to the Fe²⁺ atom at the active centre of the enzyme and additionally to neighbouring hydrophobic and ionic regions of the enzyme. The pyridinecarboxylic acids are believed to inhibit by competing with 2-oxoglutarate for both the Fe²⁺ atom and the postulated hydrophobic site (Majamaa *et al.*, 1984). There is some similarity between the inhibitory actions of THA and the pyridinecarboxylic acids. THA is a competitive inhibitor of 2-oxoglutarate, but does not compete with either procollagen or ascorbate. Since THA also complexes with Fe²⁺ it is possible that, like the pyridinecarboxylates, THA binds to the enzyme via the active Fe²⁺ atom and conceivably through additional hydrophobic interactions involving the aromatic ring system. The strong positive co-operativity of the inhibition by THA indicates that several distinct but non-independent binding sites may be involved. Inhibition by pyridine-2,5-dicarboxylate showed no evidence of co-operativity (Majamaa *et al.*, 1984).

The inactivity of the monohydroxyanthraquinone, C, against prolyl 4-hydroxylase indicates (a) that THA does not inhibit by depleting the concentration of Fe^{2+} in the medium, since compound C is also an effective chelating agent and (b) that the hydroxy-carbonyl system is unlikely to be involved with binding to the Fe^{2+} at the active centre of the enzyme. Although the Fe^{2+} -complexing activity of the catechol function is relatively poor at pH 7.4, it is conceivable that an interaction with enzyme-associated Fe^{2+} could be stabilized by other interactions with neighbouring groups in the enzyme. The ability of added Fe^{2+} to antagonize the inhibition by THA is probably attributable to the formation of inactive Fe^{2+} complexes in solution involving the hydroxy-carbonyl system.

At this stage, therefore, we believe that the inhibitory action of THA and possibly other catechols is probably best explained by a primary interaction of the dihydroxy function with Fe^{2+} at the 2-oxoglutarate-binding site of prolyl 4-hydroxylase, accompanied by secondary interactions with amino acid residues. However, alternative mechanisms may have a role to play. For example, in their studies on the complex antibiotic P-1984B, which contains a hydroxylated anthraquinone moiety flanked by two bulky oligosaccharide units, Ishimaru *et al.* (1982) suggested, although without direct evidence, that the inhibition of prolyl 4-hydroxylase by this compound might depend on its ability to scavenge a postulated activated oxygen intermediate in the catalytic reaction. However, the inhibitory mechanism of P-1894B is probably distinct from that of THA, since the former was non-competitive with respect to the synthetic peptide substrate (Pro-Pro-Gly)₅, whereas THA was apparently uncompetitive with respect to procollagen. The competitive relationship between THA and 2-oxoglutarate is not consistent with an activated oxygen-scavenging role for THA. Ishimaru *et al.* (1982) did not report on the kinetic relationship between 2-oxoglutarate and P-1894B. Presumably the quinoidal oxygen system would be important in activated oxygen-scavenging activity. The inhibition of prolyl 4-hydroxylase by the structurally related but non-quinoidal xanthenes E and F suggests that the quinoidal redox system is not important, although it is conceivable that the inhibitory mechanisms of the hydroxylated anthraquinones and xanthenes are different.

A further complexity of the interaction with THA with prolyl 4-hydroxylase is its strong positively co-operative character. This was indicated by a marked inverse relationship between K_i values and the concentration of THA in the kinetic experiments with 2-oxoglutarate: at 19.5 μM -THA the K_i was 34.2 μM , whereas at 48.9 μM -THA the value was 3.7 μM . At present it seems possible that THA interacts with at least three separate but non-independent sites on prolyl hydroxylase. Further experimental work will be necessary to establish the validity of this suggestion.

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