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Time-dependent inactivation of chick-embryo prolyl 4-hydroxylase by coumalic acid

Evidence for a syncatalytic mechanism

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From the structure–activity relationships of known competitive inhibitors, coumalic acid (2-oxo-1,2*H*-pyran-5-carboxylic acid) was deduced to be a potential syncatalytic inhibitor for chick-embryo prolyl 4-hydroxylase. The compound caused time-dependent inactivation, the reaction rate being first-order. The inactivation constant was 0.094 min^{-1}, the K_i 17 mM and the bimolecular rate constant 0.09 $M^{-1} \cdot s^{-1}$. Human prolyl 4-hydroxylase and chick embryo lysyl hydroxylase were also inactivated, though to a lesser extent. Inactivation could be prevented by adding high concentrations of 2-oxoglutarate or its competitive analogues to the reaction mixture. In Lineweaver–Burk kinetics, coumalic acid displayed S-parabolic competitive inhibition with respect to 2-oxoglutarate. The inactivation reaction had cofactor requirements similar to those for the decarboxylation of 2-oxoglutarate. Enzymic activity was partially preserved in the absence of iron, but the rescue was incomplete, owing to decreased stability of the enzyme under this condition. Coumalic acid also decreased the electrophoretic mobility of the α-subunit, but the β-subunit was not affected. Prolonged incubation of coumalic acid above pH 6.8 led to loss of its inactivating potency, owing to hydrolysis. It is concluded that the inactivation of prolyl 4-hydroxylase by coumalic acid is due to a syncatalytic mechanism. The data also suggest that the 2-oxoglutarate-binding site of the enzyme is located within the α-subunit.

INTRODUCTION

Prolyl 4-hydroxylase [procollagen-proline,2-oxoglutarate:oxygen oxidoreductase (4-hydroxylating), EC 1.14.11.2] catalyses the post-translational conversion of peptide-bound proline residues into *trans*-4-hydroxyproline, exclusively at the *N*-terminal of glycine (Cardinale & Udenfriend, 1974; Prockop *et al.*, 1976; Kivirikko & Myllylä, 1980). The genetically uncoded amino acid 4-hydroxyproline formed in the reaction is capable of participating in intramolecular hydrogen bonds, which are essential for the thermal stability of the triple helix of collagenous proteins. The reaction takes place in two consecutive steps. The energy necessary for the overall reaction is generated by the oxidative decarboxylation of 2-oxoglutarate by one atom of a dioxygen molecule. This process is thought to take the form of a ligand reaction in the co-ordination sphere of enzyme-bound Fe^{2+} (Hanauske-Abel & Günzler, 1982), yielding succinate, CO_2 and a highly reactive iron–oxygen atom complex. This ferryl ion subsequently hydroxylates an appropriate proline residue, probably by an abstraction–recombination mechanism. The decarboxylation step can proceed as an uncoupled reaction, i.e. without subsequent hydroxylation (Tuderman *et al.*, 1977; Rao & Adams, 1978; Counts *et al.*, 1978). Ascorbate serves as a specific alternative oxygen acceptor in such cycles (Myllylä *et al.*, 1984; De Jong & Kemp, 1984), and in its absence the enzyme is rapidly inactivated by self-oxidation (Myllylä *et al.*, 1978; De Jong *et al.*, 1982).

Many details of the 2-oxoglutarate-binding site have been elucidated by using competitive inhibitors and structurally related 2-oxo acids (Majamaa *et al.*, 1984, 1986). Optimal binding was shown to require a chelating moiety, presumably interacting with enzyme-bound ferrous iron, a negatively charged carboxyl function corresponding to the C-5 carboxyl group of 2-oxoglutarate, and an overall planar structure in the molecule. The aromatic nature of the compound apparently prevents its utilization as a co-substrate, as does its lack of a carboxyl moiety corresponding to the C-5 carboxyl group of 2-oxoglutarate. By using these structure–activity relationships established for prolyl 4-hydroxylase purified from chick embryos, it was possible for the first time to design a 2-oxoglutarate analogue which causes time-dependent inactivation of this enzyme, apparently by a syncatalytic mechanism.

MATERIALS AND METHODS

Materials

The synthetic substrates for prolyl 4-hydroxylase, $(Pro\text{-}Pro\text{-}Gly)_{10}$,$9H_2O$, and lysyl hydroxylase, L-I (Ala-Arg-Gly-Ile-Lys-Gly-Ile-Arg-Gly-Phe-Ser-Gly), were obtained from the Protein Research Foundation, Minoh, Osaka, Japan. Coumalic acid (2-oxo-1,2*H*-pyran-5-carboxylic acid), with a specified purity > 98%, was purchased from Fluka, Buchs, Switzerland, and the 2-oxoglutarate analogues were from the sources indicated previously (Majamaa *et al.*, 1984, 1986). Catalase was purchased from Sigma, St. Louis, MO, U.S.A., and 2-oxo[1-^{14}C]glutarate was obtained from New England Nuclear and diluted to 100000 d.p.m./0.1 μmol by

mixing with the unlabelled compound. [^{14}C]Proline-labelled protocollagen substrate was prepared from freshly isolated chick embryo tendon cells in the presence of 2,2′-bipyridyl as reported previously (Kivirikko & Myllylä, 1982).

Purification of enzymes

Prolyl 4-hydroxylase was purified from homogenized 14-day chick embryos by fractionation with $(NH_4)_2SO_4$ (0–70% satn.), affinity chromatography on poly(L-proline) coupled to Sepharose 4B, DEAE-cellulose chromatography and gel filtration, as previously reported (Tuderman *et al.*, 1975; Kedersha & Berg, 1981). Human prolyl 4-hydroxylase was purified from fresh human placentae by an identical procedure. Lysyl hydroxylase was obtained in pure form by an established method (Turpeenniemi-Hujanen *et al.*, 1980) from the 17–55%-satd.-$(NH_4)_2SO_4$ fraction of homogenized 14-day chick embryos, by using affinity chromatography on concanavalin A–Sepharose and collagen–Sepharose, and subsequent gel filtration. Both enzymes were entirely pure, as judged by SDS/polyacrylamide-gel electrophoresis.

Enzyme assays

Prolyl 4-hydroxylase and lysyl hydroxylase were inactivated in 0.2 ml of 0.05 M-Tris/HCl, pH 7.5, containing 0.05–0.2 μg of enzyme, 0.05 mM-$FeSO_4$, 2 mM-ascorbate, 0.1 mg of catalase/ml, 0.1 mM-dithiothreitol and 2 mg of bovine serum albumin/ml. The amounts of substrate added were 0.1 mg of $(Pro\text{-}Pro\text{-}Gly)_{10}$/ml or 0.5 mg of L-I/ml respectively. The reaction mixture also contained 0–16 mM-coumalic acid. The samples were incubated for 10–60 min at 37 °C.

To assay the remaining enzyme activity, the reaction volume was increased to 1 ml by addition of fresh 0.05 M-Tris/HCl containing the co-substrates, substrate, catalase, dithiothreitol and albumin in the concentrations indicated above, as well as labelled 2-oxoglutarate (final concn. 0.1 mM). The concentration of coumalic acid was thus decreased to 0–3.2 mM, and appropriate amounts were added to the control tubes at this stage to compensate for any remaining effect. The tubes were then sealed, incubated for 30 min at 37 °C, and the amount of $^{14}CO_2$ produced was determined as reported previously (Berg & Prockop, 1973).

Alternatively, prolyl 4-hydroxylase was inactivated in the absence of peptide substrate, and 60000 d.p.m. of labelled protocollagen substrate was added at the end of the inactivation period, together with 0.1 mM of unlabelled 2-oxoglutarate. The samples were then incubated for 30 min at 37 °C. The reaction was stopped by the addition of 1 ml of 12 M-HCl, and the amount of hydroxyproline synthesized was determined by the method of Juva & Prockop (1966) after hydrolysis of the samples at 120 °C for 16 h.

In the second-pulse experiment, two consecutive inactivation steps were carried out, with identical amounts of enzyme being added before and after the first step. The remaining enzyme activity was then assayed as described above. The value obtained was compared with that in samples which had gone through only one inactivation.

Further purification of coumalic acid

To exclude the possibility that the observed inactivation of prolyl 4-hydroxylase might be due to impurities in the commercially available coumalic acid, the compound was purified further by repeated recrystallization. Because of its thermal instability, it was added to either methanol or ethanol/diethyl ether (1:1, v/v), at room temperature, and it precipitated at −20 °C.

Table 1. Half-life of chick-embryo prolyl 4-hydroxylase under various conditions

Owing to oxidation of enzyme-bound Fe^{2+} ions in uncoupled decarboxylation cycles (Myllylä *et al.*, 1984; De Jong & Kemp, 1984), prolyl 4-hydroxylase is extremely rapidly inactivated in the absence of ascorbate only. The calculated half-life in the presence of coumalic acid was corrected for the spontaneous inactivation in the absence of 2-oxoglutarate.

Experimental condition	Half-life
Full reaction	> 10 h
2-Oxoglutarate omitted from reaction mixture	78 min
Ascorbate omitted from reaction mixture	seconds
Ascorbate and 2-oxoglutarate omitted from reaction mixture	20 min
Fe^{2+} and 2-oxoglutarate omitted from reaction mixture	12 min
2-Oxoglutarate omitted, saturating concentrations of coumalic acid added to reaction mixture	7 min

Hydrolysis of coumalic acid

The hydrolytic fate of coumalic acid in 0.05 M-Tris/HCl, pH 7.5, was studied by pH measurements, p.m.r. and u.v. spectroscopy. The time-dependent decrease in A_{292}, corresponding to the closed ring structure of coumalic acid, was used as an experimental parameter. The data obtained from these experiments were compared with a functional assay in which 8 mM solutions of coumalic acid were incubated at various pH values between 6.2 and 8.2 for 12 h; 0.02 M-phosphate buffer was used instead of Tris/HCl at pH values below 7.0. The solutions were then re-adjusted to pH 7.5 and used as inactivating agents in the assays described above.

Coumalic acid as a substitute for 2-oxoglutarate in the hydroxylation reaction

In order to investigate whether coumalic acid was able to maintain hydroxylation of a protocollagen substrate, 0.05–0.15 mg of chick-embryo prolyl 4-hydroxylase was incubated in 2 ml in the presence of 8 mM-coumalic acid and 150000 d.p.m. of protocollagen substrate. 2-Oxoglutarate was omitted from the reaction mixture. The concentrations of all other cofactors were identical with those indicated above.

SDS/polyacrylamide-gel electrophoresis

Enzyme (5–10 μg) was incubated in 250 μl of 0.05 M-Tris/HCl, pH 7.5, containing 0.05 mM-$FeSO_4$, 2 mM-ascorbate and 0.1 mM-dithiothreitol, for 1 h at 37 °C in the presence or absence of 8 mM-coumalic acid. Catalase and bovine serum albumin were omitted from the reaction mixture. After freeze-drying, the samples were resuspended in 100 μl of electrophoresis buffer and run

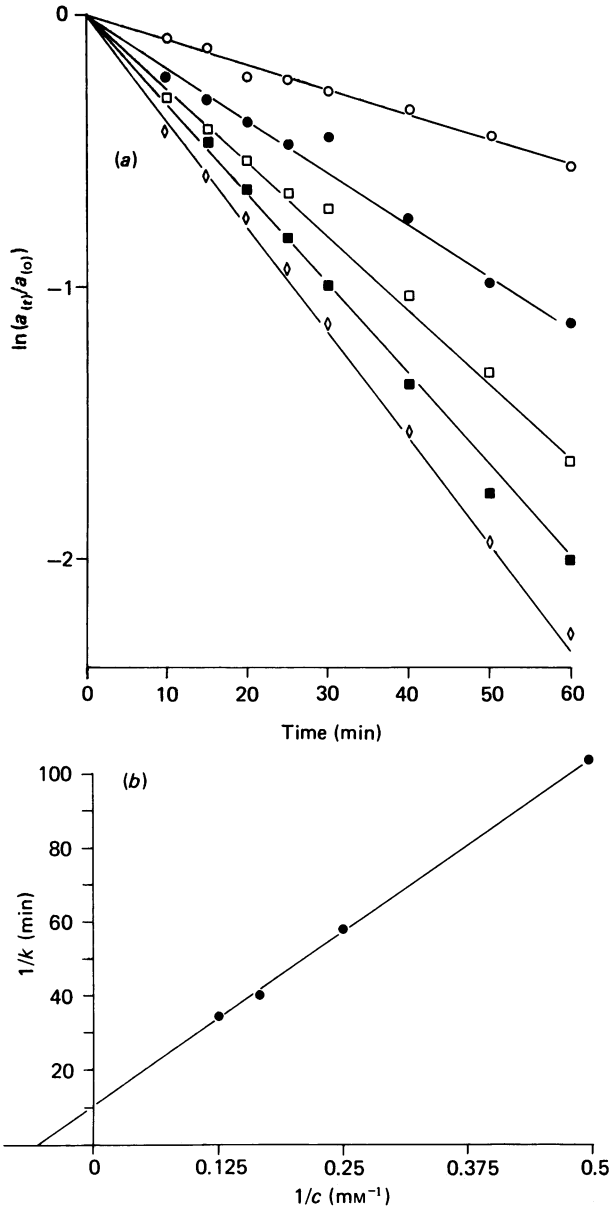

Fig. 1. Effect of coumalic acid on the activity of chick-embryo prolyl 4-hydroxylase

(*a*) Amount of enzyme activity after different preincubation periods: $a_{(0)}$ = initial enzyme activity, corresponding to 6914±468 d.p.m. of $^{14}CO_2$ formed from 2-oxo[1-^{14}C]-glutarate; $a_{(t)}$ = activity at time t (min) of preincubation. The inhibition constant k_c for a concentration c of coumalic acid is calculated from the equation $\ln(a_{(t)}/a_{(0)}) = -kt$. Concentrations of coumalic acid were 0 (○), 2 (●), 4 (□), 6 (■) and 8 (◇) mM. Each point represents the mean for five samples. (*b*) Secondary transform of Fig. 1(*a*): k, inhibition constants obtained for the individual concentrations c of coumalic acid.

on 8%-polyacrylamide slab gels. Densitometry was done on wet gels after Coomassie Blue staining.

RESULTS

Spontaneous inactivation of prolyl 4-hydroxylase

The stability of prolyl 4-hydroxylase under various conditions was studied in preliminary experiments. No

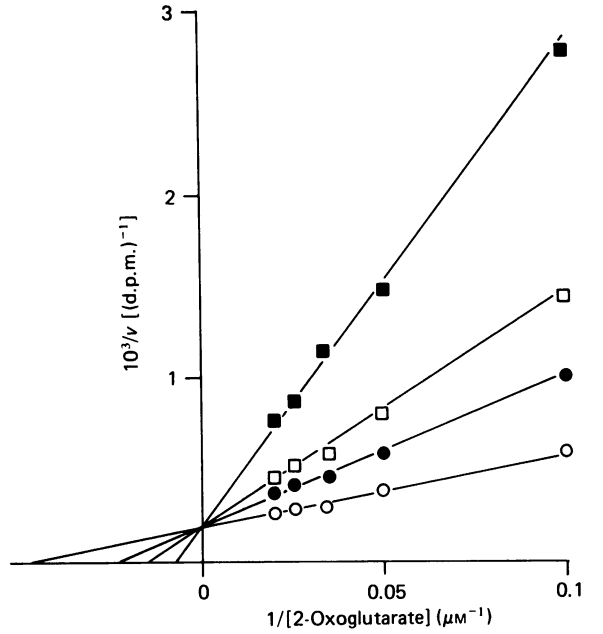

Fig. 2. Lineweaver–Burk plot of the inhibition of chick-embryo prolyl 4-hydroxylase by coumalic acid with 2-oxoglutarate as the variable substrate

Concentrations of coumalic acid were 0 (○), 4 (●), 8 (□) and 12 (■) mM.

inactivation was found under turnover conditions for up to 1 h, but spontaneous inactivation occurred if one or more of the co-substrates were omitted from the reaction mixture. If only 2-oxoglutarate was omitted, as in most coumalic acid experiments, spontaneous inactivation was not negligible, the $t_{\frac{1}{2}}$ (half-life) being 78 min (Table 1). Compounds that inhibit prolyl 4-hydroxylase competitively with respect to 2-oxoglutarate, i.e. pyridine-2,4-dicarboxylate, pyridine-2,5-dicarboxylate, 3,4-dihydroxybenzoate and 4-hydroxybenzoate, prevented the loss of enzymic activity (results not shown). Very rapid self-oxidation was observed in the absence of 2-oxoglutarate and either ascorbate or Fe^{2+} (Table 1). Similar behaviour was found if iron was present but completely chelated by 2,2′,2″-terpyridine. Under these conditions only 5% of the control value remained after 60 min, compared with 15% remaining if no Fe^{2+} was added.

Rate and constants of inactivation

Coumalic acid proved to be an inactivator of prolyl 4-hydroxylase, with first-order reaction rate, as determined by both the decarboxylation of 2-oxoglutarate (Fig. 1*a*) and hydroxyproline formation. The inactivation constants for the individual concentrations of coumalic acid were calculated from the equation $a_{(t)} = a_{(0)}e^{-kt}$, with $a_{(t)}$ = remaining enzyme activity after an inactivation period of t min. The double-reciprocal plot of k versus the concentration of coumalic acid was linear, with a correlation coefficient r^2 of > 0.999 (Fig. 1*b*). The x- and y-axis intercepts of this secondary transform represent $-1/K_i$ and $1/k_{inact.}$ respectively. The bimolecular rate constant is obtained from $k_{inact.}/K_i$. These constants were different from prolyl 4-hydroxylases of chick and human origin. For the chick enzyme, $k_{inact.}$ was 0.094 min^{-1}, K_i 17 mM and the bimolecular rate

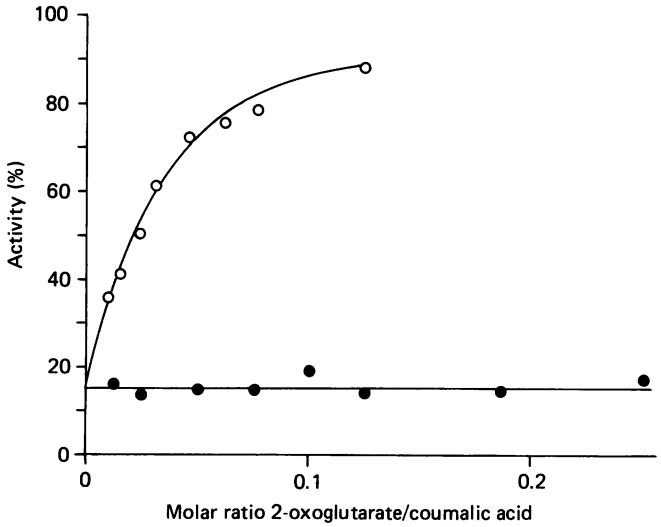

Fig. 3. Rescue of enzyme activity by 2-oxoglutarate

The co-substrate was capable of preserving the enzyme activity only if it was added before (○), but not after (●), inactivation with 8 mM-coumalic acid. The ordinate shows recovery of enzyme activity as a percentage of control value.

constant 0.09 $M^{-1} \cdot s^{-1}$. The human enzyme was less susceptible, with constants of 0.026 min^{-1}, 32 mM and 0.013 $M^{-1} \cdot s^{-1}$ respectively. The specific activities of different enzyme preparations did not influence the speed of inactivation. Preincubation of coumalic acid with the inactivation reaction solution in the absence of enzyme did not result in inactivation.

When two consecutive inactivation steps were carried out with identical amounts of enzyme, no increase in the speed of inactivation was observed. With 8 mM-coumalic acid, 17% of the activity remained after 60 min, and 17.2% after the addition of fresh enzyme and further incubation for 1 h.

Chick embryo lysyl hydroxylase was also inactivated by coumalic acid, $K_{inact.}$ being 0.025 min^{-1}, K_i 40 mM, and the bimolecular rate constant 0.01 $M^{-1} \cdot s^{-1}$, i.e. similar to the values found for human prolyl 4-hydroxylase. Further purification of coumalic acid by recrystallization, as described in the Materials and methods section, did not alter the inactivating potency of the compound, although its colour changed from brown to yellow, owing to the removal of impurities. No change in the u.v. spectrum was observed after recrystallization.

Site of the inactivation reaction

Lineweaver–Burk kinetic plots obtained by direct assay of the enzyme activity without preceding inactivation showed coumalic acid to exhibit an S-parabolic competitive type of inhibition with 2-oxoglutarate as the variable substrate (Fig. 2).

Inactivation of prolyl 4-hydroxylase by coumalic acid could be prevented if 2-oxoglutarate was added before, but not after, the inactivation period (Fig. 3). The recovery of enzyme activity increased with the 2-oxoglutarate concentration. Relatively high amounts of the co-substrate were required for quantitative rescue of the enzyme activity, in view of the almost 1000-fold difference in the K_m of 2-oxoglutarate and K_i of coumalic acid.

Table 2. Protection of prolyl 4-hydroxylase activity against coumalic acid inactivation by competitive 2-oxoglutarate analogues

RD_{50} refers to the concentration of compound yielding 50% remaining activity after 1 h incubation at 37 °C in the presence of 8 mM-coumalic acid. Remaining enzyme activity without added rescuing agent was 15% of the control value.

Compound	K_m or K_i (mM)	RD_{50} (mM)
2-Oxoglutarate	0.022	0.2
Pyridine-2,4-dicarboxylate	0.002	0.005
Pyridine-2,5-dicarboxylate	0.0008	0.015
3,4-Dihydroxybenzoate	0.005	0.08–0.18
4-Hydroxybenzoate	3.2	1.5

The action of coumalic acid could also be prevented by using competitive antagonists of 2-oxoglutarate (Table 2). The compounds tested here included the three most potent analogues known so far (Majamaa *et al.*, 1984, 1986) and also 4-hydroxybenzoate, a structural analogue of coumalic acid (Fig. 4).

Coumalic acid could not serve as a substitute for 2-oxoglutarate in the hydroxylation reaction. If it is assumed that each molecule of prolyl 4-hydroxylase catalyses one reaction cycle during the inactivation, 0.2–0.6 nmol of labelled hydroxyproline should be formed by the amount of enzyme used. This quantity should be readily detectable. No hydroxylation of proline-labelled protocollagen substrate was detected in the presence of 8 mM inactivator, however.

Cofactor-dependence of the inactivation reaction

Inactivation of prolyl 4-hydroxylase by coumalic acid had cofactor requirements similar to those for the decarboxylation of 2-oxoglutarate. Increasing concentrations of Fe^{2+} enhanced the inactivating capacity of the compound (Fig. 5*a*). In the complete absence of

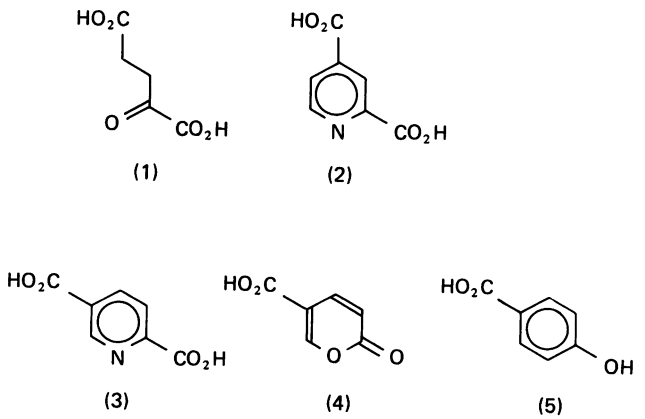

Fig. 4. Structural relationship between 2-oxoglutarate, coumalic acid and competitive inhibitors

Structures shown are (1) 2-oxoglutarate, (2) pyridine-2,4-dicarboxylate, (3) pyridine-2,5-dicarboxylate, (4) coumalic acid, (5) 4-hydroxybenzoate.

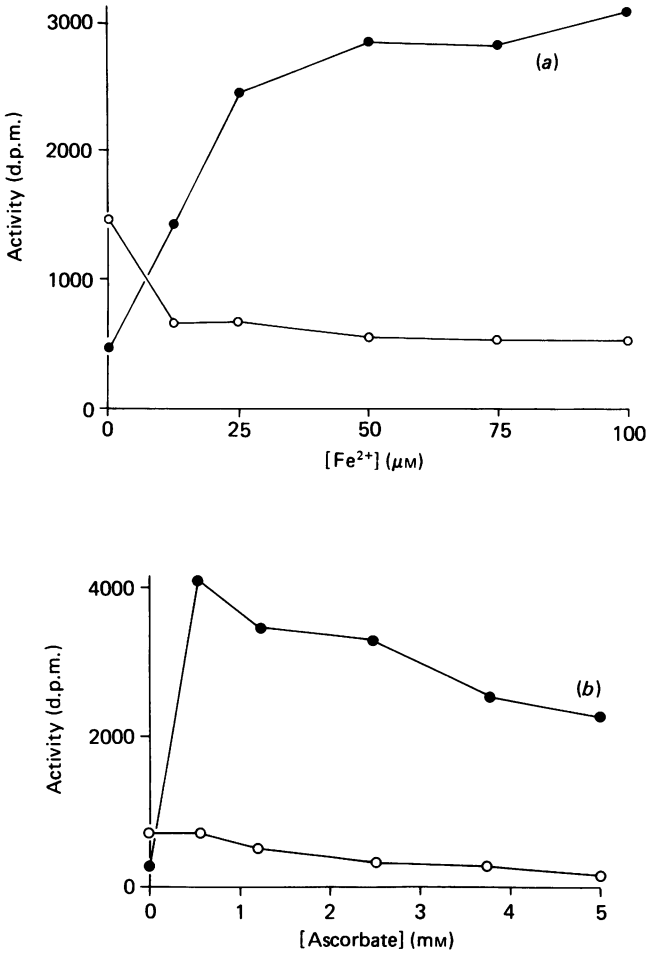

Fig. 5. Cofactor-dependence of the inactivation of chick-embryo prolyl 4-hydroxylase by coumalic acid

(*a*) Enzyme activity remaining after preincubation for 60 min in the absence (●) or presence (○) of 8 mM-coumalic acid at different concentrations of Fe^{2+}. The concentration of Fe^{2+} in the assay of activity remaining was adjusted to 0.05 mM in all the samples. (*b*) Amount of enzyme activity after preincubation for 60 min in the absence (●) or presence (○) of 8 mM-coumalic acid at different concentrations of ascorbate. The concentration of ascorbate in the assay of activity remaining was adjusted to 2 mM in all samples. The ordinate shows the amount of $^{14}CO_2$ formed from 2-oxo[1-^{14}C]glutarate.

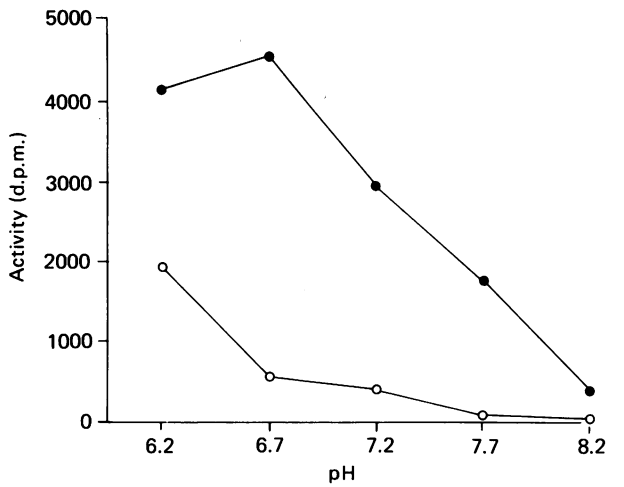

Fig. 6. Effect of pH on inactivation of chick-embryo prolyl 4-hydroxylase by coumalic acid

Enzyme activity remaining after preincubation for 60 min in the absence (●) or presence (○) of 8 mM-coumalic acid. The buffers used were 0.02 mM-sodium phosphate below pH 7 and 0.05 M-Tris/HCl above it. The pH in the assay of activity remaining was adjusted to 7.5 in all samples. The ordinate shows the amount of $^{14}CO_2$ formed from 2-oxo[1-^{14}C]glutarate.

added Fe^{2+}, causing rapid spontaneous inactivation of the enzyme, prolyl 4-hydroxylase was stabilized in the presence of coumalic acid, and both a relative and an absolute increase in remaining activity were observed as compared with the results obtained with the lowest concentration of Fe^{2+} used.

Variation of the ascorbate concentration had little effect on the inactivation process, although enzyme recovery was slightly lower at high ascorbate concentrations in both the presence and the absence of coumalic acid. The ratio remained constant (Fig. 5*b*). Complete omission of this reducing agent again caused rapid spontaneous loss of enzyme activity (Table 1), but had no influence on the inactivation caused by coumalic acid. Likewise, variation of the concentration of the peptide substrate had no effect (results not shown).

pH-dependence of the inactivation and hydrolysis of coumalic acid

The activity of coumalic acid was partially prevented if the pH of the reaction mixture was lowered, but not if it was raised (Fig. 6). This finding coincides with that of enhanced enzyme survival at low pH values and increased spontaneous inactivation at pH > 7.5 in the absence of an inactivating agent.

The hydrolysis of coumalic acid was found to proceed with a half-life of 22 h at room temperature and 7 h at 37 °C under the conditions described in the Materials and methods section. Thus approx. 9% of the coumalic acid is hydrolytically cleaved in a 1 h preincubation. These results are in agreement with the functional test performed with coumalic acid solutions incubated at different pH values for 12 h before use as inactivating agents (Table 3). It thus seems that coumalic acid is the active agent, rather than any of its hydrolysis products.

Modification of protein mobility in gel electrophoresis

Syncatalytic inactivation of prolyl 4-hydroxylase can be expected to alter the M_r of the active-site-bearing subunit, owing to covalent incorporation of the inhibitor. Incubation of chick-embryo prolyl 4-hydroxylase with 8 mM-coumalic acid for 1 h at 37 °C caused a change in the migration behaviour of the α-subunit in SDS/polyacrylamide-gel electrophoresis, but the β-subunit was unaffected. In densitometry of four samples each, the peak height of the α-subunit in the controls amounted to 31.2 ± 4.7 arbitrary units, compared with 12.0 ± 2.12 for the coumalic acid-treated samples. The peak width at half peak height was 2.61 ± 0.48 mm in the controls and 3.50 ± 0.29 mm in the treated samples; the distance between the maxima of the α- and β-subunits was 7.44 ± 0.19 and 8.02 ± 0.15 mm, respectively, changes that were significant at the 1% level in the two-sided t-test.

Table 3. Inactivating capacity of 8 mM-coumalic acid after partial hydrolysis at 37 °C for 12 h at various pH values

Sodium phosphate buffer (0.02 M) was used for hydrolysis below pH 7, and Tris/HCl (0.05 M) above this value.

pH	Activity of enzyme remaining (%)	Calculated amount of coumalic acid (mM)	(%)
6.2	30.7	7.25	90.7
6.7	59.3	3.21	40.2
7.2	70.1	2.18	27.3
7.7	67.8	2.39	29.8
8.2	64.4	2.69	33.8

DISCUSSION

The results presented here describe coumalic acid as an inactivator of chick embryo prolyl 4-hydroxylase. The reaction takes place at the binding site of 2-oxoglutarate, requires similar conditions to the decarboxylation of this co-substrate, and is first-order. These data are in accord with established criteria for syncatalytic inactivators (Rando, 1977).

The method employed to study the effects of this inactivator, i.e. preincubation of the enzyme in a small volume, followed by dilution of the mixture to the standard volume for an assay of the remaining activity, is a suitable procedure for the detection and study of enzyme-activated irreversible inhibitors (Waley, 1980). The 5-fold increase in reaction volume minimizes the effects of the inhibitor during the enzyme assay, and any remaining inactivation or competitive antagonism is accounted for by the addition of appropriate amounts of the inhibitor at the beginning of the assay period. It is therefore not necessary to separate the inactivating agent from the enzyme. Fresh cofactors are added at the end of the preincubation period, thus ensuring that the remaining enzymic activity is assayed under optimal conditions. The disadvantage of the method is the fact that it is not possible to apply very high concentrations of competitive antagonists of 2-oxoglutarate in the preincubation, because they will interfere with the determination of remaining enzyme activity regardless of the assay procedure used.

By this method, coumalic acid was found to be most effective in the absence of 2-oxoglutarate. Spontaneous inactivation of the enzyme is not negligible under these conditions, but this is a disadvantage only at first glance, since competitive inhibitors which are able to enter the binding site of 2-oxoglutarate, such as the pyridine-2-carboxylic acids, or *o*-dihydroxybenzoates, protect the enzyme from autoxidation, yielding more remaining activity than the controls. Inactivators naturally decrease the recovery of enzymic activity. This sharply contrasting behaviour helps to distinguish competitive effects from inactivation.

The fact that the effect of coumalic acid is more marked with chick-embryo prolyl 4-hydroxylase than with lysyl hydroxylase or human prolyl 4-hydroxylase is not surprising, because the structure–activity relationships from which the structure of coumalic acid was deduced as a potential inactivator (Majamaa *et al.*, 1984, 1986) were established with chick enzyme, and lysyl hydroxylase has been reported to show a lower affinity towards both 2-oxoglutarate and its competitive antagonists (Majamaa *et al.*, 1985). Our data also indicate that the K_m of 2-oxoglutarate for human prolyl 4-hydroxylase is 40 μM, compared with 22 μM for the chick enzyme, and accordingly the K_i values of the competitive 2-oxoglutarate analogues were approximately twice those reported for the chick enzyme. The differences in susceptibility to inactivation by coumalic acid thus reflect differences in the 2-oxoglutarate binding of the enzymes, indicating that the effect of the inactivator is due to a syncatalytic mechanism and not to any overall chemical reactivity on the part of the compound.

The ability of 2-oxoglutarate analogues to preserve enzymic activity parallels, on a general scale, the K_i values reported for these compounds (Table 2), again indicating that the inactivation takes place at the binding site of 2-oxoglutarate. 3,4-Dihydroxybenzoate, a compound that has been found to inhibit prolyl 4-hydroxylase competitively with respect to both 2-oxoglutarate and ascorbate (Majamaa *et al.*, 1986), displays the greatest difference between the ability to rescue enzymic activity and the reported K_i value. The observation that it is a rather poor rescuing agent suggests that it acts mainly at the stage of the enzymic reaction, when ascorbate is utilized.

The observed differences in migration behaviour in SDS/polyacrylamide-gel electrophoresis give, for the first time, clues to the location of the active centre within the enzyme, suggesting that the 2-oxoglutarate-binding site, and thus part of the active site of the enzyme, is located within the α-subunit. This finding agrees with photoaffinity labelling of the α-subunit with *N*-(4-azido-2-nitrophenyl)glycyl(Pro-Pro-Gly)$_5$, although in the latter case it had not been possible to identify the active site of the enzyme because the affinity labelling occurred at a distance of 15 amino acids from the preferred hydroxylation site (De Waal *et al.*, 1985). The difference in M_r between the two subunits is roughly 4000, corresponding to a 7.44 mm migration difference in the present experiments. This difference increased to 8.02 mm, or M_r 4200–4300, in coumalic acid-treated samples, assuming a linear relationship between M_r and migration.

The exact mechanism of the reaction of prolyl 4-hydroxylase with coumalic acid remains to be explained, including questions such as which part of the inhibitor molecule reacts with the enzyme and how many atoms of oxygen are involved. However, the mechanism apparently does not involve the formation of a ferryl ion intermediate, the conceivable hydroxylating oxygen species. In the absence of ascorbate, these species cause inactivation of prolyl 4-hydroxylase within seconds, but the inactivation by coumalic acid is not accelerated in the absence of the reducing agent. Accordingly, no maintenance of the hydroxylation reaction by coumalic acid could be detected.

When comparing the inactivation constants found for the reaction of coumalic acid with prolyl 4-hydroxylase with values reported for other systems (Kunze *et al.*, 1983; Ortiz de Montellano & Kunze, 1980), it has to be kept in mind that the reaction cycles of prolyl 4-hydroxylase are very slow (Prockop *et al.*, 1976; Kivirikko & Myllylä, 1980). Owing to the high K_i of coumalic acid, the inactivation is not very effective, but

improvement will be possible. From the study of competitive 2-oxoglutarate analogues it is known that capability of complexing enzyme-bound Fe^{2+} is a prerequisite for effective inhibition (Majamaa *et al.*, 1984, 1986). The K_i value found for 3,4-dihydroxybenzoate, a potent chelator, is almost 1000-fold lower than that for 4-hydroxybenzoate, which is not capable of chelation (Majamaa *et al.*, 1986). Coumalic acid, a structural analogue of 4-hydroxybenzoate, is not able to form chelate complexes. We predict that modification of the coumalic acid structure, forming a compound that can effectively co-ordinate to the catalytically active iron ion, will cause a similar decrease in the K_i of the inactivator.

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