Syncatalytic inactivation of prolyl 4-hydroxylase by anthracyclines

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The anthracyclines doxorubicin and daunorubicin were found to act as irreversible inhibitors of prolyl 4-hydroxylase. The reaction rate for enzyme from both chick and human origin was first order, the concentration of inhibitor giving 50% inhibition being 60 μM for both compounds after 1 h. The effect was dependent on the presence of iron ions in the reaction mixture. Inactivation could be prevented by addition of high concentrations of ascorbate, but not 2-oxoglutarate, before the inactivation period. The same results were obtained with competitive analogues of these cosubstrates. Lysyl hydroxylase from chick embryos was also susceptible to inactivation. Its activity was decreased by 50% after incubation for 1 h with a 150 μM concentration of the inhibitors. When chick-embryo prolyl 4-hydroxylase was incubated with [14-^{14}C]doxorubicin, both enzyme subunits were radioactively labelled, about 70% of the total radioactivity being found in the α-subunit. Since the anthracyclines are known to undergo a redox reaction generating semiquinone radicals with Fe^{3+} only, the results suggest that the enzyme-bound iron ion is oxidized to a tervalent intermediate in uncoupled reaction cycles. The data also suggest that both enzyme subunits contribute to the catalytic site of prolyl 4-hydroxylase.

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 in collagens and related proteins. The enzyme utilizes proline residues exclusively in -Xaa-Pro-Gly- sequences. The reaction product, the genetically uncoded amino acid 4-hydroxyproline, is responsible for the thermal stability of the triple-helical structure of collagenous proteins by virtue of the intramolecular hydrogen bonds which it forms (for reviews, see Cardinale & Udenfriend, 1974; Prockop *et al.*, 1976; Kivirikko & Myllylä, 1980).

The active enzyme consists of an $\alpha_2\beta_2$ tetramer (Prockop *et al.*, 1976). Its reaction follows an ordered Ter Ter mechanism (Myllylä *et al.*, 1977; Tuderman *et al.*, 1977) and takes place in two consecutive steps. 2-Oxoglutarate is decarboxylated by an S_N2_t-like attack of the dioxygen unit in the co-ordination sphere of the enzyme-bound ferrous ion, yielding succinate, CO_2 and a highly reactive ferryl ion. This oxygen-atom-transferring agent subsequently hydroxylates an appropriate proline residue, probably by an abstraction–recombination mechanism (Hanauske-Abel & Günzler, 1982). The decarboxylation step can proceed without subsequent hydroxylation in uncoupled reaction cycles (Tuderman *et al.*, 1977; Rao & Adams, 1978; Counts *et al.*, 1980), which require stoichiometric ascorbate consumption to reconstitute the enzyme activity (de Jong & Kemp, 1984; Myllylä *et al.*, 1984). Little is known about the oxidation state of the enzyme-bound iron ion in such cycles. The stoichiometry of the uncoupled reactions (de Jong & Kemp, 1984; Myllylä *et al.*, 1984) does not necessarily imply an enzyme–ferric iron intermediate complex. If the iron at the active site is allowed to be oxidized to the tervalent state, this is accompanied by rapid inactivation of the enzyme, which is only partially reversible by addition of high concentrations of ascorbic acid (Nietfeld & Kemp, 1981). The role of ascorbate is thus best described as an alternative substrate to peptide, rather than simply as reducing agent. Accordingly, it can be replaced by other reductants only to a very limited extent (Myllylä *et al.*, 1978; Nietfeld & Kemp, 1981).

The crucial role of the reaction product of prolyl 4-hydroxylase for the stability of the collagen triple helix makes this enzyme, owing to its well-known catalytic mechanism, a primary target for the pin-point development and identification of potentially antifibrotic agents (Hanauske-Abel & Günzler, 1982). Great progress has been made in designing inhibitory competitive analogues of 2-oxoglutarate and ascorbate (Hanauske-Abel, 1983; Majamaa *et al.*, 1984, 1986), and coumalic acid has recently been characterized as the first substance that appears to inactivate prolyl 4-hydroxylase by a syncatalytic mechanism, i.e. enzyme-activated irreversible inhibitor, acting as a 2-oxoglutarate analogue (Günzler *et al.*, 1987). The class I anthracyclines doxorubicin and daunorubicin (Fig. 1) are clinically important antitumour agents and are widely employed for the treatment of various neoplastic conditions (Chabner & Myers, 1982). Various reports have stressed the inhibitory effect of these agents on wound healing and hydroxyproline generation (Devereux *et al.*, 1980*a*,*b*; Lawrence *et al.*, 1986), a clinically relevant finding, as chemotherapeutic and surgical phases are combined in a number of cancer-treatment protocols. We now report that the class I anthracyclines irreversibly inhibit the collagen hydroxylases in a subset of catalytic cycles which are characterized by oxidation of the enzyme-bound Fe^{2+} ion to Fe^{3+} without hydroxylation. In its ferric form, the enzyme is

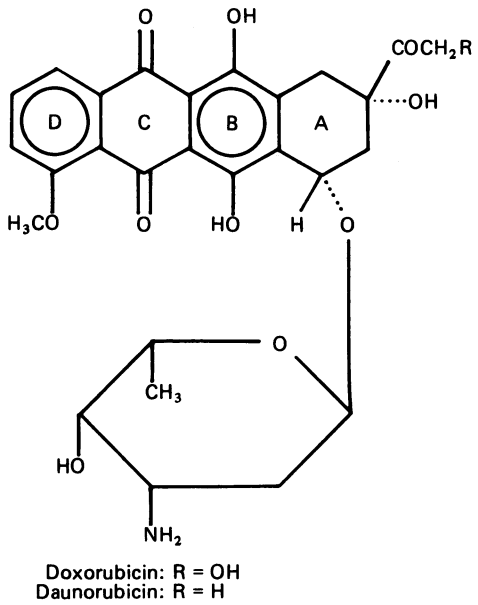


Fig. 1. Structures of the anthracyclines

then irreversibly inactivated by covalent anthracycline incorporation.

MATERIALS AND METHODS

Materials

The synthetic substrates for vertebrate prolyl 4-hydroxylase, (Pro-Pro-Gly)$_{10}$,9H$_2$O, and for lysyl hydroxylase, L-I (Ala-Arg-Gly-Ile-Lys-Gly-Ile-Arg-Gly-Phe-Ser-Gly), were purchased from the Protein Research Foundation, Minoh, Osaka, Japan, and the substrate for *Chlamydomonas reinhardii* prolyl 4-hydroxylase, poly(L-proline), was from Sigma, St. Louis, MO., U.S.A. Doxorubicin and daunorubicin, containing lactose and mannitol as carrier substances, were obtained from Farmitalia Carlo Erba, Freiburg, Germany. In preliminary experiments it was established that these carbohydrates did not influence enzyme activity. 3,4-Dihydroxybenzoate and unlabelled 2-oxoglutarate were obtained from Fluka, Buchs, Switzerland, and pyridine-2,4-dicarboxylate was from Aldrich, Steinheim, Germany. Catalase was purchased from Sigma, and [14-^{14}C]-doxorubicin and 2-oxo[1-^{14}C]glutarate were from Amersham International, Amersham, Bucks., U.K. The 2-oxo[^{14}C]glutarate was diluted to 100000 d.p.m./0.1 μmol by mixing with the unlabelled compound. [U-^{14}C]Proline-labelled protocollagen substrate was prepared from freshly isolated chick-embryo tendon cells in the presence of 2,2′-bipyridyl, as described elsewhere (Kivirikko & Myllylä, 1982).

Purification of enzymes

Prolyl 4-hydroxylase was isolated as a homogeneous protein from 14-day chick embryos by homogenization, 0–70%-satd.-$(NH_4)_2SO_4$ fractionation, affinity chromatography on poly(L-proline) coupled to Sepharose 4B, DEAE-cellulose chromatography, and gel filtration (Tuderman *et al.*, 1975; Kedersha & Berg, 1981). Human prolyl 4-hydroxylase was obtained from fresh human placentae by an identical procedure. Highly purified lysyl hydroxylase was prepared by an established method (Turpeenniemi-Hujanen *et al.*, 1980) from a 17–55%-satd.-$(NH_4)_2SO_4$ fraction of homogenized 14-day chick embryos, by affinity chromatography on concanavalin A–Sepharose and collagen–Sepharose. Prolyl 4-hydroxylase from the green alga *Chlamydomonas reinhardii* was obtained as described by Kaska *et al.* (1987).

Enzyme assays

The experiments in which the rate of inactivation of prolyl 4-hydroxylase was studied were performed by incubating 2 μg of enzyme at 37 °C in 0.5 ml of 50 mM-Tris/HCl, pH 7.5, containing 0.1 mM-2-oxoglutarate, 0.05 mM-$FeSO_4$, 1 mM-ascorbate (but 2 mM in Expt. 3 of Table 1), 0.1 mg of catalase/ml, 0.1 mM-dithiothreitol, 2 mg of bovine serum album/ml (but 1 mg/ml in Expt. 2 of Table 1), and 0.1 mg of (Pro-Pro-Gly)$_{10}$/ml. The reaction mixture also contained 0–0.2 mM of the compound used as an inactivator. Samples (50 μl) were drawn from this solution at 5 or 10 min intervals between 10 and 60 min and added to 0.95 ml of a solution containing all the components of a similar reaction mixture (except the inactivator) in the concentrations indicated above, together with 2-oxo[^{14}C]glutarate (final concn. 0.1 mM). These samples were then used to assay the remaining enzyme activity, as described below. The inactivation constant k was calculated from the formula $\ln(a_t/a_0) = -kt$ by the simple least-squares method, a_t/a_0 being the ratio of enzyme activity remaining after inactivation for t min (a_t) to initial activity (a_0).

Other inactivation experiments with prolyl 4-hydroxylase were carried out for 60 min at 37 °C in 0.1 ml (if the anthracycline concentration exceeded 100 μM) or 0.2 ml of the above reaction mixture. In some experiments the concentration of 2-oxoglutarate, ascorbate or the peptide substrate was varied, and in some experiments the mixture also contained competitive inhibitors, as indicated in the corresponding Figure legends. After the inactivation period, the reaction volume was increased to 1 ml by adding all the components of the reaction mixture (except the inactivator) in the concentrations indicated above, together with 2-oxo[^{14}C]glutarate (final concn 0.1 mM). The remaining enzyme activity was then determined.

The remaining enzyme activity was assayed by incu-

Table 1. Dependence of apparent bimolecular rate constants on ascorbate and inhibitor concentrations

k was determined as described in the Materials and methods section; C refers to the concentration of doxorubicin. Expts. 1 and 2 were performed in the presence of 1 mM-ascorbate, and Expt. 3 in the presence of 2 mM-ascorbate.

Expt. 1		Expt. 2		Expt. 3	
C (μM)	k/C	C (μM)	k/C	C (μM)	k/C
50	2.17	40	1.00	25	1.00
100	3.42	80	3.96	50	1.23
150	3.09	120	3.78	75	2.24
200	3.39	200	3.03	100	2.20
				125	2.93

bating the samples at 37 °C for 30 min and determining the amount of $^{14}CO_2$ produced as reported by Berg & Prockop (1973). The dilution step performed before this assay (see above) decreased the highest anthracycline concentration to 10 μM in experiments on the rate of inactivation or 20 μM in the other experiments. These concentrations cause no inactivation (see the Results section). Nevertheless, corresponding concentrations were added to the control samples to correct for any remaining competitive effects.

Lineweaver–Burk kinetics were established under conditions identical with those for the assay of the remaining enzyme activity.

The influence of anthracyclines on the activity of lysyl hydroxylase was studied under identical conditions, except that 5 mg of the synthetic peptide L-I/ml was used instead of $(Pro\text{-}Pro\text{-}Gly)_{10}$. In the experiments with *Chlamydomonas reinhardii* prolyl 4-hydroxylase, the peptide substrate was poly(L-proline), the $FeSO_4$ concentration was 200 μM, the buffer was Hepes (50 mM, pH 6.8), and the incubation temperature 30 °C, these differences being due to the optimal conditions needed by this enzyme (Kaska *et al.*, 1987).

Anthracyclines as substitutes for co-substrates

To investigate the possibility of the anthracyclines being able to maintain hydroxylation of a protocollagen substrate in the absence of 2-oxoglutarate, 0.05–0.15 mg of chick-embryo prolyl 4-hydroxylase was incubated in a volume of 2 ml for 30 min at 37 °C in the presence of 100 μM-doxorubicin and 150000 d.p.m. of protocollagen substrate. 2-Oxoglutarate was omitted from the reaction mixture. The concentrations of all the other cofactors were identical with those indicated above. The reaction was stopped by the addition of 2 ml of 12 M-HCl, and the amount of hydroxyproline formed was analysed after hydrolysis for 16 h at 120 °C by an established method (Juva & Prockop, 1966). The ability of the anthracyclines to act as substitutes for ascorbate was studied by using the 2-oxo[1-^{14}C]glutarate-decarboxylation assay as described above. Ascorbate was omitted from the reaction mixture, and doxorubicin or daunorubicin was added in concentrations of 0–0.5 mM.

Labelling of the active site of prolyl 4-hydroxylase with [14-^{14}C]doxorubicin

A sample consisting of 200 pmol of prolyl 4-hydroxylase, 200 pmol of phosphorylase *a*, 400 pmol of bovine serum albumin, 200 pmol of ovalbumin and 200 pmol of soya-bean trypsin inhibitor was incubated in 100 μl of 50 mM-Tris/HCl, pH 7.5, in the presence of 0.05 mM-$FeSO_4$, 0.1 mM-2-oxoglutarate, 1 mM-ascorbate, 0.1 mM-dithiothreitol and 3.75 μCi of [14-^{14}C]doxorubicin (53 mCi/mmol) for 2 h at 37 °C. The experiment was terminated by freezing and freeze-drying of the samples. SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) was performed on 8%-polyacrylamide gels. The labelled slab gel was prepared for autofluorography by treatment with 30 g of 2,5-diphenyloxazole in 200 ml of dimethyl sulphoxide for 3 h, washing in water for 1 h, and drying. An identical sample, prepared by using unlabelled doxorubicin, was stained with Coomassie Brilliant Blue.

In order to demonstrate Fe^{2+}-dependence of the labelling and the effects of high ascorbate concentrations, 42 pmol of prolyl 4-hydroxylase was incubated in 250 μl of 50 mM-Tris/HCl, pH 7.5, containing 0 or 0.05 mM-$FeSO_4$, 0.1 mM-unlabelled 2-oxoglutarate, 1 mM or 10 mM-ascorbate, 0.1 mM-dithiothreitol and 110 pmol of ovalbumin for 1 h at 37 °C in the presence of 0.667 μCi of [14-^{14}C]doxorubicin (sp. radioactivity 53 mCi/mmol). The radioactive samples and controls using unlabelled doxorubicin were analysed by SDS/polyacrylamide-gel electrophoresis as described above.

To determine the quantity of [14-^{14}C]doxorubicin incorporated into prolyl 4-hydroxylase, 1 μg of the enzyme was incubated for 1 h at 37 °C in 0.2 ml of 50 mM-Tris/HCl, pH 7.5, in the presence of 50 μM-$FeSO_4$, 1.0 mM-2-oxoglutarate, 1 mM-ascorbate, 0.1 mg of catalase/ml, 0.1 mM-dithiothreitol, 2 mg of bovine serum albumin/ml and 10 μM- or 50 μM-[14-^{14}C]doxorubicin (56 mCi/mmol). Inactivation of prolyl 4-hydroxylase was determined by assaying the activity remaining in a 5 μl sample. Prolyl 4-hydroxylase (20 μg) was added as a carrier to the reaction mixture, and prolyl 4-hydroxylase was then immediately separated from the other components by affinity chromatography in a 0.3 ml concanavalin A–Sepharose column (Chen-Kiang *et al.*, 1977) equilibrated with a solution containing 10 mM-$MnCl_2$, 0.2 M-NaCl, 20 mM-Tris/HCl, pH 7.5 at 4 °C, 0.1 M-glycine and 10 μM-dithiothreitol. The column was washed with 6 ml of the equilibration buffer and eluted with 3 ml of the equilibration buffer containing 30% (w/v) methyl α-D-glucoside. Unlabelled doxorubicin was added to the eluate (final concn. 100 μM), and the sample was exhaustively dialysed against a solution of 8 M-urea, 10% (w/v) SDS and 1.25 M-Tris/HCl, pH 6.8. The radioactivity was then determined by scintillation counting in 10 ml of Insta-Gel (Packard).

RESULTS

Lineweaver–Burk kinetic experiments

Doxorubicin inhibited prolyl 4-hydroxylase competitively with respect to 2-oxoglutarate (Fig. 2*a*), the secondary transformations of the Lineweaver–Burk plots being non-linear. The apparent K_i value, determined in the presence of saturating concentrations of all the co-substrates, was 40 μM. When doxorubicin was used in concentrations not exceeding 20 μM, competitive inhibition was also found with respect to ascorbate (Fig. 2*b*). Higher concentrations distorted the competitive picture, yielding lines in Lineweaver–Burk plots which intersected with the negative part of the ordinate. This phenomenon is likely to be due to an enhanced inactivation of prolyl 4-hydroxylase by anthracyclines in the presence of low ascorbate concentrations (see below). When Fe^{2+} was the varied substrate, non-competitive inhibition was found at low doxorubicin concentrations, whereas at high doxorubicin concentrations a phenomenon that resembled substrate inhibition by Fe^{2+} ions was observed (Fig. 2*c*).

Inactivation of collagen hydroxylases

Doxorubicin and daunorubicin proved to be inactivators of prolyl 4-hydroxylase from chick and human sources and of chick-embryo lysyl hydroxylase. These two anthracyclines displayed virtually identical properties. In agreement with previous studies (Günzler *et al.*, 1987), spontaneous loss of prolyl 4-hydroxylase activity owing to autoxidation was not negligible. Application of anthracyclines in concentrations not

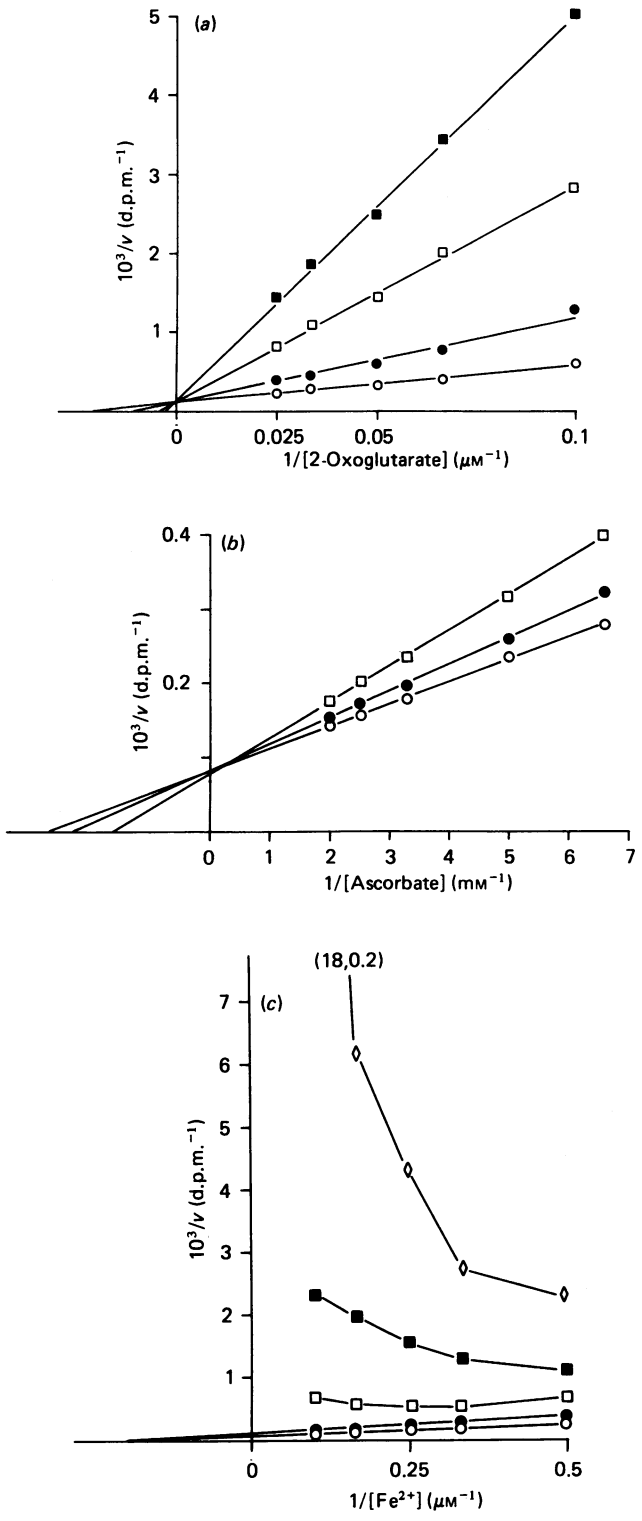


Fig. 2. Competitive inhibition of chick-embryo prolyl 4-hydroxylase by doxorubicin

(*a*) Use of 2-oxoglutarate as variable substrate. Concentrations of doxorubicin were 0 (○), 40 μM (●), 60 μM (□) and 70 μM (■). (*b*) Use of ascorbate as variable substrate. Concentrations of doxorubicin were 0 (○), 10 μM (●) and 15 μM (□). The linear regression coefficient r^2, obtained from the method of simple least squares, was > 0.99 for all concentrations. The calculated intercepts with the ordinate were 0.082×10^{-3}, 0.082×10^{-3} and 0.078×10^{-3} d.p.m.$^{-1}$ respectively. Higher concentrations of doxorubicin yielded negative ordinate intercepts (see the Results section). (*c*) Use of Fe^{2+} as variable substrate. The concentrations of doxorubicin were 0 (○), 25 μM (●), 50 μM (□), 75 μM (■) and 100 μM (◇). The lowest concentration of doxorubicin behaves non-competitively. The calculated ordinate intercepts for 0 and 25 μM-doxorubicin were 0.32×10^{-3} and 0.46×10^{-3} d.p.m.$^{-1}$ respectively, and the corresponding abscissa intercepts −0.25 and −0.246 μM^{-1}. The linear regression coefficient r^2, obtained from simple least squares, was > 0.99 in both cases. High concentrations of both Fe^{2+} and doxorubicin yield low enzyme activity, probably owing to enhanced inactivation.

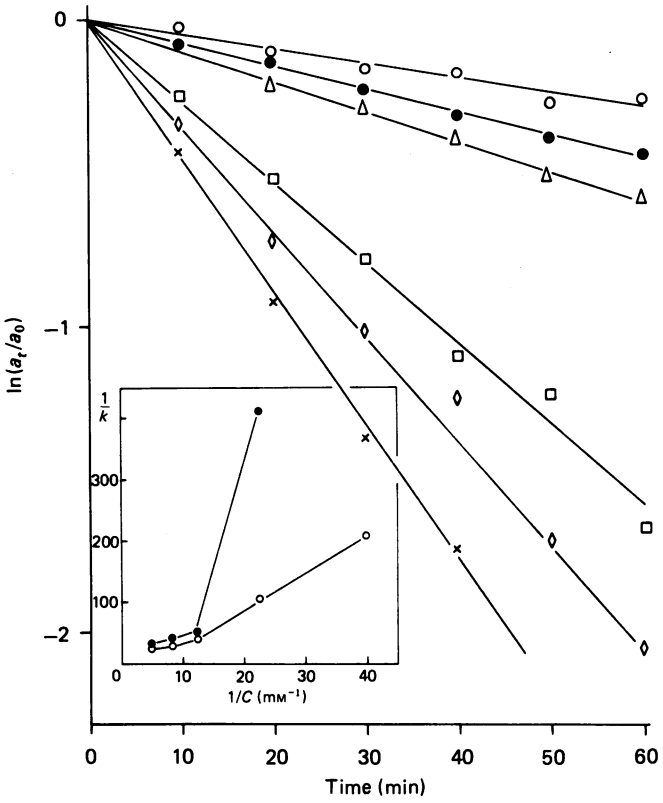


Fig. 3. Time-dependence of the inactivation of chick-embryo prolyl 4-hydroxylase by doxorubicin

The concentrations of doxorubicin were 0 (●), 25 μM (○), 40 μM (△), 80 μM (□), 120 μM (◇), or 200 μM (×). Each point represents the mean for five individual samples. A slight but reproducible protection of prolyl 4-hydroxylase against spontaneous autoxidation was observed at the lowest concentration of the inhibitor used. The inset shows the non-linearity of the secondary transformation ($1/k$ versus $1/C$). Values for k are shown both corrected for the spontaneous loss of enzyme activity (●) and without such correction (○).

exceeding 25 μM caused no inactivation, but resulted in a slight but reproducible protection of the enzyme from self-inactivation (Fig. 3). Inactivation was, however, observed at higher concentrations. The IC_{50} (concentration of inhibitor yielding 50% inhibition) after 1 h for prolyl 4-hydroxylase from either chick embryos or human placentae was 60 μM. The rate of the inactivation reaction, determined as $\ln(a_t/a_0)$, was of first order, but the secondary transformations ($1/k$ versus 1/concen-

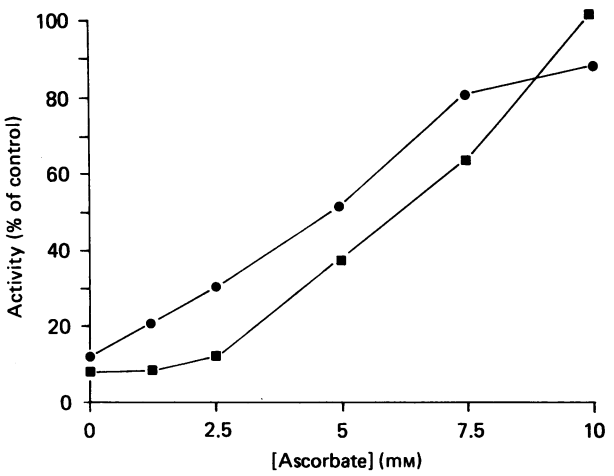


Fig. 4. Effect of high ascorbate concentrations on the inactivation of chick-embryo prolyl 4-hydroxylase

Doxorubicin (●) and daunorubicin (■) (both 100 μM) were used as inactivating agents. The [ascorbate] scale refers to the concentration of this cosubstrate during the preincubation period (see the Materials and methods section). Its concentration was adjusted to 2 mM in the assay of activity remaining.

tration) were non-linear, regardless of whether or not the observed k values were mathematically corrected for the spontaneous loss of enzyme activity owing to autoxidation (Fig. 3).

Chick-embryo lysyl hydroxylase was slightly less susceptible to inactivation, the IC_{50} values after incubation for 1 h being 150 μM for doxorubicin and 200 μM for daunorubicin. Prolyl 4-hydroxylase from the green alga *Chlamydomonas reinhardii* was neither inactivated nor reversibly inhibited by doxorubicin at concentrations up to 1 mM.

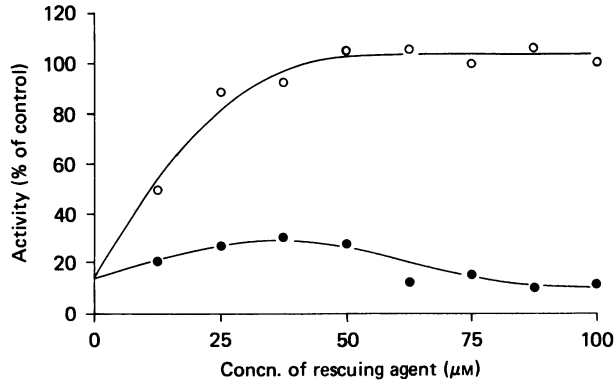


Fig. 5. Effect of competitive inhibitors of prolyl 4-hydroxylase on the inactivation of chick-embryo prolyl 4-hydroxylase

The enzyme activity could be rescued by the addition of 3,4-dihydroxybenzoate (○), a competitive inhibitor with respect to ascorbate, but not by pyridine-2,4-dicarboxylate (●), a 2-oxoglutarate analogue. Doxorubicin (100 μM) was used as the inactivating agent.

Mechanism of the inactivation reaction

The inactivation could be prevented almost completely by the addition of 10 mM-ascorbate to the preincubation mixture (Fig. 4). Protection was also observed with 25 μM-3,4-dihydroxybenzoate (Fig. 5), a competitive inhibitor with respect to ascorbate (Majamaa *et al.*, 1986), but not with 100 μM-pyridine-2,4-dicarboxylate (Fig. 5), a competitive inhibitor with respect to 2-oxoglutarate (Majamaa *et al.*, 1984), or with 1 mM-2-oxoglutarate (results not shown).

Inactivation could also be minimized by omission of Fe^{2+} from the preincubation mixture (results not shown). The rescue was incomplete, however, because preparations of purified prolyl 4-hydroxylase contain different amounts of iron (Pänkäläinen & Kivirikko, 1971; Prockop *et al.*, 1976; Tuderman *et al.*, 1977; Nietfeld & Kemp, 1980). If no iron was added to the reaction mixture, doxorubicin was able to increase the recovery of enzyme activity as compared with the controls. High concentrations of Fe^{2+} increased the effectiveness of the inactivation. Variation of the concentration of the peptide substrate had no effect on the inactivation (results not shown).

Labelling of the active site of prolyl 4-hydroxylase with [14-^{14}C]doxorubicin

Radioactive label was found in the autofluorographs of slab gels of prolyl 4-hydroxylase incubated with [14-^{14}C]doxorubicin, whereas the control proteins present in identical concentration, or in twice the concentration in the case of bovine serum albumin, incorporated only negligible amounts of the label (Fig. 6*a*). Bands corresponding to the control proteins could be clearly seen if the film was exposed to such an extent that the band corresponding to the α-subunit of prolyl 4-hydroxylase was over-exposed, but even under these conditions the radioactivity in the control proteins did not exceed 10% that of the enzyme, when measured by densitometry (results not shown). About 70% of the total radioactivity in the enzyme was found in the α-subunit and 30% in the β-subunit.

The addition of 10 mM-ascorbate strikingly decreased the incorporation, and a similar effect was obtained when Fe^{2+} was omitted from the preincubation mixture (Fig. 6*b*). The labelling could not be prevented completely in the absence of iron, possibly because of the presence of this ion in the enzyme preparation. This finding agrees with the result of the functional test.

In an additional experiment an attempt was made to quantify the relationship between the degree of inactivation of the enzyme and the amount of labelled doxorubicin incorporated. Prolyl 4-hydroxylase was incubated with 10 μM- or 50 μM-doxorubicin for 1 h as described in the Materials and methods section, and the quantity of doxorubicin incorporated was calculated from its specific radioactivity. Incubation with 10 μM-doxorubicin gave no inactivation, and the quantity of doxorubicin found in the enzyme was 0.07 pmol/pmol of tetramer. Incubation with 50 μM-doxorubicin resulted in an inactivation by 40–49% in three samples, the quantity of doxorubicin incorporated being 0.75–1.2 pmol/pmol of tetramer. These values agree well with those expected, as the enzyme tetramer contains two catalytic sites (de Waal *et al.*, 1985), and thus complete inactivation probably requires binding of two molecules of doxorubicin per molecule of tetramer.

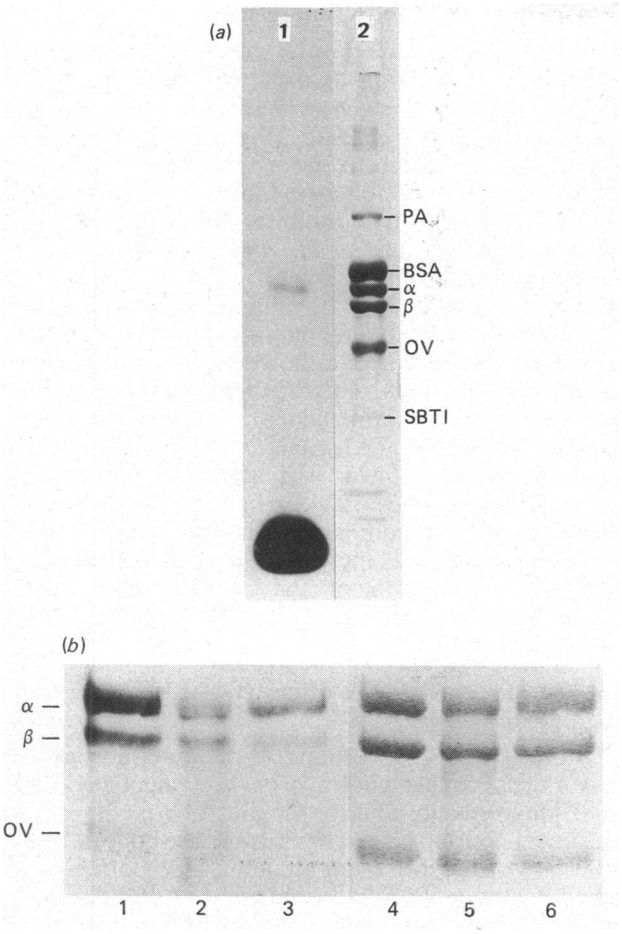


Fig. 6. Labelling of chick-embryo prolyl 4-hydroxylase with [14-^{14}C]doxorubicin

(*a*) Lane 1 shows an autofluorograph of a sample of 200 pmol of phosphorylase *a* (PA), 400 pmol of bovine serum albumin (BSA), 200 pmol of tetrameric ($\alpha_2\beta_2$) prolyl 4-hydroxylase, separated into α- and β-subunits by the electrophoresis procedure, 200 pmol of ovalbumin (OV), and 200 pmol of soya-bean trypsin inhibitor (SBTI), incubated with radioactive doxorubicin and analysed by SDS/polyacrylamide-slab-gel electrophoresis as described in the Materials and methods section. Lane 2 shows the corresponding Coomassie-Blue-stained sample. (*b*) Autofluorograph after SDS/polyacrylamide-slab-gel electrophoresis of 42 pmol of tetrameric ($\alpha_2\beta_2$) prolyl 4-hydroxylase and 110 pmol of ovalbumin (OV) labelled with 0.66 μCi of [14-^{14}C]doxorubicin in the presence of 1 mM-ascorbate (lane 1), 10 mM-ascorbate (lane 2) and 1 mM-ascorbate, but no Fe^{2+} added to the reaction mixture (lane 3). Lanes 4–6 show identical Coomassie-Blue-stained samples.

Co-substrate-substitution experiments

The anthracyclines were not able to replace either 2-oxoglutarate or ascorbate in the enzymic reaction (results not shown).

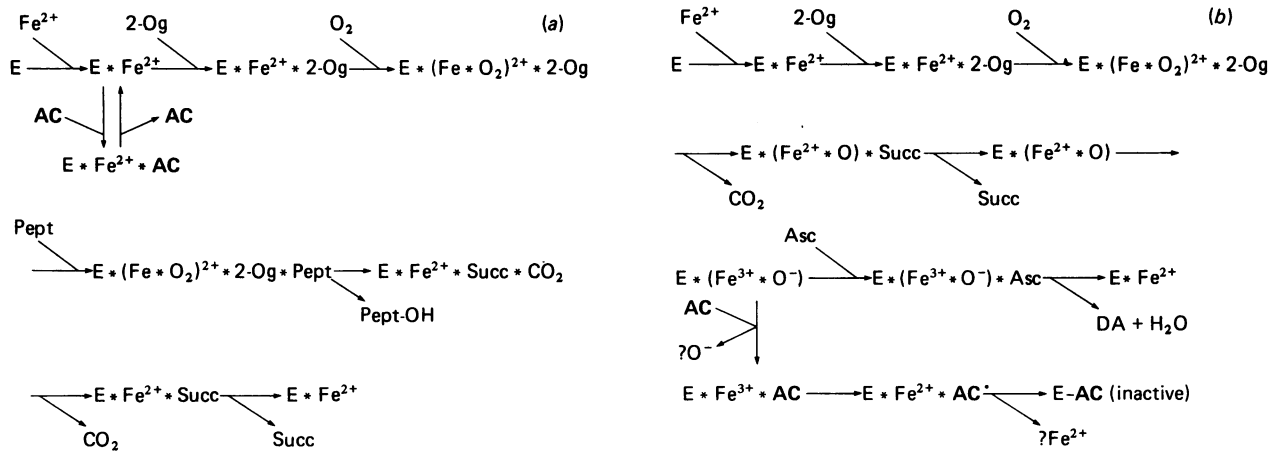
DISCUSSION

The results presented here demonstrate that class I anthracycline antibiotics are powerful agents for inactivating prolyl 4-hydroxylase of both chick and human origin, and also chick-embryo lysyl hydroxylase. They are capable of forming chelate complexes with both Fe^{3+} and Fe^{2+} ions (May *et al.*, 1980; Eliot *et al.*, 1984; Zweier, 1984), whose chemical properties are determined by the redox-amphoteric nature of the ligands. Although the anthracyclines can be reduced to semiquinone radicals (Land *et al.*, 1983), Fe^{2+} adducts are stable (Zweier, 1984), and thus reduction of Fe^{3+} to the bivalent state by doxorubicin has been observed (Zweier, 1984). Re-oxidation may occur in the presence of atmospheric oxygen (Zweier, 1984), and these redox cycles are accelerated by various enzymes (Davies *et al.*, 1983; Fisher *et al.*, 1985; Davies & Doroshow, 1986). The simultaneously produced highly reactive oxygen species, of which superoxide anion, H_2O_2, and hydroxyl radical have been identified (Davies *et al.*, 1983; Doroshow & Davies, 1986), are held responsible for the marked cardiotoxicity of these drugs (Eliot *et al.*, 1984; Doroshow & Davies, 1986).

In agreement with these chemical properties, the anthracycline antibiotics apparently interact with the iron-dependent enzyme prolyl 4-hydroxylase in a complex way. In full reaction cycles (Scheme 1) they act as 2-oxoglutarate antagonists, as demonstrated by the competitive inhibition observed with respect to this cosubstrate. Because the enzyme-bound iron remains in the bivalent state, no radical formation takes place under these conditions, and thus there is no inactivation. Accordingly, neither 2-oxoglutarate nor its competitive analogue pyridine-2,4-dicarboxylate (Majamaa *et al.*, 1984) was able to prevent inactivation.

On the other hand, the anthracyclines are also able to bind to the active site of the enzyme during uncoupled reaction cycles (Scheme 1), as demonstrated by the competitive mode of inhibition with respect to ascorbate, the specific substrate in these cycles (de Jong & Kemp, 1984; Myllylä *et al.*, 1984). At this stage inactivation takes place, which can be prevented in the presence of high concentrations of ascorbate or low concentrations of its competitive analogue 3,4-dihydroxybenzoate (Majamaa *et al.*, 1986). Because of the chemical nature of the anthracyclines, and on the basis of previous data (Majamaa *et al.*, 1986), it must be assumed that the uncoupled reaction cycles proceed via a transient enzyme–ferric iron intermediate complex. Such oxidation of the enzyme-bound iron is not an irreversible process, as activity of an Fe^{3+} enzyme can be reconstituted by the addition of ascorbate (de Jong *et al.*, 1982). Oxidation of a class I anthracycline molecule by Fe^{3+} present at the active site of the enzyme probably generates a ring-B radical which reacts with the protein, inactivating the enzyme. In agreement with this hypothesis, doxorubicin and daunorubicin were not able to serve as substitutes for ascorbate in the enzymic reaction, in spite of their ability to reduce Fe^{3+} (Zweier, 1984).

The multiple possibilities of interaction of the anthracycline antibiotics with prolyl 4-hydroxylase are probably responsible for the stabilization of enzyme at low anthracycline concentrations and the non-linear behaviour of the secondary transformations of the inactivation plot (Fig. 3). Previous data indicate that competitive inhibitors of prolyl 4-hydroxylase with respect to 2-oxoglutarate stabilize the enzyme against autooxidation (Günzler *et al.*, 1987). Non-linearity of the secondary transformations may also be due to the impossibility of completely omitting ascorbate from the reaction mixture. Consequently, the enzyme was at least partly protected



Scheme 1. Scheme of the reactions of anthracyclines (AC) with prolyl 4-hydroxylase

(*a*) Full and (*b*) uncoupled reaction catalysed by prolyl 4-hydroxylase (Kivirikko & Myllylä, 1987). E, enzyme protein; 2-Og, 2-oxoglutarate; Pept, peptide substrate; Pept-OH, hydroxylated substrate; Succ, succinate; Asc, ascorbate; DA, dehydroascorbate; AC·, anthracycline semiquinone radical. Asterisks (*) symbolize reversible association, the dash (–) covalent binding. The question marks in (*b*) indicate that the fate of these species in the anthracycline reaction remains to be clarified.

from the action of the anthracyclines under all experimental conditions.

The inactivation reaction involves covalent linkage of anthracycline to the enzyme protein. Hypothetically, the incorporation of [14-^{14}C]doxorubicin may occur at the iron- or ascorbate-binding site of prolyl 4-hydroxylase, or both. The observed labelling of both the α- and β-subunits suggests that the active site of the enzyme is co-operatively built up of both these polypeptide chains. The α-subunit appears to contribute a major part to the catalytic site of the enzyme, as it probably also contains the peptide-binding region (de Waal *et al.*, 1985) and the 2-oxoglutarate attachment site (Günzler *et al.*, 1987; de Waal *et al.*, 1987).

An inactivation of prolyl 4-hydroxylase by anthracycline radicals or reactive oxygen species generated in solution without enzymic participation must be considered on theoretical grounds, but is highly unlikely, for several independent reasons. (1) The strongly reducing conditions of the reaction mixture, 2 mM-ascorbate and 0.1 mM-dithiothreitol, maintains the iron in its bivalent state, thus preventing the first step in the redox cycles, i.e. oxidation of anthracyclines by Fe^{3+} outside the active site of the enzyme. Moreover, inactivation of prolyl 4-hydroxylase by anthracyclines shows the greatest increase between 2 μM- and 10 μM-Fe^{2+}, a range in which enzyme activity, and thus the number of uncoupled reaction cycles, is linearly dependent on the iron concentration (Fig. 2*c*). (2) The assumption of inactivation of prolyl 4-hydroxylase by a reactive oxygen species generated in solution by reductive activation of the anthracyclines is not compatible with the observation of incorporation of labelled doxorubicin into the enzyme protein. The presence of high concentrations of catalase in the reaction mixture detracts further from this concept. (3) The assumption of inactivation by anthracycline semiquinone radicals reductively generated in solution without participation of the enzyme is not in accord with the observed incorporation of labelled doxorubicin predominantly into prolyl 4-hydroxylase among the proteins studied. (4) An additional argument in favour of a specific action of the anthracyclines comes from the observation that *Chlamydomonas reinhardii* prolyl 4-hydroxylase was not susceptible to inactivation. Although this enzyme is also an iron-dependent dioxygenase, distinct differences have been found between the algal and vertebrate prolyl 4-hydroxylases (Kaska *et al.*, 1987). Doxorubicin apparently has no affinity for the algal enzyme, as it caused no reversible inhibition. The complete lack of inactivation of this enzyme makes it highly unlikely that the inactivation could be produced to any significant extent by anthracycline radicals or reactive oxygen species, generated by redox cycles involving iron and anthracycline molecules in the reaction mixture.

It is generally accepted that the mechanism of action of a class I anthracycline is related to reduction of its C ring to a semiquinone radical by low-potential flavin and iron–sulphur centres of various oxidoreductases (Chabner & Myers, 1982; Fisher *et al.*, 1985), with the compound acting as an oxidant. The results presented here, however, can only be understood rationally if the anthracycline acts as a reductant (Zweier, 1984) for the collagen hydroxylases in their ferric form by virtue of oxidation of its B ring.

The finding that class I anthracyclines are potent irreversible inactivators of the collagen hydroxylases is helpful in understanding the inhibitory effect of such compounds on hydroxyproline generation *in vivo*. Clinically, extravasation of these compounds produces very slowly healing lesions not amenable to autologous skin grafting (Chabner & Myers, 1982). The tissue concentrations of ascorbate are about 2.5 mM in normal skin and may increase to 6.8 mM in wounded tissue (Gould, 1968). Both concentrations are insufficient to prevent inactivation of prolyl 4-hydroxylase by anthracyclines. Even single-dose administration of anthracyclines produces a striking decrease in wound-chamber hydroxyproline content, collagen-fibre diameter and wound-breaking strength for as long as 20 days (Lawrence *et al.*, 1986; Devereux *et al.*, 1980*a*,*b*), a length of time that may be required for the synthesis *de novo* of active collagen hydroxylases suggested by our results.

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