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Modification of vertebrate and algal prolyl 4-hydroxylases and vertebrate lysyl hydroxylase by diethyl pyrocarbonate

Evidence for histidine residues in the catalytic site of 2-oxoglutarate-coupled dioxygenases

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A search for conserved amino acid residues within the cDNA-derived amino acid sequences of 2-oxoglutarate-coupled dioxygenases revealed the presence of two distinct motifs, spaced 49–71 amino acids apart, toward the *C*-terminal regions of these proteins. Each of the two common motifs contains an invariant histidine residue at a conserved position. The 2-oxoglutarate-coupled dioxygenases function in diverse processes, including the post-translational hydroxylation of proline and lysine residues in vertebrate collagens and the biosynthesis of microbial cephalosporins, yet they have a common reaction mechanism, which requires the binding of Fe^{2+}, 2-oxoglutarate, O_2 and ascorbate at the catalytic site. The two regions of homology, and specifically the identical histidines, potentially represent functionally important sites related to their catalytic activity. Modification of histidine residues by diethyl pyrocarbonate inactivated vertebrate and algal prolyl 4-hydroxylase and vertebrate lysyl hydroxylase, indicating that histidine residues function in the catalytic site of these 2-oxoglutarate-coupled dioxygenases. Inactivation was prevented by the presence of co-substrates, but not by the peptide substrate. It is proposed that the histidine residues in the conserved motifs may function as Fe^{2+}-binding ligands.

INTRODUCTION

Vertebrate prolyl 4-hydroxylase (EC 1.14.11.2) and lysyl hydroxylase (EC 1.14.11.4) catalyse the formation of 4-hydroxyproline and hydroxylysine in collagens and related proteins by the hydroxylation of proline and lysine residues, respectively, in peptide linkages. These enzymes are very similar in their catalytic properties; the reactions require, in addition to their polypeptide substrate, Fe^{2+}, 2-oxoglutarate, O_2 and ascorbate, and follow an ordered Ter Ter mechanism (for reviews, see [1–3]). The kinetic constants of the enzymes for their co-substrates and competitive inhibitors are also very similar [1–3]. In spite of their related reaction mechanisms, these enzymes show great structural differences. Vertebrate prolyl 4-hydroxylase is composed of two non-identical subunits constituting an $\alpha_2\beta_2$ tetramer with a mass of 250 kDa [1–3]. The catalytic sites are located mainly in the α subunits [1–3], but some parts of the catalytic sites may be co-operatively built up from both types of subunit [4]. The β subunit has been shown to be identical with the enzyme protein disulphide-isomerase [5,6]. Lysyl hydroxylase is a dimer consisting of identical subunits and having a molecular mass of 180 kDa [1,2]. Complete cDNA-derived amino acid sequences have recently been determined for both types of subunit of human and chick prolyl 4-hydroxylase [5,7–10], and for lysyl hydroxylase [11,12]. Comparison of the primary structures of these proteins was expected to provide information about amino acid residues involved in the catalytic sites, possibly in the form of large conserved domains. Initial comparison of the sequences, however, has revealed little similarity between the primary structures of the vertebrate hydroxylases [11], suggesting that any conserved elements are small and hence difficult to recognize.

Recently a number of low-molecular-mass (35–45 kDa) 2-oxoglutarate-coupled dioxygenases have been identified. Although the molecular masses of the target molecules for these reactions may vary from a few hundred Da to more than 1000 kDa, their co-substrate and cofactor requirements are identical with those of the vertebrate hydroxylases. These enzymes include prolyl 4-hydroxylase from green algae, which hydroxylates prolines in proteins destined for incorporation into the cell wall [13,14], the bifunctional deacetoxycephalosporin C (DAOC) and deacetylcephalosporin C (DAC) synthase and the monofunctional DAOC and DAC synthases, which expand the ring of penicillin N and convert it into DAC in bacteria and fungi [15,16], bacterial clavaminate synthase [17], and several plant hydroxylases [18].

The two catalytic sites of the vertebrate prolyl 4-hydroxylase tetramer are presumed to comprise a set of separate locations for the binding of the peptide substrate and the various co-substrates [1–3,19]. The Fe^{2+} is probably co-ordinated with the enzyme by three side chains [1–3,19]. We have here compared the primary structures of several 2-oxoglutarate-coupled dioxygenases with each other and with those of a related dioxygenase that requires Fe^{2+}, O_2 and ascorbate, isopenicillin N synthase [20–24], as well as of three 35 kDa plant proteins of unknown function [9,11,15,16,18,21,25–28]. These comparisons indicate the presence of two conserved histidine residues, which might act as binding sites for Fe^{2+} to these proteins. To elucidate the role of histidine residues in the catalytic sites, we have studied the effect of chemical modification with diethyl pyrocarbonate on the activity of vertebrate and algal prolyl 4-hydroxylase and vertebrate lysyl hydroxylase. Our data define histidine residues of importance for the catalytic function of these enzymes and suggest the presence of histidine residues in the catalytic sites of 2-oxoglutarate-coupled dioxygenases.

MATERIALS AND METHODS

Materials

Diethyl pyrocarbonate and poly(L-proline) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. The synthetic

Abbreviations used: DAOC, deacetoxycephalosporin C; DAC, deacetylcephalosporin C.

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peptide (Pro-Pro-Gly)$_{10}$,9H$_2$O was obtained from the Protein Research Foundation, Minoh, Osaka, Japan. 2-Oxo[1-^{14}C]-glutarate was purchased from Amersham International, Amersham, Bucks., U.K. It was diluted to 100000 d.p.m./0.1 μmol by mixing with the unlabelled compound. [^{3}H]Lysine-labelled non-hydroxylated procollagen was prepared from freshly isolated chick-embryo tendon cells as described in detail elsewhere [29].

Purification of enzymes

Vertebrate prolyl 4-hydroxylase was isolated from 15-day whole chick embryos by a procedure consisting of affinity chromatography on poly(L-proline)–Sepharose, ion-exchange chromatography and gel filtration [30]. Purification of lysyl hydroxylase from 15-day whole chick embryos was carried out by a protocol consisting of affinity chromatographies on concanavalin A–Sepharose and collagen–Sepharose and chromatography on a hydroxyapatite column [31]. Prolyl 4-hydroxylase was partially purified from *Chlamydomonas reinhardii* by ion-exchange chromatography and gel filtration [13].

Enzyme assays

The activity of vertebrate prolyl 4-hydroxylase was assayed by determination of the release of $^{14}CO_2$ during the hydroxylation-coupled stoichiometric decarboxylation of 2-oxo[1-^{14}C]glutarate and with the synthetic peptide (Pro-Pro-Gly)$_{10}$ as the substrate [29]. Algal prolyl 4-hydroxylase activity was assayed similarly with poly(L-proline) as the substrate [13]. Lysyl hydroxylase activity was assayed by measuring the formation of radioactive hydroxylysine in [^{3}H]lysine-labelled non-hydroxylated procollagen substrate [29].

Inactivation of the enzyme activities with diethyl pyrocarbonate

The enzymes were inactivated with diethyl pyrocarbonate diluted with ethanol. The ethanol concentration in the reaction mixture never exceeded 5% (v/v). The enzyme solution in 50 mM-Tris/HCl buffer, pH 6.8, or 50 mM-phosphate buffer, pH 6.8, was incubated on ice with 4 μl of diluted diethyl pyrocarbonate. To the reference sample 4 μl of ethanol was added. At various time intervals, samples (5–30 μl) were withdrawn from the incubation mixture and assayed for the enzyme activity.

Treatment of the inactivated enzyme with hydroxylamine

In order to study the reversibility of the diethyl pyrocarbonate inactivation, the inactivated enzyme was treated with hydroxylamine. After incubation with diethyl pyrocarbonate, the excess of diethyl pyrocarbonate was bound to imidazole (2.5 mM) added to the reaction mixture. The reaction mixture was dialysed against 10 mM-Tris/HCl/100 mM-NaCl, pH 7.5, and hydroxylamine was added to a final concentration of 2.5 mM. The sample was incubated at 22 °C for up to 40 min and was then dialysed against the above solution. In the control sample ethanol was added instead of diethyl pyrocarbonate.

Other assays

Amino acid sequences were analysed by using the MacVector global and local sequences-alignment programs (International Biotechnologies, New Haven, CT, U.S.A). Protein was assayed by the method of Bradford [32], with BSA as standard. Spectral analyses of diethyl pyrocarbonate-treated proteins were carried out by monitoring the absorbance from 300 nm to 220 nm on a spectrophotometer in the dual-wavelength/double-beam mode (Shimadzu UV 3000; Shimadzu Corp., Kyoto, Japan).

RESULTS

Comparison of primary structures

In order to search for possible conserved regions in 2-oxoglutarate-coupled dioxygenases and closely related enzymes, the amino acid sequences of human and chick prolyl 4-hydroxylase and lysyl hydroxylase were compared with those of several enzymes from non-vertebrate species. Two conserved histidine-containing sequences, termed His-1 and His-2 motifs, were found in all these enzymes, the distance between the two motifs ranging from 49 to 71 residues (Fig. 1). In the His-1 motif of human and chick lysyl hydroxylase, 7 out of the 9 amino acids are identical and one additional amino acid is similar to those in the other enzymes or in three of the 35 kDa plant proteins described below (Fig. 1). In the His-1 motif in the α subunit of human and chick prolyl 4-hydroxylase, only three amino acids are identical and three additional ones are similar to those in the other enzymes, but it is noteworthy that the first four residues of this motif are all either identical or similar. In the His-2 motif of lysyl hydroxylase 12 out of 20 amino acids present in the motif of this enzyme are identical and four additional ones are similar to those in the other proteins, whereas the corresponding numbers for the His-2 motif in the α subunit of prolyl 4-hydroxylase are 10 and 6 respectively (Fig. 1). While this manuscript was in preparation, the primary sequence of a plant hyoscyamine 6β-hydroxylase was reported [18]. Those authors noted homologies

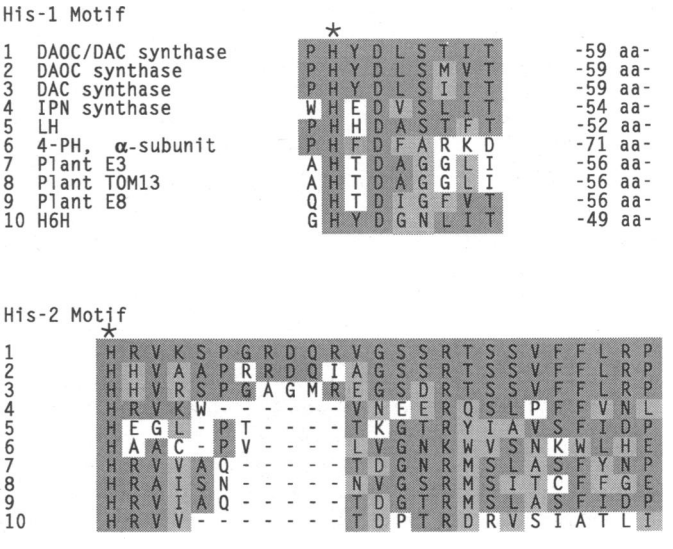

Fig. 1. Comparison of amino acid sequences showing the proposed His-1 and His-2 motifs

Results are shown for DAOC/DAC synthase from *Cephalosporium acremomium* [15], DAOC synthase from *Streptomyces clavuligerus* [16], DAC synthase from *Streptomyces clavuligerus* [25], isopenicillin N (IPN) synthase from *Penicillium chrysogenum* [21], lysyl hydroxylase (LH) from chick [11], prolyl 4-hydroxylase (4-PH) α-subunit from human [9], plant E3 from avocado [26], plant TOM13 [27], plant E8 [28] from tomato and hyoscyamine 6β-hydroxylase (H6H) from *Hyoscyamus niger* [18]. Amino acids identical for at least two hydroxylase (nos. 1–6) or one hydroxylase (nos. 1–6) and one plant protein (nos. 7–10) are dark-stippled, whereas corresponding similar ones are light-stippled. The invariant histidine residues in each motif are indicated by asterisks. The numbers are the numbers of amino acids (aa) between the two motifs. Similar amino acids: Gly = Ala = Ser; Ala = Val; Val = Ile = Leu = Met; Ile = Leu = Met = Phe = Tyr = Trp; Lys = Arg = His; Asp = Glu = Gln = Asn; Ser = Thr = Glu = Asn.

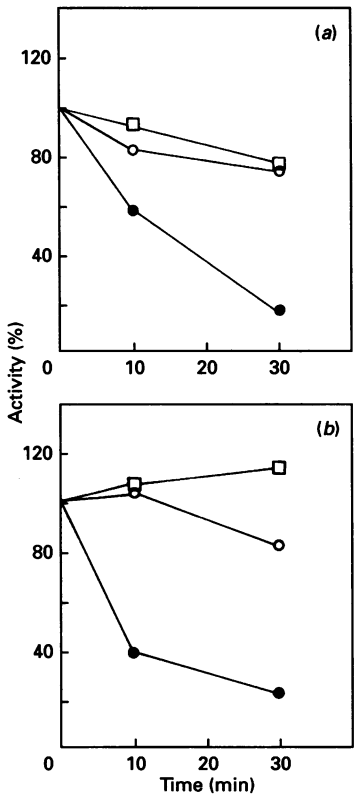

Fig. 2. Inactivation of chick (*a*) and algal (*b*) prolyl 4-hydroxylase by diethyl pyrocarbonate

The enzymes were incubated without (●) or with (○) cofactor mixture in the presence of 1.4 mM-diethyl pyrocarbonate; (□) enzyme incubation in the presence of cofactor mixture but omitting diethyl pyrocarbonate. The enzyme activities were assayed at 0, 10 and 30 min.

between hyoscyamine 6β-hydroxylase, several microbial 2-oxoglutarate-coupled dioxygenases and other plant proteins with unknown functions, although they were not able to detect homologies to vertebrate 2-oxoglutarate-coupled dioxygenases [18].

Three 35 kDa plant proteins of unknown function were also found to contain the two His motifs (Fig. 1). These are a ripening-related protein E3 from avocado [26], and the ethylene-regulated and ripening-induced proteins TOM13 [27] and E8 [28] from tomato. One of these proteins, TOM13, appears to be 1-aminocyclopropane-1-carboxylic acid oxidase, on the basis of studies with antisense RNA [33]. Furthermore, TOM13 has been reported to have a 33% amino acid sequence identity and 58% amino acid sequence similarity to flavanone 3β-hydroxylase, a plant protein known to be a 2-oxoglutarate-coupled dioxygenase [33].

Modification of vertebrate and algal prolyl 4-hydroxylases and vertebrate lysyl hydroxylase by diethyl pyrocarbonate

Chick prolyl 4-hydroxylase was rapidly inactivated by incubation with 1.4 mM-diethyl pyrocarbonate at pH 6.8 on ice. After incubation for 30 min, the enzyme was found to be inactivated by 90% (Fig. 2*a*). Similar results were obtained with algal prolyl 4-hydroxylase, the enzyme activity decreasing in 30 min by 80% (Fig. 2*b*). Chick lysyl hydroxylase was also very sensitive, incubation for 30 min with 1.4 mM-diethyl pyrocarbonate inactivating the enzyme by 60% (results not shown).

The spectra of the non-inactivated and inactivated enzymes were monitored at 220–300 nm, as shown for chick prolyl 4-hydroxylase in Fig. 3. The inactivated enzymes showed only one peak in the difference absorbance at 240 nm, whereas none occurred at 279 nm. These results indicate that diethyl pyrocarbonate had modified histidine residues, whereas reaction with tyrosine residues could be excluded [34]. Diethyl pyrocarbonate can react with a variety of other nucleophilic residues, including thiol, arginyl and amino groups, which are reactive only in their unprotonated form, however. Previous studies on the pH-dependence of the rate of reaction of diethyl pyrocarbonate with these residues indicate that at pH 6.8, the pH used in this study, only the unprotonated imidazole or histidine residue is reactive, making the compound very selective [34].

Treatment of inactivated chick prolyl 4-hydroxylase with 2.5 mM-hydroxylamine at 22 °C for 40 min increased its activity 2–3-fold (results not shown). Reversal by higher concentrations could not be studied, owing to irreversible inactivation of the enzyme by hydroxylamine.

Protection of the enzymes from inactivation by diethyl pyrocarbonate

The co-substrates and the peptide substrates were tested for their ability to protect chick and algal prolyl 4-hydroxylase and chick lysyl hydroxylase from inactivation by diethyl pyrocarbonate. A marked protection of all three enzyme activities was found when all the co-substrates and the peptide substrate were present, as shown for chick and algal prolyl 4-hydroxylase in Fig. 2.

The effects of individual components of the reaction mixture were studied further by omitting one or more than one of them. For chick prolyl 4-hydroxylase, 2-oxoglutarate and ascorbate appeared to be the most effective compounds in protecting the enzyme from the diethyl pyrocarbonate inactivation, as omission of 2-oxoglutarate or ascorbate from the reaction mixture containing the inactivator and all the co-substrates and the peptide substrate decreased the enzyme activity in various experiments to about 20–30% and 30–50%, respectively, of that found in the presence of all the components. The addition of 2-oxoglutarate without ascorbate and the peptide substrate could not be studied, owing to the rapid self-inactivation of the enzyme by the uncoupled 2-oxoglutarate decarboxylation under these conditions, but addition of ascorbate as the only protecting compound was found to give enzyme activity which was about 70% of that recorded in the presence of all the compounds (results not shown). To exclude the possibility that the protection by ascorbate was due to a direct reaction of ascorbate with diethyl pyrocarbonate, an additional experiment was performed in which the ascorbate concentration in the co-substrate mixture was decreased to 1 mM (3.3 times K_m) and the diethyl pyrocarbonate concentration was increased to 2.5 mM. Only a minor decrease in the extent of protection (from 75% to 60%) was found under these conditions, compared with that found in the presence of 2 mM-ascorbate and 1.4 mM-diethyl pyrocarbonate. Fe^{2+} did not protect the enzyme alone, but a combination of Fe^{2+} with a 5 μM-pyridine-2,5-dicarboxylate, a competitive inhibitor with respect to 2-oxoglutarate [35], increased the enzyme activity from 10% (with no protecting compound or with pyridine-2,5-dicarboxylate alone) to 25%, and a combination of Fe^{2+} with ascorbate increased the enzyme activity from 10% (70% with ascorbate alone) to about 90%. The peptide substrate gave no protecting effect alone, and its omission from the complete reaction mixture gave no decrease in the magnitude of protection.

The data obtained with algal prolyl 4-hydroxylase were basically similar, but omission of ascorbate alone from the complete mixture had a less distinct effect on the level of protection, which decreased to about 70%, and correspondingly ascorbate alone gave only 40% protection of enzyme activity.

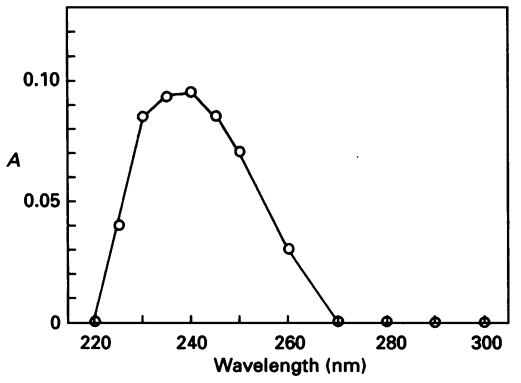

Fig. 3. Spectral analysis of chick prolyl 4-hydroxylase inactivated by diethyl pyrocarbonate.

A 70 μg sample of purified chick proly 4-hydroxylase was incubated with 1.4 mM-diethyl pyrocarbonate in 50 mM-phosphate buffer, pH 6.8, for 30 min on ice. The spectrum of prolyl 4-hydroxylase incubated without diethyl pyrocarbonate was subtracted. The spectrum was monitored from 300 to 220 nm.

The peptide substrate had again no protective effect. Lysyl hydroxylase also showed a similar pattern, but here omission of ascorbate alone decreased the level of protection only to about 80%, and addition of ascorbate alone gave no consistent protection.

DISCUSSION

Comparison of the cDNA-derived amino acid sequence between the α subunits of human [9] and chick [10] prolyl 4-hydroxylase and between human and chick lysyl hydroxylase [11,12] indicates that the *C*-terminal regions are the most conserved areas between species in both enzymes. For the α subunit of human and chick prolyl 4-hydroxylase, the overall identity is 87%, but for residues 375–517 (residue 517 is the *C*-terminus) it is 98%, and for human and chick lysyl hydroxylase, which have an overall identity of 78%, the identity is 97% for residues 620–708 (residue 708 is the *C*-terminus). It is therefore highly likely that the *C*-terminal regions of these proteins are important for their catalytic activity. In the present study, we have identified two conserved histidine motifs in these *C*-terminal regions which are shared with all the other microbial and plant 2-oxoglutarate-coupled dioxygenases and closely related enzymes for which primary sequences are known. The His-1 motif begins in human prolyl 4-hydroxylase at residue 411, and the His-2 motif ends at residue 502, the corresponding residues in human lysyl hydroxylase being 637 and 708.

The experiments with diethyl pyrocarbonate suggest the involvement of histidine residue(s) in the catalytic sites of vertebrate and algal prolyl 4-hydroxylase and vertebrate lysyl hydroxylase. All three enzymes were effectively inactivated by this compound, and in all cases the presence of the co-substrates protected the enzymes from inactivation. It is not known, however, if His-1 and His-2 motifs are found in the algal prolyl 4-hydroxylase, because the primary sequence is not yet available for this enzyme.

The precise role of the individual co-substrates in the protection against inactivation by diethyl pyrocarbonate is difficult to evaluate. In the mechanisms of the prolyl 4-hydroxylase and lysyl hydroxylase reactions, the individual components become bound to the enzyme in the order Fe^{2+}, 2-oxoglutarate, O_2 and the peptide substrate, in which the order of binding of O_2 and the peptide substrate is uncertain and in which Fe^{2+} is not released between most catalytic cycles [1–3]. In the absence of the peptide substrate, the enzymes catalyse an uncoupled decarboxylation of 2-oxoglutarate, ascorbate being needed as a highly specific reducing agent after such uncoupled cycles [36,37]. The binding sites on the enzymes for both 2-oxoglutarate (in the complete reaction) and ascorbate (after the uncoupled cycles) probably involve two *cis*-positioned equatorial co-ordination sites of the catalytic-site iron atom, but some of the amino acid side chains also contribute to these sites [35,38].

In the present experiments 2-oxoglutarate and ascorbate appeared to offer most of the protection observed against the inactivation by diethyl pyrocarbonate. The failure to observe any protection by Fe^{2+} alone is not surprising, as Fe^{2+} will not remain bound to the enzymes under non-turnover conditions [1–3]. The ability of ascorbate to protect even in the absence of any other compound may seem surprising, as ascorbate might not become bound to the enzymes in the absence of Fe^{2+}. Nevertheless, the purified enzyme preparations appear to contain trace amounts of Fe^{2+}, as a significant enzyme activity is often obtained even without added Fe^{2+} [3]. It is noteworthy that addition of Fe^{2+} potentiated the degree of protection obtained by ascorbate, and addition of Fe^{2+} together with a competitive inhibitor with respect to 2-oxoglutarate also offered a partial protection in the absence of ascorbate. The peptide substrate, which can bind to the enzymes even in the absence of the co-substrates, forming a dead-end complex [3], offered no protection, and its omission from the complete reaction mixture caused no decrease in protection. Thus the histidines influenced by diethyl pyrocarbonate are not likely to be involved in binding to the peptide within the active site.

The iron at the catalytic sites of 2-oxoglutarate-coupled dioxygenases is probably co-ordinated with the enzymes by three side chains [19]. Spectroscopic analyses of isopenicillin N synthase suggest the presence of three histidine ligands [39]. The present data would be consistent with the involvement of the His-1 and His-2 motifs in the Fe^{2+}-binding sites of the enzymes, but other roles for these histidines, such as direct contribution to the 2-oxoglutarate- or ascorbate-binding sites, are not excluded.

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