The catalytic mechanism of the hydroxylation reaction of peptidyl proline and lysine does not require protein disulphide-isomerase activity

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Prolyl 4-hydroxylase, an $\alpha_2\beta_2$ tetramer, catalyses the formation of 4-hydroxyproline in collagens. The β subunit is known to be identical with the enzyme protein disulphide-isomerase and to possess disulphide-isomerase activity even when present in the prolyl 4-hydroxylase tetramer. We here report that lysyl hydroxylase, a homodimer, and algal prolyl 4-hydroxylase, a monomer, do not contain detectable protein disulphide-isomerase activity. Since the hydroxylase reaction mechanisms are similar, the data suggest that the protein disulphide-isomerase activity of the vertebrate prolyl 4-hydroxylase β subunit is unlikely to be involved in the catalytic mechanism of the hydroxylation reaction.

INTRODUCTION

Prolyl 4-hydroxylase (EC 1.14.11.2) catalyses the formation of 4-hydroxyproline in collagens and collagen-like proteins by the hydroxylation of certain peptidyl proline residues [1–3]. The reaction requires the peptide substrate, Fe^{2+} ion, 2-oxoglutarate, molecular oxygen and ascorbate and follows an ordered Ter Ter mechanism [4,5]. The enzyme is a tetramer ($\alpha_2\beta_2$) consisting of two different types of inactive monomer with M_r values of 64000 (α subunit) and 60000 (β subunit) [1–3]. Recently the β subunit has been found to be identical with the enzyme protein disulphide-isomerase (EC 5.3.4.1) and to have protein disulphide-isomerase activity both when present in the prolyl 4-hydroxylase tetramer and as an isolated subunit [6,7]. Protein disulphide-isomerase catalyses the re-arrangement of disulphide bonds in various proteins *in vitro*, and is now regarded as the catalyst *in vivo* for disulphide-bond formation in the biosynthesis of various secretory proteins, including procollagens [8–11]. In addition, it is known that protein disulphide-isomerase retains prolyl 4-hydroxylase structure in its native conformation *in vitro* [12]. It is not known, however, whether protein disulphide-isomerase activity has any role in the hydroxylation reaction. To elucidate this aspect, we studied whether two other hydroxylases with similar reaction mechanisms express any protein disulphide-isomerase activity. One of these is lysyl hydroxylase, which is very similar to prolyl 4-hydroxylase in its catalytic properties [1–3,13–17] but differs in substrate specificity and structure, consisting of only one type of monomer [1–3,18,19]. The other enzyme is prolyl 4-hydroxylase from a green alga, which hydroxylates proline residues in proteins incorporated into the cell wall. The algal enzyme requires the same co-substrates, but differs from the vertebrate prolyl 4-hydroxylase in being a monomer antigenically related to the α subunit of the vertebrate enzyme [20,21].

EXPERIMENTAL

Materials

RNA and RNAase (type XII-A) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Urea (ultrapure grade) was purchased from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.). Randomly cross-linked RNAase was prepared as described previously [22].

Isolation of enzymes

Lysyl hydroxylase from 15-day whole chick embryos [19] and prolyl 4-hydroxylase from the green alga *Chlamydomonas reinhardtii* [20,21] were purified by procedures described in the references cited. Vertebrate prolyl 4-hydroxylase was isolated from chick embryos [23].

Algal protein disulphide-isomerase was isolated from a wall-less mutant strain (CW15) of *Chlamydomonas reinhardtii*. The cells were lysed in a buffer containing 0.1 M-sodium phosphate, pH 7.5, 0.1 M-NaCl, 5 mM-EDTA and 0.1% (v/v) Triton X-100, and the supernatant was collected after centrifugation (15000 g, 20 min). Partial purification was achieved by precipitation with $(\text{NH}_4)_2\text{SO}_4$ (85% satn.), followed by gel-filtration (Bio-Gel A1.5M) and ion-exchange (DEAE-Sephacel) chromatographies, with an elution gradient of 0.1–0.7 M-NaCl.

Assays

Protein disulphide-isomerase activity was assayed by a method involving measurement of the rate of regeneration of incorrectly disulphide-linked RNAase to the native active form. The extent of RNAase re-activation was determined by measuring the degradation of RNA, by monitoring the change in A_{260} relative to A_{280} on a spectrophotometer in the dual-wavelength mode [24]. The incubations were carried out in a final volume of

Fig. 1. Spectrophotometric assay of protein disulphide-isomerase activity of purified lysyl hydroxylase (a) and purified algal prolyl 4-hydroxylase from *Chlamydomonas reinhardtii* (b)

Lysyl hydroxylase (2.1 m-units) and prolyl 4-hydroxylase (12.3 m-units) from *C. reinhardtii* were assayed for protein disulphide-isomerase activity as described in the Experimental section, and the change in absorbance was measured spectrophotometrically for 3 min. Trace 1 shows the absorbance change obtained in the absence of added enzyme, trace 2 that obtained with 0.8 m-unit of purified prolyl 4-hydroxylase from chick embryos, and trace 3 that obtained with lysyl hydroxylase and algal prolyl 4-hydroxylase, respectively.

100 μ l containing 10 μ g of randomly cross-linked RNA, 2 μ M-dithiothreitol, 1 mM-EDTA and 50 mM-Tris/HCl, pH 7.5 (30 $^{\circ}$ C). The enzyme reaction was run for 15 min. The re-activation of randomly cross-linked RNA was linear for at least 15 min with the enzyme concentrations used here.

The activities of prolyl 4-hydroxylase from *Chlamydomonas reinhardtii* [20,21] and from chick embryos [23], and of lysyl hydroxylase from chick embryos [23], were assayed by measuring the decarboxylation of 2-oxo[1- ^{14} C]glutarate as described in the references cited. One unit of enzyme activity is defined as the amount of enzyme required to synthesize 1 μ mol of hydroxyproline (hydroxyllysine)/min under the conditions described previously [20,23].

The protein content of the enzyme preparations was measured by peptide absorbance at 230 nm, with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Lysyl hydroxylase and algal prolyl 4-hydroxylase did not contain any detectable protein disulphide-isomerase activity, in contrast with the finding concerning prolyl 4-hydroxylase from vertebrate sources (Fig. 1). When equivalent units or even more of lysyl hydroxylase or algal prolyl 4-hydroxylase were assayed, no detectable protein disulphide-isomerase activity could be measured. The absence of protein disulphide-isomerase activity in these two enzymes suggests that this activity is unlikely to be involved in the catalytic mechanism of the hydroxylation reaction. The alga *Chlamydomonas reinhardtii* does contain protein disulphide-isomerase, but this enzyme does not co-purify with the algal prolyl 4-hydroxylase. The algal protein with protein disulphide-isomerase activity corresponds in gel-filtration chromatography to an apparent M_r of about 130 000, whereas prolyl 4-hydroxylase has an apparent M_r of 40 000–50 000 (Fig. 2). Protein disulphide-isomerase has previously been detected in higher plants [25,26]; however, this is the first report that the enzyme is present in algae.

Binding studies with suicide inactivators and other compounds [3,15,16,27,28] indicate that the α subunit

Fig. 2. Protein disulphide-isomerase activity from the green alga *Chlamydomonas reinhardtii*, after gel-filtration chromatography

A sample (5 ml) of 15000 g supernatant, containing 50 mg of protein, was applied to a column of Bio-Gel A 1.5 m. Fractions were collected and analysed for protein (○) and protein disulphide-isomerase (PDI) activity (●). Arrow indicates elution of algal prolyl 4-hydroxylase. The column was calibrated with the following standards: Blue Dextran (BD); bovine serum albumin (BSA); ovalbumin (OV); cytochrome c (CyC).

probably contributes to most parts of the large catalytic site of the vertebrate prolyl 4-hydroxylase. The ascorbate-binding site, however, may be co-operatively built up of both the α and β subunits [16]. The catalytic sites of protein disulphide-isomerase have been proposed to be the two -Cys-Gly-His-Cys- sequences present in the protein [9,29]. As shown in the present study, this catalytic activity is not required for the hydroxylation reaction. It is possible, however, that some amino acid sequences corresponding to those in the β subunit are required for hydroxylation. This suggestion is supported by the finding that the algal prolyl 4-hydroxylase monomer appears to contain some sequences similar to those of the β subunit, because its activity is partially inhibited by a monoclonal antibody to the β subunit of human prolyl 4-hydroxylase [20]. It will be necessary to determine the

nucleic acid and amino acid sequences of lysyl hydroxylase and the algal prolyl 4-hydroxylase in order to identify regions present in these enzymes which show sequence identity with protein disulphide-isomerase.

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