Protein Disulfide-Isomerase Retains Procollagen Prolyl 4-Hydroxylase Structure in Its Native Conformation†

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ABSTRACT: Protein disulfide-isomerase was isolated as a homogeneous protein from 15-day-old chick embryos. The enzyme has a molecular weight of 56 000 in SDS–polyacrylamide gel electrophoresis. Its K_m value for randomly cross-linked ribonuclease, a protein used as a substrate for the enzyme, was 0.3 μM, and the K_m value for DTT was 1.0 μM. Its optimum pH was 7.5 and its optimum temperature, 33 °C. The maximal velocity of pure protein disulfide-isomerase from chick embryos under optimal conditions was about 29 000 units/g. Protein disulfide-isomerase was able to activate purified prolyl 4-hydroxylase 2- to 3-fold, the activation being higher for enzyme stored for a longer time. This activation is probably due to the repairing of disulfide exchanges occurring in the prolyl 4-hydroxylase structure during purification and storage. Prolyl 4-hydroxylase activity was very stable in microsomes, however, and protein disulfide-isomerase was unable to increase the microsomal prolyl 4-hydroxylase activity, suggesting that prolyl 4-hydroxylase retains its native conformation in microsomes. Protein disulfide-isomerase was able to reactivate prolyl 4-hydroxylase inactivated by mild H_2O_2 treatment. The activity obtained after this treatment and protein disulfide-isomerase incubation corresponded to the amount of prolyl 4-hydroxylase tetramer found after H_2O_2 treatment. The data suggest that protein disulfide-isomerase is able to activate only the tetramer part of the enzyme preparation. Similar findings were obtained after the dissociation of prolyl 4-hydroxylase by DTT. It thus seems probable that protein disulfide-isomerase may be an enzyme that is able to retain the prolyl 4-hydroxylase structure in its native conformation, in which it has maximum enzyme activity, but it seems to lack the ability to associate the enzyme monomers into the active tetrameric form.

Disulfide bonds are found in most extracellular and many intracellular proteins. In small proteins such as ribonuclease, the primary structure is sufficient to define the tertiary structure, so that the disulfide bonds carry no structural information (Epstein et al., 1963; Anfinsen, 1972), but in some proteins such as insulin and collagen, the folding of the molecule into its native structure is very slow without a disulfide-bonded precursor, implying that at least in some cases appropriate disulfide bond formation is essential either to generate or to maintain the biologically active conformation (Freedman et al., 1984).

The formation of disulfide bonds in vitro in proteins occurs via a series of thiol–disulfide interchange reactions (Wetlaufer & Ristow, 1973). Oxidation of reduced ribonuclease, for instance, occurs as a two-step process: first, there is a rapid loss of free thiol groups, indicating the formation of randomly cross-linked disulfide bonds; second, there is a slower appearance of ribonuclease activity due to isomerization of the randomly cross-linked, nonnative disulfides to native ones [for reviews, see Freedman and Hawkins (1977) and Freedman and Hillson (1980)]. The isomerization of disulfides is rapid at pH 8–9 but is too slow at more physiological conditions to be the process occurring in vivo. Protein disulfide-isomerase (EC 5.3.4.1), an enzyme catalyzing isomerization reactions, is a thiol dependent that is able to catalyze thiol–disulfide interchange in many proteins. It has been isolated from bovine, mouse, and rat liver and from mouse spleen (Freedman et al., 1984). It has been shown to be a homodimer consisting of subunits of a molecular weight of about 55 000 by SDS–polyacrylamide gel electrophoresis[1] and is widely distributed in animal tissues, being bound to the microsomal membrane (Mills et al., 1983; Lambert & Freedman, 1985).

There are also findings referring to the association of this enzyme with collagen biosynthesis. Protein disulfide-isomerase activity has been measured in various cells that synthesize collagen and has been found to correlate with the rate of collagen synthesis in these cells (Brockway et al., 1980; Myllylä et al., 1983). Furthermore, it has been shown that protein disulfide-isomerase accelerates the arrangement of the polypeptide chains of type I procollagen in the trimeric conformation (Forster & Freedman, 1984).

Prolyl 4-hydroxylase is an enzyme catalyzing 4-hydroxyproline formation as a posttranslational event in collagen biosynthesis (Kivirikko & Myllylä, 1980, 1984) and is located within the cisternae of the rough endoplasmic reticulum, being either free within the cisternae or loosely bound to the inner membrane. It is a tetramer consisting of two types of enzymatically inactive monomer with molecular weights of about 64 000 and 60 000, respectively. Intrachain disulfide bonds seem to be essential for the monomers to maintain the native structure necessary for their association into the tetrameric form, whereas the presence of interchain disulfide bonds does not seem likely [see Kivirikko & Myllylä (1980)].

Protein disulfide-isomerase has a wide specificity for protein disulfide substrates and has been proposed as the enzyme catalyzing disulfide bond formation in vivo [see Freedman et al. (1984)]. There is still uncertainty about its physiological role, however. Protein disulfide-isomerase and prolyl 4-hydroxylase are located in the same compartment of the cell, and therefore it is of interest to study whether the former has any effect on the formation of the native structure of this

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[1] Abbreviations: DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PDI, protein disulfide-isomerase; H_2O_2, hydrogen peroxide; EDTA, ethylenediaminetetraacetic acid.

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enzyme or on the association of inactive monomers into the active tetrameric form. In order to be sure that the effect is caused specifically by protein disulfide-isomerase, the purified enzyme is utilized in the experiments reported here. The particular aim of this work was to study whether protein disulfide-isomerase is able to reassociate the dissociated prolyl 4-hydroxylase into the native enzyme, in order to identify one possible biological function for protein disulfide-isomerase in vivo.

Experimental Procedures

Materials. Ribonucleic acid and ribonuclease (type XII-A) were obtained from Sigma Chemical Co. (St. Louis, MO), and randomly cross-linked ribonuclease was prepared as earlier (Myllylä & Oikarinen, 1983). Urea of ultrapure grade was purchased from Bethesda Research Laboratories (New Isenburg, FRG), all urea solutions being freshly prepared and deionized.

[^{14}C]Proline was obtained from Amersham International (Amersham, Bucks, U.K.). Fertilized eggs of White Leghorn chickens were purchased from Siipikarjanhoitajien liittro r.y. (Hämeenlinna, Finland).

The protocollagen substrate (unhydroxylated procollagen) was prepared biosynthetically in freshly isolated tendon cells of 16-day-old chick embryos as described in detail elsewhere [see Kivirikko and Myllylä (1982)].

Enzyme Assays. Protein Disulfide-Isomerase. The activity of protein disulfide-isomerase was assayed by a method including measurement of the rate of regeneration of incorrectly disulfide-linked ribonuclease to the native, active form. The extent of ribonuclease reactivation was determined by estimating the degradation of ribonucleic acid, monitoring the change in A_{260} relative to A_{280} on a spectrophotometer in dual-wavelength mode (Ibbetson & Freedman, 1976). The incubations under standard conditions were carried out in a final volume of 100 μL containing 10 μg of randomly cross-linked ribonuclease, 2 μM DTT, 1 mM EDTA, and 50 mM Tris-HCl, pH 7.5 (30 °C). The enzyme reaction was run for 15 min at 30 °C. The reactivation of randomly cross-linked ribonuclease was linear for at least 15 min with the enzyme concentrations used here.

Prolyl 4-Hydroxylase. Prolyl 4-hydroxyiase activity was assayed by measuring the formation of radioactive 4-hydroxyproline in the [^{14}C]proline-labeled protocollagen substrate [see Kivirikko and Myllylä (1982)].

Purification of Enzymes. Protein Disulfide-Isomerase. The enzyme was purified by a procedure based on that described earlier (Carmichael et al., 1977; Lambert & Freedman, 1983), but with many modifications. All procedures were carried out at 0–4 °C, and enzyme samples were stored at 0–4 °C without freezing between the various purification steps. All centrifugations were carried out at 15000*g* at 4 °C.

A total of 150–200 15-day-old chick embryos was homogenized in a solution of 0.1 M sodium phosphate buffer pH 7.5, 0.1 M NaCl, 5 mM EDTA, and 1% (v/v) Triton X-100 using a Waring Blendor at full speed, twice for 30 s with a 1-min interval (1 mL of solution/g of embryo). The homogenate was allowed to stand with occasional stirring for about 1 h and was then centrifuged for 30 min.

Ammonium sulfate fractionation was carried out by slowly stirring solid ammonium sulfate into the supernatant to a final concentration of 55% saturation. After the centrifugation, further ammonium sulfate was added to the supernatant of the previous fractionation to give a final saturation of 85%. The supernatant was discarded, and the pellet was suspended in 250 mL of 25 mM sodium citrate buffer, pH 5.2, 0.1 M NaCl, and 1 mM EDTA (buffer A) and then dialyzed overnight against the same buffer (2 × 20 L). The dialyzed enzyme fraction was applied to a column of CM-Sephadex C-50 (5 cm × 40 cm) previously equilibrated with buffer A and eluted with the same buffer. The unbound material was collected, diluted 1:2.5 with H_2O, and trisodium citrate, NaCl, and EDTA were added to give buffer B (25 mM sodium citrate pH 6.2, 0.1 M NaCl and 1 mM EDTA).

The unbound, diluted material was applied to a DEAE-Sephacel column (2.5 cm × 15 cm) equilibrated with buffer B, and the bound material was eluted with a linear gradient of 0.1–0.6 M NaCl in the same buffer. Fractions of 8 mL were collected, and those containing most of the enzyme activity were pooled and concentrated by ultrafiltration in an Amicon cell with a PM-10 membrane to a final volume about 2 mL.

The concentrated enzyme was applied to a column of Sephacryl S-300 superfine (1.5 cm × 95 cm) equilibrated with buffer B and eluted with the same buffer at a flow rate of 2–3 mL/h. Fractions of 2 mL were collected, and those having the highest specific activities were pooled and concentrated. This constituted the purified protein disulfide-isomerase.

Procollagen Prolyl 4-Hydroxylase. Prolyl 4-hydroxylase was purified from 15-day-old chick embryos by a purification procedure consisting of an affinity chromatography on poly(L-proline), DEAE-cellulose chromatography, and gel filtration (Tuderman et al., 1975; Kedersha & Berg, 1981). The enzyme was pure as judged by SDS–polyacrylamide gel electrophoresis.

Other Procedures. Preparation of Microsomes. The microsomes were isolated from whole-chick embryos by differential centrifugation in 0.01 M Tris-HCl, pH 7.4 (4 °C), buffer containing 0.25 M sucrose. Twenty 15-day-old chick embryos were first briefly homogenized in a Waring Blendor homogenizer at half-speed (1 mL of solution/1 g of embryo), twice for 5 s with a 1-min interval. The homogenate was then centrifuged at 1500 rpm for 15 min in order to remove insoluble material. The crude homogenate was further homogenized with a Teflon glass homogenizer (1200 rpm, 10 strokes). After centrifugation at 17000*g* for 20 min, the supernatant was centrifuged at 105000*g* for 2 h. The pellet was suspended into the Tris–sucrose buffer and recentrifuged as before. The pellet of microsomes was suspended into 4 mL of the buffer and stored at −20 °C for up to 10 months.

V8 Proteinase Mapping. V8 proteinase peptide mapping of protein bands from SDS–polyacrylamide slab gels was carried out by the method of Cleveland et al. (1977).

SDS Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis under reduced conditions was performed with 10% acrylamide as the separating gel (Weber & Osborn, 1975). The gels were stained with Coomassie brilliant blue or silver.

Protein Determination. The protein content of each fraction was measured throughout the purification procedures by peptide absorbance at 225 nm with bovine serum albumin as a standard or by the absorbance of aromatic amino acids at 280 nm.

Results

Isolation of Protein Disulfide-Isomerase from Chick Embryos. Protein disulfide-isomerase was purified from 15-day-old whole chick embryos by the purification procedure, which consists of two ammonium sulfate fractionations, cation-exchange chromatography, anion-exchange chromatography, and gel filtration. The enzyme was not bound to the CM-Sephadex C-50 cation exchanger under the conditions used but was bound to the DEAE-Sephacel anion exchanger at pH 6.2 and was eluted with 0.2 M NaCl (see Figure 1).

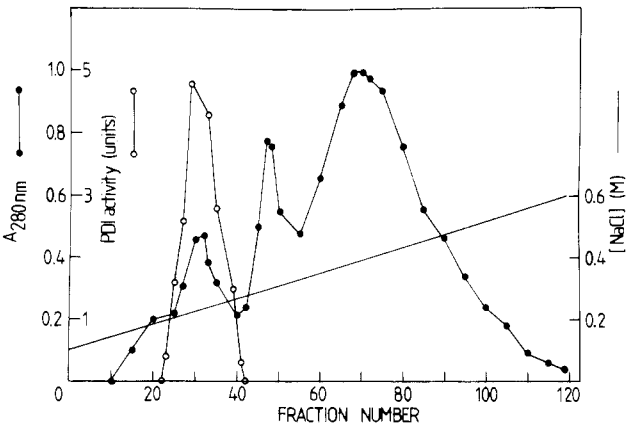

FIGURE 1: Elution of protein disulfide-isomerase from the DEAE-Sephacel C-50 cation-exchange column. Conditions as described under Experimental Procedures. Fractions 22–40 were pooled and constituted the pool run in gel filtration.

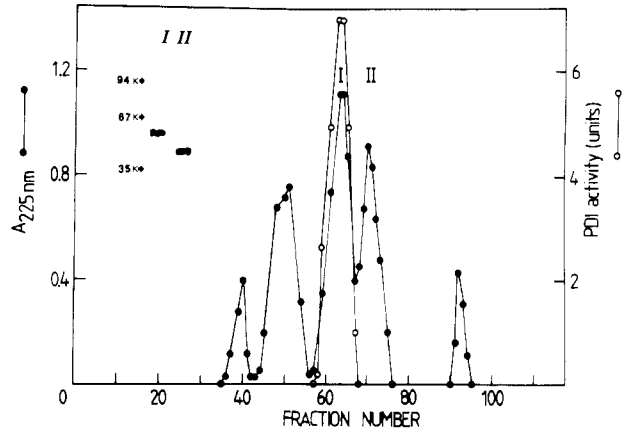

FIGURE 2: Separation of protein disulfide-isomerase by gel filtration. The pool obtained from DEAE-Sephacel was run on Sephacryl S-300. The chromatography conditions are described under Experimental Procedures. Fractions 60–65 constituted the purified enzyme. I corresponded to a molecular weight of 56 000 and II to 45 000 in SDS–polyacrylamide gel electrophoresis (see inset of figure).

The eluted enzyme pool was separated into five protein peaks in gel filtration, only one of which gave any disulfide-isomerase activity (Figure 2). The pooled enzyme fraction (peak I, Figure 2) contained only one protein band in SDS–polyacrylamide gel electrophoresis, corresponding to a molecular weight of 56 000. In some experiments the gel filtration was run with Bio-Gel A1.5m, in which the protein peak having the enzyme activity consisted of two proteins with molecular weights of 56 000 and 45 000, respectively (peaks I and II, Figure 2).

Starting with 150–200 chick embryos, the homogenate contained a total of about 300 g of soluble protein. The amount of soluble protein in the pooled enzyme fraction was 1.5 g after cation-exchange chromatography and 23 mg after the DEAE-Sephacel column. The purification procedure yielded 2 mg of homogeneous protein disulfide-isomerase, purified about 2500-fold in terms of the ratio of its specific activity to that of the homogenate. The real purification ratio is in fact much higher still, as it is found that free thiol and disulfide groups reactivate a randomly cross-linked ribonuclease, a compound used as a substrate for protein disulfide-isomerase, resulting in the high nonspecific enzyme activity observed in crude homogenate [see Freedman et al. (1984)].

Optimization of the Reaction Conditions for Protein Disulfide-Isomerase. Protein disulfide-isomerase activates randomly cross-linked ribonuclease by isomerizing the incorrect disulfides to correct ones. The presence of a thiol group is required for this reaction. Many thiols and thiol/disulfide systems are suitable for this purpose, the most potent one being dithiothreitol (Freedman & Hillson, 1980; Hillson & Freedman, 1980). Hillson and Freedman (1980) report K_m values of 5.4 and 1.7 μM for dithiothreitol and randomly cross-linked ribonuclease, respectively, using 10 μM dithiothreitol and 3.6 μM randomly cross-linked ribonuclease under standard conditions. These assay conditions seemed not to be the optimal ones in our experiments, however, since the reduction of dithiothreitol concentration resulted in an increase of protein disulfide-isomerase activity. It was therefore necessary to optimize the reaction conditions again, whereupon a K_m value of 0.3 μM was obtained for the randomly cross-linked ribonuclease and 1.0 μM for dithiothreitol. The optimum pH was 7.5, and the optimum temperature 33 °C (Table I). The maximal velocity (V_m) of pure protein disulfide-isomerase obtained under optimal conditions was about 29 000 U/g.

Effect of Protein Disulfide-Isomerase on the Activity of Prolyl 4-Hydroxylase. Activation of Purified Prolyl 4-Hydroxylase by Protein Disulfide-Isomerase (Table II). Procollagen prolyl 4-hydroxylase is a labile enzyme that is easily inactivated, especially when stored in dilute solutions [see Kivirikko and Myllylä (1982)]. The exact mechanism of this inactivation is not known, and it thus seemed to be of interest to study whether rearrangements of disulfide bridges are involved. For this purpose, batches of freshly prepared and stored prolyl 4-hydroxylase were incubated with protein

Table I: Effect of pH and Temperature on the Activity of Protein Disulfide-Isomerase[a]

pH	protein disulfide-isomerase act. units/g	protein disulfide-isomerase act. %[c]	temp[b] (°C)	protein disulfide-isomerase act. units/g	protein disulfide-isomerase act. %[c]
6.5	83	2.7	20	1272	43.0
7.0	1133	36.6	25	1937	65.5
7.25	2034	65.8	30	2683	90.7
7.5	3092	100	33	2959	100
7.75	2875	93.0	37	2470	83.5
8.0	2442	79.0	40	1661	56.1
8.5	2884	93.3	45	622	21.0

[a] The activity found without the enzyme at each pH and temperature was subtracted as representing the background activity. The values shown are means of two measurements in the pH experiments and four measurements in the temperature experiments. [b] The pH of the assay buffer was adjusted to 7.5 at the temperature studied. [c] The highest activity was taken at 100.

Table II: Activation of Purified Prolyl 4-Hydroxylase by Protein Disulfide-Isomerase

prepn of prolyl 4-hydroxylase	amt of prolyl 4-hydroxylase (ng)	prolyl 4-hydroxylase act. (dpm) without PDI	prolyl 4-hydroxylase act. (dpm) after PDI[a]	activation (x-fold)
stored[b]	35	1408	4158	3.0
	50	2285	7428	3.3
freshly prepared	35	3395	6321	1.9
	50	5012	9569	1.9
freshly prepared and incubated for 24 h at 4 °C[c]	50	2198	6174	2.8

[a] The amount of protein disulfide-isomerase corresponded to 2 ng of the pure enzyme protein. [b] Stored at −20 °C for up to 1 year. [c] Prolyl 4-hydroxylase was incubated for 24 h at 4 °C at a concentration of 1.4 μg/mL before incubation with or without PDI for 20 min at 37 °C. Prolyl 4-hydroxylase activity was then measured as described under Experimental Procedures.

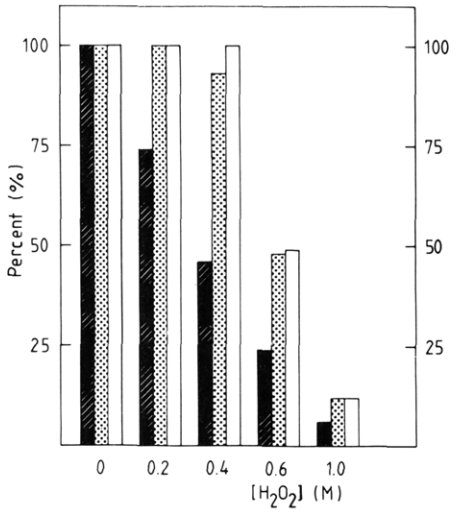

FIGURE 3: Effect of protein disulfide-isomerase on intact and H_2O_2-treated microsomes containing prolyl 4-hydroxylase activity. Microsomes were incubated in the presence of H_2O_2 for 30 min at 0 °C (total volume 15 μL). An 85-μL portion of 50 mM Tris-HCl buffer, pH 7.5, at 37 °C was then added, and H_2O_2 was removed on a bed of Sephadex G-25 (Penefsky, 1977). The material eluted was diluted 1:45 with the above Tris-HCl buffer, and 15–50 μL of the solution was incubated with or without disulfide-isomerase in the presence of 4 μM DTT for 20 min at 37 °C and prolyl 4-hydroxylase activity measured. Cross-hatched bar, prolyl 4-hydroxylase activity without PDI, taking the activity without H_2O_2 as 100; dotted bar, prolyl 4-hydroxylase activity after incubation with PDI; open bar, percentage of prolyl 4-hydroxylase tetramers after H_2O_2 treatment. Values are based on data given by Nietfeld et al. (1981).

disulfide-isomerase in parallel experiments in order to see whether the latter was able to increase the enzyme activity and whether there was any difference in the increase between these two prolyl 4-hydroxylase preparations. Protein disulfide-isomerase enhanced the freshly prepared, pure prolyl 4-hydroxylase activity about 1.9-fold. The same prolyl 4-hydroxylase preparation was also incubated at a dilute concentration for 24 h at 4 °C to give a preparation possessing about 40% of the activity of the freshly prepared prolyl 4-hydroxylase. When this inactivated preparation was treated with disulfide-isomerase, a 2.8-fold activation was measured. Even higher activation ratios were observed when prolyl 4-hydroxylase preparations stored for about 1 year were incubated with disulfide-isomerase (Table II).

Effect of Protein Disulfide-Isomerase on Microsomal Prolyl 4-Hydroxylase. Prolyl 4-hydroxylase was found to be stable in microsomes, and only a very slight inactivation (less than 10%) was seen after storage in a dilute solution for up to 10 months at −20 °C. Protein disulfide-isomerase was unable to increase the microsomal prolyl 4-hydroxylase activity (Figure 3), in contrast to the finding for purified prolyl 4-hydroxylase.

Treatment of the microsomes with H_2O_2 reduced the prolyl 4-hydroxylase activity, 1 M H_2O_2 giving 6%, 0.6 M H_2O_2 24%, 0.4 M H_2O_2 48%, and 0.2 M H_2O_2 74% of the initial prolyl 4-hydroxylase activity. On the basis of data published by Nietfeld et al. (1981), it can be calculated that after these kinds of inactivations the prolyl 4-hydroxylase is dissociated so that 12%, 50%, 100%, and 100% of the enzyme is in tetrameric form, respectively (Figure 3). Protein disulfide-isomerase was unable to reactivate the prolyl 4-hydroxylase after total inactivation, but a clear reactivation was seen when milder H_2O_2 treatment was used. Prolyl 4-hydroxylase activity after activation by protein disulfide-isomerase, calculated as a percentage of the starting activity, corresponds very well to the percentage of the tetramer form present in enzyme preparation after H_2O_2 treatment (Figure 3).

Dithiothreitol reduced microsomal prolyl 4-hydroxylase activity, 0.45 mM DTT causing total inactivation of the enzyme activity. It was not possible to increase the enzyme activity by incubation with protein disulfide-isomerase after total or partial inactivation of prolyl 4-hydroxylase activity (not shown). Similar results were obtained with pure chicken prolyl 4-hydroxylase.

Discussion

Protein disulfide-isomerase was isolated from chick embryos as a homogeneous protein having a molecular weight of 56 000 in SDS–polyacrylamide gel electrophoresis and in this way resembling the enzyme isolated from other sources [see Freedman et al. (1984)]. The use of Bio-Gel A0.5m instead of Sephacryl S-300 as the gel filtration step gave an additional protein band of molecular weight about 45 000. When the purified enzyme was stored at −20 °C for several months, a protein band of molecular weight 52 000 was found to appear. V8-proteinase digestion of this band and one with a molecular weight of 45 000 gave peptide maps very similar to that of protein disulfide isomerase (not shown), suggesting that they may both be degradation products of the enzyme. A similar component with smaller molecular weight, probably a degradation product of the enzyme, was observed in the enzyme preparation from chick embryo tendon (Brockway & Freedman, 1984).

The K_m value for the reoxidized ribonuclease used as a substrate in the assay of protein disulfide isomerase was about 5 times smaller than that published by others (Lambert & Freedman, 1983; Brockway & Freedman, 1984), as also was the K_m value for dithiothreitol. The substrate for the enzymic reaction was prepared by denaturing ribonuclease after reduction in 8 M urea solution and allowing it to reoxidize randomly at room temperature (Anfinsen & Haber, 1961). An ultrapure-grade urea solution produced a very sensitive substrate for protein disulfide-isomerase, whereas urea of analytical grade gave poor activation of the ribonuclease. In addition, the sensitivity of the substrate varied in a narrow range from one preparation to another. The conditions for protein disulfide-isomerase were therefore optimized separately for every substrate preparation. On the basis of these findings it seems probable that the differences in K_m values may be mainly due to the different reoxidized ribonucleases used. Furthermore, the low specific activity reported for pure protein disulfide-isomerase from rat liver (Lambert & Freedman, 1983) may be due to the use of a less sensitive ribonuclease rather than low catalytic activity of the liver enzyme.

It was found here that various disulfides and thiols are able to reactivate a reoxidized ribonuclease to an active form. Consequently the protein disulfide-isomerase activity measured in crude enzyme preparations is artificially high, and only preparations that are already highly purified give correct activity values. The purification of the enzyme achieved in this study is thus probably much higher than that indicated.

Protein disulfide-isomerase activated purified prolyl 4-hydroxylase. Similarly, it activated purified lysyl hydroxylase, another intracellular enzyme of collagen biosynthesis (not shown). The activation is apparently due to enzymic character of protein disulfide-isomerase, since as little as 20 ng/mL of enzyme protein is enough to cause this effect. The activation ratio varied between enzyme preparations for both hydroxylases, being higher in enzymes stored for a long time. Prolyl 4-hydroxylase and lysyl hydroxylase are easily inactivated in vitro (Kivirikko & Myllylä, 1982), and although the mechanism of inactivation is not known, the fact that protein disulfide-isomerase is able to reactivate them indicates that some

kind of disulfide exchange must occur.

The data obtained from H_2O_2 studies suggest that protein disulfide-isomerase is unable to reassociate the dissociated prolyl 4-hydroxylase to the active form but is able to activate the tetrameric part of the enzyme. The activation is probably due to rearrangement reactions in which the incorrect disulfide bonds formed by H_2O_2 treatment are replaced by native disulfide pairings, or in which some of the disulfide bonds are reduced to free thiol groups that may be essential to prolyl 4-hydroxylase activity. Total inactivation of prolyl 4-hydroxylase was observed when the enzyme was incubated with 0.45 mM dithiothreitol, and no reactivation could be brought about with protein disulfide-isomerase. It had been shown (Tuderman et al., 1977) that incubation of prolyl 4-hydroxylase with 0.45 mM dithiothreitol at 37 °C for 30 min dissociates the enzyme almost totally into its subunits in the cell but that after a subsequent 5-h incubation of the cells without dithiothreitol about half of the enzyme protein is again in the form of the active tetramer. The inability of protein disulfide-isomerase to accelerate this reassociation and the fact that it is unable to catalyze reassociation of the dissociated enzyme to the active form after H_2O_2 treatment suggest that its function in vivo cannot be to assemble the subunits into tetramers. Protein disulfide-isomerase is able to activate prolyl 4-hydroxylase only after the association of monomeric subunits randomly with each other, as happened after mild H_2O_2 treatment (Nietfeld et al., 1981). This suggests that at least in the case of prolyl 4-hydroxylase protein disulfide-isomerase only catalyzes the isomerization of disulfide bonds.

Prolyl 4-hydroxylase has been located within the cisternae of the rough endoplasmic reticulum, being either free within the cisternae or loosely bound to the inner membrane [see Kivirikko & Myllylä (1980, 1984)]. The fact that the enzyme activity found in microsomes was surprisingly stable and could not be increased by protein disulfide-isomerase suggests that prolyl 4-hydroxylase retains its native conformation in this case and no disulfide exchanges occur during storage. This is probably due to the presence of protein disulfide-isomerase in the microsomal membrane and due to association of prolyl 4-hydroxylase with the membrane of rough endoplasmic reticulum, which keeps it in the active conformation and prevents disulfide exchanges.

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