A Single Polypeptide Acts Both as the β Subunit of Prolyl 4-Hydroxylase and as a Protein Disulfide-Isomerase*

(Received for publication, January 21, 1987)

Juha Koivut, Raiji Myllylä, Tarja Helaakoski,
Taina Pihlajaniemi, Kaisa Tasanen, and
Kari I. Kivirikko

From the Collegen Research Unit, University of Oulu,
Department of Medical Biochemistry, SF-90220
Oulu, Finland

A single polypeptide is shown to act both as the β subunit of the proline hydroxylase (EC 1.14.11.2) and as a protein disulfide-isomerase (EC 5.3.4.1). When isolated from chick embryos or rat liver, the β subunit of prolyl 4-hydroxylase and the enzyme protein disulfide-isomerase have identical molecular weights and peptide maps as produced by digestion with Staphylococcus aureus V8 protease. The apparent molecular weights of both proteins isolated from human placental tissue are slightly higher, and the human β subunit and one of its peptides have molecular weights about 500 higher than the protein disulfide-isomerase and its corresponding peptide. Experiments with polyclonal and monoclonal antibodies also suggest a structural identity between the two proteins. The β subunit isolated from the prolyl 4-hydroxylase tetramer has protein disulfide-isomerase activity similar to protein disulfide-isomerase itself, and even the β subunit when present in the prolyl 4-hydroxylase tetramer has one-half of this activity.

Proline hydroxylase (EC 1.14.11.2) catalyzes the formation of 4-hydroxyproline in collagens by the hydroxylation of certain proline residues in peptide linkages. The active enzyme is a tetramer (αβαβ) consisting of two types of monomer with molecular weights of about 64,000 (α subunit) and 60,000 (β subunit). These monomers do not have prolyl 4-hydroxylase activity (for review, see Refs. 1-4). The enzyme protein is found in cells not only as the active tetramer but also in a monomeric form that corresponds to the β subunit in a number of molecular properties (1-7). This monomer has been shown to act as a precursor of the β subunit in the assembly of the enzyme tetramer (see Ref. 4). It has not been known, however, whether this monomeric protein also has some additional functions, since it is present in many cells in large excess over the active tetramers (1-7).

Protein disulfide-isomerase (EC 5.3.4.1) catalyzes the rearrangement of disulfide bonds in various proteins in vitro (for reviews, see Refs. 8-10), including prolyl 4-hydroxylase (11) and type I and II procollagens (12). Protein disulfide-isomerase, with a reported molecular weight of about 57,000, has been suggested as the in vitro catalyst of disulfide bond formation (8-13). We have recently found that the cDNA sequences of the β subunit of human prolyl 4-hydroxylase are very similar to those recently reported for rat protein disulfide-isomerase (14), the degree of homology between these two proteins being in the whole coding region 84% at the level of nucleotide sequences and 94% at the level of cDNA-derived amino acid sequences. Furthermore, Southern blot analyses of human genomic DNA with a cDNA probe for the prolyl 4-hydroxylase β subunit indicated the presence of only one gene containing these sequences.1 We here report comparisons between the β subunit isolated from the prolyl 4-hydroxylase tetramer and the purified protein disulfide-isomerase from the same species to study the structural and functional relationship of these two proteins. Our data suggest that a single polypeptide acts as both these proteins and thus the excess β subunit protein of prolyl 4-hydroxylase present in various cells (1-7) is the enzyme protein disulfide-isomerase.

EXPERIMENTAL PROCEDURES

Isolation of Enzymes—Human and chick embryo prolyl 4-hydroxylase was purified to homogeneity by affinity chromatography on poly-L-proline (poly-L-proline) linked to agarose followed by ion-exchange chromatography on DEAE-cellulose and gel filtration (15, 16). To isolate the β subunit, the enzyme tetramer was incubated in the presence of 10 mM dithiothreitol at 37°C for 30 min in 50 mM Tris-HCl buffer, pH 7.5. The dithiothreitol was removed by centrifugation in Sephadex G-25 column (17) and the β subunit was isolated by concanavalin A-agarose chromatography (6). Protein disulfide-isomerase was isolated from human placentas and chick embryos by ammonium sulfate fractionation (55-80%) followed by ion-exchange chromatographies on CM-Sephadex C-50 and DEAE-Sephacel (10). Gel filtration on Sephacryl S-300 was used as a final purification step to give a homogeneous protein (11).

Assay of Enzyme Activities—Prolyl 4-hydroxylase activity was assayed by measuring the stoichiometric decarboxylation of 2-oxo-[1-14C]glutarate in the reaction (see Ref. 18) and protein disulfide-isomerase activity by the ribonuclease-linked method (19).

Electrophoretical Methods—SDS-PAGE was performed under reducing conditions using 10% acrylamide as the separating gel (19). Linear peptide maps from purified proteins were obtained using the method of Cleveland et al. (20). About 40 μg of chick embryo- or human placental prolyl 4-hydroxylase and 20 μg of chick embryo- or human placental protein disulfide-isomerase were analyzed by SDS-PAGE and the gel was stained briefly with Coomassie Brilliant Blue. The β subunit of prolyl 4-hydroxylase and the protein disulfide-isomerase were then cut from the gel and placed in wells of a second SDS-PAGE consisting of a 4.5% stacking gel and a 16% separating gel. The slices were surrounded with a solution of 125 mM Tris-HCl (pH 6.8), 0.1% SDS, and 20% glycerol and then overlayed with 20 μl of the above solution containing 10% glycerol, 0.04% bromphenol blue, and 4 μg of S. aureus V8 protease. Current was applied until the dye just passed the interphase between the stacking and separating gels and was continued after a pause of 45 min. The gels were then stained with either Coomassie Brilliant Blue (19) or silver (21) to visualize the peptides.

Western Blot Analysis of Purified Proteins—The enzymes isolated

1 Pihlajaniemi, T., Helaakoski, T., Tasanen, K., Myllylä, R., Huhtala, M.-L., Koivut, J., and Kivirikko, K. I. (1987) EMBO J., in press.
2 The abbreviation used is: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
from human placental tissue were separated by SDS-PAGE and blotted onto nitrocellulose, and the blot was incubated with antibodies. Bound antibody was recognized with anti-IgG antibody linked to peroxidase (see Refs. 13 and 22).

**Antibodies**—Polyclonal antibodies to human placental prolyl 4-hydroxylase and to purified human placental and chick embryo protein disulfide-isomerase were prepared in rabbits (13). Two monoclonal antibodies to human placental prolyl 4-hydroxylase, termed 3G3 and 5B5, were specific for the \( \beta \) subunit of the enzyme (22).

**RESULTS AND DISCUSSION**

The molecular weights, as determined by SDS-PAGE, for the prolyl 4-hydroxylase \( \beta \) subunit and the protein disulfide-isomerase polypeptide chain were found to be both slightly higher (by about \( M_r \), 1500) in the case of human placental tissue than in chick embryos (Fig. 1A). Protein disulfide-isomerase and the prolyl 4-hydroxylase \( \beta \) subunit purified from rat liver had the same apparent molecular weight as their counterpart from chick embryos (not shown). The molecular weights determined for the two polypeptides from chick embryo and rat liver were identical, while those from human placental tissue differed by about \( M_r \), 500, protein disulfide-isomerase having a slightly lower molecular weight (Fig. 1A). The peptide maps for the two peptides from chick embryos produced by digestion with \( S. \) aureus V8 protease were identical and from human placental tissue almost identical, except for one peptide which consistently showed a slightly lower apparent molecular weight (by about \( M_r \), 500) in protein disulfide-isomerase than in the \( \beta \) subunit (Fig. 1B). The reasons for this minor difference are unknown, but it may be due to differences in glycosylation or some other post-translational modification of the proteins in the placental tissue.

Polyclonal antibodies prepared against the human prolyl 4-hydroxylase stained human protein disulfide-isomerase on Western blots and those prepared to human and chick embryo protein disulfide-isomerase stained the human \( \beta \) subunit (Fig. 2). In addition, two different monoclonal antibodies to the human \( \beta \) subunit stained the human protein disulfide-isomerase (Fig. 2). Polyclonal antibodies to human prolyl 4-hydroxylase, immunoadsorbed by human protein disulfide-isomerase, did not stain either human protein disulfide-isomerase or the \( \beta \) subunit of human prolyl 4-hydroxylase on Western blots. This result demonstrates that there were no \( \beta \) subunit-specific epitopes which were not shared by protein disulfide-isomerase and thus supports the view of a structural identity between the two proteins.

Various polyclonal and monoclonal antibodies which were inhibitory against the activity of the human prolyl 4-hydroxylase tetramer did not inhibit the activity of human protein disulfide-isomerase, even though the ratio of concentration of the antibody to concentration of the enzyme protein was higher in the cases of the protein disulfide-isomerase than prolyl 4-hydroxylase activity assays (Table I). Such antibodies included two polyclonal antibodies prepared to either human or chick protein disulfide-isomerase that slightly inhibited the activity of human prolyl 4-hydroxylase (Table I). Additional experiments with several different antibody concentrations gave the same result (not shown). The differences between prolyl 4-hydroxylase and protein disulfide-isomerase in this respect may be due to differences in the structures of their catalytic sites. The catalytic site of prolyl 4-hydroxylase comprises a set of separate locations for the binding of the peptide substrate and the various cosubstrates (23), both enzyme subunits contributing to the structure of the catalytic site.

Thus the antibodies may bind at various sites of this large catalytic center. The catalytic site of protein disulfide-isomerase is simpler and probably comprises a small area of the polypeptide chain (14). Accordingly, only antibodies which bind to this small area may inhibit the protein disulfide-isomerase activity.

A mixture of the \( \alpha \) and \( \beta \) subunits of the dissociated human prolyl 4-hydroxylase tetramer and the pure \( \beta \) subunit isolated from such samples had protein disulfide-isomerase activity (Table II), which was in both cases identical in amount to that of the protein disulfide-isomerase when expressed per mg of the \( \beta \) subunit. The intact prolyl 4-hydroxylase tetramer

---

9 V. Günzler, H. M. Hanuske-Abel, R. Myllylä, A. Hanuske, and K. I. Kivirikko, unpublished data.
Our results demonstrate that a single polypeptide possesses two entirely different enzymatic functions. The polypeptide has protein disulfide-isomerase activity both when it is present as the monomer and when it is present in the prolyl 4-hydroxylase tetramer. In the latter case it also catalyzes the 4-hydroxylation of proline residues, contributing together with the α subunit to the structure of the catalytic center of the prolyl 4-hydroxylase tetramer.

Acknowledgments—We thank L. Aijalä and S. Vilmi for technical assistance and Drs. V. Günzler and H. Hanauke-Abel for helpful discussions.

REFERENCES
1. Cardinale, G. J., and Udenfriend, S. (1974) Adv. Enzymol. 41, 245–300
2. Prokop, D. J., Berg, R. A., Kivirikko, K. I., and Uitto, J. (1980) in Biochemistry of Collagens (Ramachandran, G. N., and Reddi, A. H., eds) pp. 163–273, Plenum Publishing Corp., New York
3. Kivirikko, K. I., and Myllylä, R. (1980) in The Enzymology of Post-Translational Modifications of Proteins (Fredman, R. B., and Hawkins, H. C., eds) pp. 53–104, Academic Press, London
4. Kivirikko, K. I., and Myllylä, R. (1985) Ann. N. Y. Acad. Sci. 460, 187–201
5. McGill, J. D., O’D., Langness, U., and Udenfriend, S. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 1585–1589
6. Chen-Kiang, S., Cardinale, G. J., and Udenfriend, S. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4420–4424
7. Berg, R. A., Kao, W. W.-Y., and Kedersha, N. L. (1980) Biochem. J. 189, 491–499
8. Anfinsen, C. B., and Schragers, H. A. (1975) Adv. Protein Chem. 29, 205–300
9. Freedman, R. B., and Hillson, D. A. (1980) in The Post-Translational Modifications of Proteins (Fredman, R. B., and Hawkins, H. C., eds) pp. 157–212, Academic Press, London
10. Hillson, D. A., Lambert, N., and Freedman, R. B. (1984) Methods Enzymol. 107, 281–294
11. Koivu, J., and Myllylä, R. (1986) Biochemistry 25, 5982–5986
12. Koivu, J., and Myllylä, R. (1987) J. Biol. Chem. 262, 6159–6164
13. Kivirikko, K. I., and Myllylä, R. (1987) Methods Enzymol. 144, 86–114
14. Edman, J. C., Ellis, L., Blacher, R. W., Roth, R. A., and Rutter, W. J. (1985) Nature 317, 267–270
15. Tuderman, L., Kuutti, E.-R., and Kivirikko, K. I. (1975) Eur. J. Biochem. 52, 5–16
16. Kedersha, N., and Berg, R. A. (1981) Collagen Relat. Res. 1, 345–353
17. Penefsky, H. S. (1977) J. Biol. Chem. 252, 2901–2909
18. Kivirikko, K. I., and Myllylä, R. (1982) Methods Enzymol. 82, 245–304
19. Weber, K., and Osborne, M. (1978) in The Proteins (Neurath, H., and Hill, R. L., eds) Vol. 1, pp. 173–223, Academic Press, New York
20. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) J. Biol. Chem. 252, 1103–1106
21. Wray, W., Boulikas, T., Wray, V. P., and Hancock, R. (1981) Anal. Biochem. 118, 197–203
22. Hovhnya, M., Myllylä, R., Fiura, J., Kivirikko, K. I., and Tryggvason, K. (1984) Eur. J. Biochem. 141, 477–482
23. Hanauke-Abel, H. M., and Günzler, V. (1982) J. Theor. Biol. 94, 421–455