Co-expression of the α subunit of human prolyl 4-hydroxylase with BiP polypeptide in insect cells leads to the formation of soluble and insoluble complexes

Soluble α -subunit–BiP complexes have no prolyl 4-hydroxylase activity

Johanna VEIJOLA, Taina PIHLAJANIEMI and Kari I. KIVIRIKKO*

Collagen Research Unit, Biocenter and Department of Medical Biochemistry, University of Oulu, FIN-90220 Oulu, Finland

Prolyl 4-hydroxylase (EC 1.14.11.2) catalyses the post-translational formation of 4-hydroxyproline in collagens. The vertebrate enzymes are $\alpha_2\beta_2$ tetramers, their β subunit being identical to protein disulphide isomerase (PDI). The function of the PDI- β subunit in prolyl 4-hydroxylases is not fully understood, but it seems to be that of keeping the highly insoluble α subunits in solution. We report here that expression of the α subunit of human type I prolyl 4-hydroxylase in insect cells together with BiP polypeptide leads to the formation of both soluble and insoluble α -subunit–BiP complexes. Formation of the soluble complexes was evident from (1) a marked increase in the amount of the α subunit in the soluble fraction of the cell

homogenates when expressed together with BiP, (2) immunoprecipitation experiments and (3) demonstration of the presence of some of the complexes by polyacrylamide gel electrophoresis under non-denaturing conditions. Formation of the insoluble complexes was suggested by an increase in the amount of BiP in the insoluble fraction when expressed together with the α subunit. Nevertheless the soluble α -subunit–BiP complexes had no prolyl 4-hydroxylase activity. This indicates that the function of the PDI- β subunit in the prolyl 4-hydroxylase tetramer is not only that of keeping the α subunits in solution but appears to be more specific, probably that of keeping them in a catalytically active, non-aggregated conformation.

INTRODUCTION

Prolyl 4-hydroxylase (EC 1.14.11.2) catalyses the co-translational and post-translational hydroxylation of proline residues in -Xaa-Pro-Gly- sequences in collagens and other proteins with collagen-like amino acid sequences. The 4-hydroxyproline residues formed in the reaction are essential for the folding of the newly synthesized collagen polypeptide chains into triple-helical molecules, and thus this modification plays a central role in the biosynthesis of all collagens (reviewed in [1–4]). The enzyme from all the vertebrate sources studied is either an $[\alpha(I)]_2\beta_2$ or an $[\alpha(II)]_2\beta_2$ tetramer, called type I or type II enzyme respectively [4,5], whereas the enzyme from the nematode *Caenorhabditis elegans* is an $\alpha\beta$ dimer [6]. The α subunits, which have now been cloned from man [7], mouse [5], rat [8], chicken [9] and *C. elegans* [6], contribute to most if not all parts of the catalytic sites of the enzymes [1–3,10]. The β subunit has been cloned from many sources [1–3,11–14] and found to be a highly unusual multifunctional polypeptide that is identical to the enzyme protein disulphide isomerase (PDI) [12,15,16]. Other suggested functions for this polypeptide are to act as a major cellular thyroid hormone-binding protein [17], a chaperone-like polypeptide that non-specifically binds various peptides within the lumen of the endoplasmic reticulum [13,18–23], the smaller subunit of the microsomal triglyceride transfer protein [24,25] and a dehydroascorbate reductase [26].

The function of the PDI- β subunit in prolyl 4-hydroxylases is not fully understood. When any of the prolyl 4-hydroxylase α subunits is expressed in insect cells alone, the recombinant polypeptide forms insoluble aggregates with no prolyl 4-hydroxylase activity [5,6,27]. Also, when an α subunit is expressed in a cell-free translation/translocation system of dog pancreas microsomes [28,29] depleted of PDI, BiP polypeptide and other

soluble endoplasmic reticulum proteins, the α subunit aggregates [28]. Thus one function of the PDI- β subunit seems to be to keep the α subunits in a non-aggregated conformation. This function resembles the role of certain chaperones in some proteins [14,27], but no data are available to indicate whether the PDI- β subunit can be replaced by other chaperones such as BiP. PDI activity at the two independently acting -Cys-Gly-His-Cys- catalytic sites of the PDI- β polypeptide [30,31] is not required for prolyl 4-hydroxylase assembly or activity [31].

To study whether an α subunit of prolyl 4-hydroxylase can form complexes with BiP under conditions in which insufficient amounts of the PDI- β polypeptide are available for tetramer formation, we expressed the α subunit of human type I prolyl 4-hydroxylase in insect cells together with BiP. Our data indicate the formation of both soluble and insoluble α -subunit–BiP complexes, but they had no prolyl 4-hydroxylase activity.

EXPERIMENTAL

Recombinant baculoviruses

The recombinant baculoviruses coding for the α subunit of human type I prolyl 4-hydroxylase ($\alpha 59$ virus) and the human PDI- β polypeptide have been described earlier [27]. The baculovirus encoding rat BiP was a gift from Dr. Stephen Fuller, EMBL, Heidelberg, Germany.

Antibodies

Rabbit antibodies to the human PDI- β polypeptide were prepared by using either affinity-purified [32,33] recombinant human prolyl 4-hydroxylase [27] (antibody K60) or a mixture of de-

natured and incompletely digested CNBr peptides of the purified recombinant PDI- β polypeptide [27,32,33] (antibodies K37 and K38) as the antigen. Rabbit antibodies to the α subunit of human type I prolyl 4-hydroxylase (antibodies K17 and K18) were raised against aggregated, denatured recombinant α subunit [27] and purified by means of a few detergent washings and a preparative SDS/PAGE followed by copper staining [34]. Rabbit antibodies to rat BiP (antibodies K39 and K40) were prepared by using SDS/PAGE slices containing denatured, recombinant BiP as an antigen. IgG immunoglobulins were isolated from the antisera [34] and purified with a negative-affinity column containing a crude mixture of proteins from insect cells infected with a baculovirus coding for the α subunit of *C. elegans* prolyl 4-hydroxylase [6].

Cell culture and baculovirus infection

Sf9 insect cells (Invitrogen) were cultured in TNM-FH medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (BioClear) and 2 mM L-glutamine (Gibco) as monolayers at 27 °C. To produce recombinant proteins, insect cells seeded at a density of 6×10^6 cells per 9 cm culture dish were infected at a multiplicity of 2–5 with each of the recombinant viruses $\alpha 59$, PDI- β or BiP alone. The multiplicity of infection for the viruses was reduced to 1–2 when the cells were co-transfected with different recombinant viruses. The cells were harvested 48 h after infection, washed twice with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4, homogenized in a buffer consisting of 0.1 M NaCl, 0.1 M glycine, 10 μ M dithiothreitol, 0.1% Triton X-100 and 0.01 M Tris, pH 7.8, and centrifuged at 10000 g for 20 min at 4 °C. Aliquots of the resulting supernatants were analysed by SDS/PAGE (8% gel) under reducing conditions or non-denaturing 7.5% PAGE and assayed for enzyme activity and protein concentration. The remaining cell pellets were washed once with the homogenizing buffer, solubilized in 1% SDS and analysed by reducing SDS/PAGE (8% gel).

Cross-linking experiments

Insect cells were cultured and infected as above by using a multiplicity of infection of 1 for each virus. For the cross-linking studies the cells were harvested 48 h after infection, washed twice with a solution of 0.13 M NaCl and 20 mM Bicine (Sigma), pH 8.0, divided into two aliquots with the washing buffer and resuspended in a lysis buffer, which consisted of 0.15 M NaCl, 5 mM KCl, 5 mM MgCl₂, 0.2% digitonin, 1 mM PMSF, 1 mM N-ethylmaleimide, 1 μ g/ml leupeptin and 1 μ g/ml pepstatin with or without 100 μ g/ml dithiobis(succinimidyl propionate) (DSP). The samples were then incubated on ice for 30 min, the excess of the cross-linker was inactivated with 10 mM glycine for 10 min on ice, nuclei were removed by centrifugation at 500 g for 10 min at 4 °C, and the lysates were analysed by SDS/PAGE (8% gel) under either non-reducing or reducing conditions or by non-denaturing 7.5% PAGE.

Metabolic labelling

Insect cells were cultured and infected as above. The medium was removed 36 h after infection, and the infected cells were washed gently with a solution of 0.15 M NaCl and 20 mM phosphate, pH 7.4. They were then starved for 40 min in a methionine-free Grace's insect cell medium (Gibco) supplemented with 5% (v/v) dialysed fetal calf serum (Gibco) and labelled with 60 μ Ci/ml L-[³⁵S]methionine (Amersham) for 4 h at 27 °C. The labelling was stopped by chilling on ice, and the cells and supernatants were separated by centrifugation. The labelled cells were washed with

the ice-cold washing buffer, lysed as above, with or without the DSP cross-linking reagent, and the lysates were divided into aliquots and processed for immunoprecipitation.

Immunoprecipitation

The cell lysates were precleared twice in the presence of pre-immune sera (50 μ l/ml lysate) and *Staphylococcus aureus* Cowan I cells (Fluka). The samples were then immunoprecipitated with approx. 30 μ g of IgG per ml of lysate overnight at 4 °C. The precipitates were collected by incubating the samples with beads of Protein A–Sepharose (Pharmacia) for 1 h at 4 °C. The beads and the bound antibody–protein complexes were washed twice with a solution of 0.1% Nonidet P40 and 10 mM Tris, pH 7.5, once with a solution of 0.15 M NaCl, 0.1% Nonidet P40, 1 mM EDTA, 0.25% gelatin, 0.02% sodium azide and 50 mM Tris, pH 7.5, and once with a solution of 0.15 M NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris, pH 7.5. Antibody–antigen interaction was destroyed by adding 1 volume of a solution of 100 mM dithiothreitol, 2% SDS, 0.1% Bromophenol Blue and 50 mM Tris, pH 6.8, to the samples and incubating them at 100 °C for 3 min. The samples were then analysed by reducing SDS/PAGE (8% gel), dried and autoradiographed.

Other assays

Prolyl 4-hydroxylase activity was assayed by a method based on the hydroxylation-coupled decarboxylation of 2-oxo[1-¹⁴C]-glutarate [32]. Protein concentrations were estimated with a Bio-Rad protein assay kit using BSA as a standard. Non-denaturing 7.5% PAGE was performed in a running buffer containing 0.192 M glycine and 25 mM Tris, pH 8.3, at 15 V for 22 h at 4 °C. For Western analyses, proteins were transferred onto a poly(vinylidene fluoride) membrane (Immobilon P) at 80 V for 50 min in a cooled chamber (4 °C).

RESULTS

Co-expression with BiP increases the solubility of the prolyl 4-hydroxylase α subunit in insect cells

To study whether the α subunit of human prolyl 4-hydroxylase forms complexes with BiP under conditions in which only minor amounts of the *Spodoptera frugiperda* PDI- β polypeptide are available for tetramer formation, the α subunit was expressed in insect cells with and without the rat BiP polypeptide. The cells were harvested 48 h after the baculovirus infection, homogenized in a buffer containing 0.1% Triton X-100 and centrifuged. The cell pellets were further solubilized in 1% SDS, and the proteins soluble in 0.1% Triton X-100 and 1% SDS were analysed by SDS/PAGE under reducing conditions followed by either Coomassie Blue staining (Figures 1A and 1C) or Western blotting with an antibody to the human α subunit (Figure 1B). As reported previously [6,7,27], when the α subunit was expressed in insect cells alone, none could be extracted from the cell homogenates with 0.1% Triton X-100 (Figures 1A and 1B, lanes 1), and efficient extraction required the use of 1% SDS (Figure 1C, lane 1). When the α subunit was expressed together with the PDI- β subunit, a soluble enzyme tetramer was formed, and hence considerable amounts of the α subunit were found in the fraction soluble in Triton X-100 (Figures 1A and 1B, lanes 4). Nevertheless large amounts of the α subunit also remained non-associated and were found in the fraction soluble in 1% SDS (Figure 1C, lane 4). When the α subunit was expressed together with BiP, significant amounts of the α subunit were again found in the

Figure 1 Analysis of the expression of recombinant polypeptides in insect cells by SDS/PAGE under reducing conditions

(A, B) Proteins soluble in 0.1% Triton X-100; (C) proteins solubilized from the remaining cell pellets with 1% SDS. The samples in (A) and (C) were analysed by staining with Coomassie Blue and those in (B) by Western blotting with the polyclonal rabbit antibody K17 to the α subunit of human prolyl 4-hydroxylase. Lane 1 in each panel shows polypeptides from cells infected with a virus coding for the α subunit of human prolyl 4-hydroxylase, lane 2 with a virus coding for rat BiP, lane 3 co-infected with viruses coding for the α subunit of human prolyl 4-hydroxylase and rat BiP, lane 4 co-infected with viruses coding for the α subunit of human prolyl 4-hydroxylase and human PDI and lane 5 co-infected with viruses coding for human PDI and rat BiP. The positions of the molecular mass markers (kDa) are indicated on the left and the positions of BiP, human α subunit and PDI by arrows on the right.

fraction soluble in Triton X-100 (Figures 1A and 1B, lanes 3), suggesting that the α subunit became associated with BiP to form soluble complexes. Comparison of the amounts of BiP in the 1% SDS-soluble fraction of cells expressing BiP either alone (Figure 1C, lane 2) or together with the α subunit (Figure 1C, lane 3) indicated that a larger amount of BiP is found in this fraction in the presence of the α subunit. This suggests that the α subunit also formed insoluble complexes with BiP.

Experiments were also performed in which the cells were lysed in the presence of DSP, a homobifunctional cross-linker that can be cleaved under reducing conditions. When the 0.2% digitonin-soluble proteins from cells expressing the α subunit together with

Figure 2 Analysis by SDS/PAGE under reducing conditions of the [35 S]methionine-labelled polypeptides present in the immunoprecipitates obtained with various antibodies

Insect cells were metabolically labelled with [35 S]methionine and lysed in a digitonin buffer in the absence (–) or presence (+) of 100 μ g/ml DSP, as described in the Experimental section. The lysates of the cells co-expressing the α subunit of human prolyl 4-hydroxylase and BiP (lanes 1 and 2), the α subunit and the PDI- β (lanes 3 and 4) or BiP and the PDI- β polypeptide (lanes 5 and 6) were divided into aliquots and processed for immunoprecipitation. (A) Lysates were immunoprecipitated with antibody K40 to rat BiP (lanes 1, 2, 5 and 6) or antibody K60 to the human PDI- β polypeptide (lanes 3 and 4). (B) Lysates were immunoprecipitated with antibody K17 to the α subunit of human prolyl 4-hydroxylase (lanes 1–4) or with antibody K60 to the human PDI- β polypeptide. The gels were exposed overnight. The positions of the molecular mass markers (kDa) are indicated on the left and the positions of the BiP, α subunit and PDI polypeptides are shown by arrows.

BiP were analysed by SDS/PAGE under non-reducing conditions, essentially all the proteins remained at the top of the gel, indicating extensive cross-linking. When these samples were reduced before the electrophoresis, and the electrophoresis was performed under reducing conditions, the data were very similar to those shown in Figures 1A and 1B (lanes 3) except that the band corresponding to the α subunit was even more prominent than in samples homogenized and extracted without the cross-linker (results not shown).

Immunoprecipitation of the α -subunit-BiP complexes

Insect cells were infected with the various viruses as above and labelled with [35 S]methionine during the last 4 h of the culture. They were harvested 40 h after baculovirus infection, lysed in a buffer containing 0.2% digitonin, in the absence or presence of DSP, and the cell lysates were immunoprecipitated with various antibodies. The immunoprecipitates were then analysed by SDS/PAGE under reducing conditions, followed by fluorography.

When the α subunit was expressed together with the PDI- β subunit, significant amounts of the former were precipitated with antibodies to the latter (Figure 2A, lane 3), and the presence of the cross-linker had no enhancing effect (Figure 2A, lane 4)

Figure 3 Analysis of the formation of complexes between recombinant polypeptides expressed in insect cells by PAGE under non-denaturing conditions

Insect cells expressing various recombinant polypeptides were extracted with a buffer containing 0.2% digitonin, and soluble proteins were analysed by 7.5% PAGE under non-denaturing conditions in a running buffer of 0.192 M glycine and 0.025 M Tris, pH 8.3, at 15 V for 20 h at 4 °C. The samples were analysed by Western blotting with antibody K17 to the α subunit of human prolyl 4-hydroxylase (**A**) or with antibody K38 to the human PDI- β polypeptide (**B**). In (**A**) lane 1 shows polypeptides from cells expressing the α subunit of human prolyl 4-hydroxylase alone, lane 2 rat BiP alone, lane 3 human PDI- β polypeptide alone, lane 4 the α subunit and BiP together and lane 5 the α subunit and the PDI- β polypeptide together. In (**B**) lane 1 shows polypeptides from cells expressing BiP alone, lane 2 the PDI- β polypeptide alone, lane 3 the α subunit and the PDI- β polypeptide together, and lane 4 the PDI- β polypeptide and BiP together. The arrows indicate the human enzyme tetramer ($H_{\alpha}H_{\beta}$), the α -subunit-BiP complex ($H_{\alpha}BiP$) and the non-associated PDI- β polypeptide (H_{β}). The faint immunostained band seen in lane 3 in (**B**) but not in lane 5 in (**A**) probably represents the PDI- β polypeptide dimer (unlabelled arrow). The non-associated α subunit is insoluble in 0.2% digitonin and is not seen.

because the prolyl 4-hydroxylase tetramer formed can be expected to be stable even in the absence of DSP. When the enzyme tetramer produced by co-expression of the α subunit with the PDI- β subunit was precipitated with antibodies to the former, no band corresponding to the latter was seen in the immunoprecipitates (Figure 2B, lanes 3 and 4) because the appearance of the label in the PDI- β subunit of the enzyme tetramer is delayed owing to its dilution in the preformed pool of free PDI- β subunit polypeptide [35]. When the α subunit was expressed together with BiP, significant amounts of the former were precipitated with antibodies to the latter (Figure 2A, lanes 1 and 2), especially in the presence of the cross-linker. Correspondingly, significant amounts of labelled BiP were precipitated with antibodies to the α subunit (Figure 2B, lanes 1 and 2) even though the appearance of the label in BiP can be expected to be delayed, as with the PDI- β subunit above. The results obtained with both the α subunit and BiP antibodies thus demonstrate formation of a complex between the α subunit and BiP. In contrast, no complex formation

Table 1 Prolyl 4-hydroxylase activity in Triton X-100 extracts of cells expressing various recombinant polypeptides

The values are given in d.p.m. per 100 μ g of extractable cell protein (means of duplicate values), and as percentages of the value obtained by co-expression of the human prolyl 4-hydroxylase α subunit with human PDI- β subunit.

Recombinant polypeptides expressed	Prolyl 4-hydroxylase activity	
	(d.p.m. per 100 μ g)	(%)
None	230	2
α subunit alone	440	4
PDI- β polypeptide alone	< 100	< 1
BiP alone	< 100	< 1
α subunit and PDI- β polypeptide	12300	100
α subunit and BiP	200	2

was seen between the PDI- β polypeptide and BiP, as no PDI- β subunit was found in immunoprecipitates obtained with the BiP antibodies in the corresponding double-infection experiments (Figure 2A, lanes 5 and 6), and no BiP was seen in immunoprecipitates obtained with the PDI antibody (Figure 2B, lanes 5 and 6).

Demonstration of formation of the α -subunit-BiP complex by non-denaturing PAGE

Insect cells were infected with various recombinant baculoviruses, harvested 48 h after infection, homogenized in a buffer containing 0.2% digitonin and centrifuged. The soluble proteins were then analysed by PAGE performed under non-denaturing conditions, followed by Western blotting with an antibody to either the human α subunit (Figure 3A) or the human PDI- β polypeptide (Figure 3B). When the α subunit was expressed alone, no staining was detected (Figure 3, lane 1), because the non-associated α subunit forms insoluble complexes [5,6,27]. When the α subunit was expressed together with BiP, diffuse staining was seen close to the top of the gel and a weak though distinct band occurred with a mobility slightly greater than that of the prolyl 4-hydroxylase tetramer (Figure 3A, lane 4). Nevertheless the amounts of complex detected were much smaller than that of prolyl 4-hydroxylase tetramer formed by co-expression of the α subunit with the PDI- β subunit (Figure 3A, lane 5), possibly because most of the α -subunit-BiP complexes dissociated into the non-associated α subunit (which forms a precipitate) and BiP under the conditions used here. No complex formation could be detected when the PDI- β polypeptide was expressed together with BiP (Figure 3B, lane 4).

The soluble α -subunit-BiP complexes have no prolyl 4-hydroxylase activity

To study whether the soluble α -subunit-BiP complexes have any prolyl 4-hydroxylase activity, 0.1% Triton X-100 extracts of cell homogenates were assayed by a method based on measurement of the hydroxylation-coupled decarboxylation of 2-oxo-[1-¹⁴C]glutarate [32]. An extract of cells expressing the α subunit together with the PDI- β subunit gave 12300 d.p.m. per 100 μ g of extractable cell protein in this assay, whereas the value for an extract of non-infected cells was less than 2% of this (Table 1). As reported previously [6], a slight increase in the latter

value to about 4% was seen when the α subunit was expressed alone (Table 1), possibly owing to the formation of small amounts of a hybrid prolyl 4-hydroxylase between the human α subunit and the *S. frugiperda* PDI- β polypeptide. The value obtained when the α subunit was expressed with BiP was even lower than that seen when it was expressed alone (Table 1). Additional experiments with an assay based on the formation of hydroxy[14 C]proline in a biologically prepared [14 C]proline-labelled protein substrate consisting of non-hydroxylated pro- α chains of type I procollagen [33] confirmed these findings (results not shown).

DISCUSSION

Several recent studies have suggested that the PDI- β polypeptide catalyses protein folding by being both a disulphide isomerase and a chaperone [13,18–23]. The role of this polypeptide as the β subunit of prolyl 4-hydroxylases seems to be related to its chaperone function, as this role is to keep the α subunits in a non-aggregated conformation [5,6,27,28]. The site in the PDI- β polypeptide that non-specifically binds various peptides within the lumen of the endoplasmic reticulum has been localized to a single 26-amino acid tryptic peptide close to the C-terminus of the bovine PDI- β polypeptide [18]. Deletion of this sequence from the human polypeptide prevents prolyl 4-hydroxylase tetramer formation (P. Koivunen, T. Helaakoski, A.-P. Kvist, T. Pihlajaniemi and K. I. Kivirikko, unpublished work), supporting the suggestion that the role of this polypeptide as a protein subunit is related to the chaperone function.

We set out in the present work to study whether the PDI- β polypeptide in the prolyl 4-hydroxylase tetramer can be replaced functionally by another molecular chaperone, namely BiP. It has previously been shown that when the α subunit of the type I prolyl 4-hydroxylase tetramer was expressed in a dog pancreas microsomal system under conditions that prevented disulphide bond formation, the product that was synthesized formed aggregates that were associated with BiP [28]. Nevertheless no data were available to indicate whether the α subunit associates with BiP in the cell and under conditions that allow disulphide bond formation. It was similarly not known whether any α -subunit-BiP complexes that may be formed possess prolyl 4-hydroxylase activity.

The present results indicate that the α subunit of human type I prolyl 4-hydroxylase can form both soluble and insoluble complexes with BiP. The formation of soluble complexes was evident from data indicating that (1) expression of the α subunit with BiP markedly increased the amount of this subunit in the 0.1% Triton X-100-soluble protein fraction of the cell homogenate, (2) immunoprecipitation with antibodies to the α subunit also precipitated BiP, and antibodies to BiP also precipitated the α subunit, and (3) some of the complexes could be detected by PAGE performed under non-denaturing conditions. The formation of insoluble α -subunit-BiP complexes was suggested by results indicating that expression of BiP together with the α subunit increased the amount of BiP in the fraction insoluble in Triton X-100 but soluble in 1% SDS. In contrast, no complex formation could be detected between the PDI- β polypeptide and BiP either in immunoprecipitation or by non-denaturing PAGE.

Although most, if not all, of the sequences contributing to the two catalytic sites of the prolyl 4-hydroxylase tetramer are located in the α subunits, no enzyme activity has been found in these subunits when a tetramer is dissociated into its subunits *in vitro* [1–3]. This has been assumed to be due to the fact that the free α subunit immediately forms insoluble aggregates [3]. The present data indicate that the α subunit has no prolyl 4-

hydroxylase activity even when it is present in a soluble complex with BiP. The lack of activity is not likely to be due to a complete absence of native-type disulphide bonding, as the α subunit has only five cysteine residues and as numerous proteins, some of which contain many disulphide bonds, have been expressed in insect cells in a fully active form [36]. Furthermore the α subunit forms a fully active prolyl 4-hydroxylase tetramer in insect cells with a double-mutant PDI- β subunit in which both -Cys-Gly-His-Cys- catalytic site sequences have been inactivated by converting them into -Ser-Gly-His-Cys- [31]. The results thus indicate that the function of the PDI- β subunit in the prolyl 4-hydroxylase tetramer is not merely that of keeping the α subunits in solution but it seems to be more specific, probably that of keeping them in a catalytically active conformation.

We thank Ms. Liisa Äijälä for her expert technical assistance. This work was supported by grants from the Medical Research Council of the Academy of Finland.

REFERENCES

- Kivirikko, K. I., Myllylä, R. and Pihlajaniemi, T. (1989) *FASEB J.* **3**, 1609–1617
- Kivirikko, K. I., Helaakoski, T., Tasanen, K., Vuori, K., Myllylä, R., Parkkonen, T. and Pihlajaniemi, T. (1990) *Ann. N. Y. Acad. Sci.* **580**, 132–142
- Kivirikko, K. I., Myllylä, R. and Pihlajaniemi, T. (1992) in *Post-Translational Modifications of Proteins* (Harding, J. J. and Crabbe, M. J. C., eds.), pp. 1–51, CRC Press, Boca Raton, FL
- Prockop, D. J. and Kivirikko, K. I. (1995) *Annu. Rev. Biochem.* **64**, 403–434
- Helaakoski, T., Annunen, P., Vuori, K., MacNeil, I. A., Pihlajaniemi, T. and Kivirikko, K. I. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4427–4431
- Veijola, J., Koivunen, P., Annunen, P., Pihlajaniemi, T. and Kivirikko, K. I. (1994) *J. Biol. Chem.* **269**, 26746–26753
- Helaakoski, T., Vuori, K., Myllylä, R., Kivirikko, K. I. and Pihlajaniemi, T. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4392–4396
- Hopkinson, I., Smith, S. A., Donne, A., Gregory, H., Franklin, T. J., Grant, M. E. and Rosamond, J. (1994) *Gene* **149**, 391–392
- Bassuk, J. A., Kao, W. W.-Y., Herzer, P., Kedersha, N. L., Seyer, J., DeMartino, J. A., Daugherty, B. L., Mark, G. E. III and Berg, R. A. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7382–7386
- Lamberg, A., Pihlajaniemi, P. and Kivirikko, K. I. (1995) *J. Biol. Chem.* **270**, 9926–9931
- Edman, J. C., Ellis, L., Blacher, R. W., Roth, R. A. and Rutter, W. J. (1985) *Nature (London)* **317**, 267–270
- Pihlajaniemi, T., Helaakoski, T., Tasanen, K., Myllylä, R., Huhtala, M.-L., Koivu, J. and Kivirikko, K. I. (1987) *EMBO J.* **6**, 643–649
- Noiva, R. and Lennarz, W. J. (1992) *J. Biol. Chem.* **266**, 19645–19649
- Freedman, R. B., Hirst, T. R. and Tuite, M. F. (1994) *Trends Biochem. Sci.* **19**, 331–336
- Koivu, J., Myllylä, R., Helaakoski, T., Pihlajaniemi, T., Tasanen, K. and Kivirikko, K. I. (1987) *J. Biol. Chem.* **262**, 6447–6449
- Parkkonen, T., Pihlajaniemi, T. and Kivirikko, K. I. (1988) *Biochem. J.* **256**, 1005–1011
- Cheng, S., Gong, Q., Parkinson, C., Robinson, E. A., Appella, E., Merlino, G. T. and Pastan, I. (1987) *J. Biol. Chem.* **262**, 11221–11227
- Noiva, R., Freedman, R. B. and Lennarz, W. J. (1993) *J. Biol. Chem.* **268**, 19210–19217
- Cai, H., Wang, C.-C. and Tsou, C.-L. (1994) *J. Biol. Chem.* **269**, 24550–24552
- Otsu, M., Omura, F., Yoshimori, T. and Kikuchi, M. (1994) *J. Biol. Chem.* **269**, 6874–6877
- Puig, A. and Gilbert, H. F. (1994) *J. Biol. Chem.* **269**, 7764–7771
- Puig, A., Lyles, M. M., Noiva, R. and Gilbert, H. F. (1994) *J. Biol. Chem.* **269**, 19128–19135
- Rupp, K., Birnbach, U., Lundström, J., Nguyen Van, P. and Soling, H.-D. (1994) *J. Biol. Chem.* **269**, 2501–2507
- Wetterau, J. R., Combs, K. A., Spinner, S. N. and Joiner, B. J. (1990) *J. Biol. Chem.* **265**, 9800–9807
- Wetterau, J. R., Combs, K. A., McLean, L. R., Spinner, S. N. and Aggerbeck, L. P. (1991) *Biochemistry* **30**, 9728–9735
- Wells, W. W., Xu, D. P., Yang, Y. and Rocque, P. A. (1990) *J. Biol. Chem.* **265**, 15361–15364
- Vuori, K., Pihlajaniemi, T., Marttila, M. and Kivirikko, K. I. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7467–7470
- John, D. C. A., Grant, M. E. and Bulleid, N. J. (1993) *EMBO J.* **12**, 1587–1595
- John, D. C. A. and Bulleid, N. J. (1994) *Biochemistry* **33**, 14018–14025

- 30 Vuori, K., Myllylä, R., Pihlajaniemi, T. and Kivirikko, K. I. (1992) *J. Biol. Chem.* **267**, 7211–7214
- 31 Vuori, K., Pihlajaniemi, T., Myllylä, R. and Kivirikko, K. I. (1992) *EMBO J.* **11**, 4213–4217
- 32 Kivirikko, K. I. and Myllylä, R. (1982) *Methods Enzymol.* **82**, 245–304
- 33 Kivirikko, K. I. and Myllylä, R. (1987) *Methods Enzymol.* **144**, 96–114
- 34 Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- 35 Kedesha, N. L., Tkacz, J. S. and Berg, R. A. (1985) *Biochemistry* **24**, 5952–5960
- 36 Kidd, M. and Emery, V. C. (1993) *Appl. Biochem. Biotechnol.* **42**, 137–159

Received 24 October 1995/20 December 1995; accepted 21 December 1995