Intracellular dissociation and reassembly of prolyl 4-hydroxylase: the α -subunits associate with the immunoglobulin-heavy-chain binding protein (BiP) allowing reassembly with the β -subunit

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Prolyl 4-hydroxylase (P4-H) consists of two distinct polypeptides; the catalytically more important α -subunit and the β -subunit, which is identical to the multifunctional enzyme protein disulphide isomerase. The enzyme appears to be assembled *in vivo* into an $\alpha_2\beta_2$ tetramer from newly synthesized α -subunits associating with an endogenous pool of β -subunits. Using a cell-free system, we have shown previously that enzyme assembly is redox-dependent and that assembled α -subunits are intramolecularly disulphide-bonded [John and Bulleid (1994) *Biochemistry* 33, 14018–14025]. Here we have studied this assembly process within intact cells by expressing both subunits in COS-1 cells. Newly synthesized α -subunits were shown to assemble

with the β -subunit, to form insoluble aggregates, or to remain soluble but not associate with the β -subunit. Treatment of cells with dithiothreitol (DTT) led to dissociation of P4-H into subunits and on removal of DTT the enzyme reassembled. This reassembly was ATP-dependent, suggesting an interaction with an ATP-dependent chaperone. This was confirmed when immunoglobulin-heavy-chain binding protein (BiP) and α -subunits were co-immunoprecipitated with antibodies against the α -subunit and BiP, respectively. These results indicate that unassembled α -subunits are maintained in an assembly-competent form by interacting with the molecular chaperone BiP.

INTRODUCTION

Prolyl 4-hydroxylase (P4-H), an enzyme localized to the endoplasmic reticulum (ER), catalyses the co- and post-translational hydroxylation of proline residues in Xaa-Pro-Gly sequences of collagens and proteins with collagen-like amino acid sequences [1]. Vertebrate P4-H consists of two distinct polypeptides; the catalytically more important, glycosylated α -subunit (64 kDa) and the β -subunit (60 kDa) which is identical to the multifunctional enzyme protein disulphide isomerase (PDI) [2]. The enzyme appears to be assembled *in vivo* into an $\alpha_2\beta_2$ tetramer from newly synthesized α -subunits associating with an endogenous pool of β -subunits [3]. Attempts to reassociate the isolated subunits *in vitro* to form an active enzyme tetramer have been unsuccessful [4,5] due to the aggregation tendency and consequent insolubility of the α -subunit on dissociation from the β -subunit [6]. This may indicate one possible role for the β -subunit (PDI) in maintaining the α -subunit in a non-aggregated form, a role proposed for PDI as a component of the microsomal triacylglycerol transfer protein complex [7]. However, it has been established that P4-H is retained within the ER by means of the β -subunit C-terminal -KDEL sequence [8]. Intramolecular disulphide bonds are formed within the α -subunit [9] and are required for tetramer stability, as the complex can be dissociated *in vitro* and *in vivo* using the reducing agent dithiothreitol (DTT) [3,4,10,11]. The above findings suggest that the assembly of P4-H *in vivo* depends upon disulphide bond formation and the presence of chaperone proteins to prevent aggregation of newly synthesized α -subunit.

We have previously employed a cell-free system to study the events occurring during P4-H assembly [12] where mRNA coding

for the α -subunit was translated in the presence of microsomal membranes containing endogenous PDI. Under translation conditions which prevented disulphide bond formation, newly synthesized α -subunits formed insoluble aggregates and complexes with the immunoglobulin-heavy-chain binding protein (BiP). Translations carried out under conditions that promoted disulphide bond formation yielded a product that was not associated with BiP but formed a complex with the endogenous β -subunit (PDI). In addition, when microsomal vesicles were depleted of PDI, BiP and other soluble ER proteins, no complex formation was observed and the α -subunit aggregated even under conditions that promoted disulphide bond formation. Therefore, the solubility of the α -subunit appears to be dependent upon the formation of intramolecular disulphide bonds, its association with PDI and possibly on the presence of other soluble ER lumen proteins during α -subunit synthesis. Site-directed mutagenesis of α -subunit cysteine residues has revealed that Cys-276 and Cys-293 are required for α - β complex formation [9], that α -subunits present in α - β complexes are intramolecularly disulphide-bonded and that Cys-486 and Cys-511 are necessary for formation of enzymically active tetramers [13].

In this study we have examined the assembly of P4-H from its constituent subunits when expressed in COS-1 cells after transient transfection. We have shown that DTT-mediated dissociation of P4-H tetramers *in vivo* was reversible and that recovery of α - β complexes occurred after both co- and post-translational treatment with DTT. The recovery was ATP-dependent and a proportion of α -subunits were associated with BiP. These results demonstrate that α -subunit disulphide bonds are required for complex formation and that *in vivo*, complex formation is

Abbreviations used: BiP, immunoglobulin-heavy-chain binding protein; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ER, endoplasmic reticulum; EndoH, endoglycosidase H; P4-H, prolyl 4-hydroxylase; PDI, protein disulphide isomerase.

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reversible by the manipulation of redox conditions. The possible role of BiP in P4-H assembly and the role of PDI as enzyme subunit and catalyst of α -subunit disulphide bond formation are discussed.

MATERIALS AND METHODS

Materials

All mammalian cell culture reagents were purchased from Gibco-BRL (U.K.). COS-1 cells were purchased from the ECACC collection (PHLS, Porton Down, U.K.). Endoglycosidase H (EndoH) was purchased from Boehringer Mannheim (Lewes, U.K.), cycloheximide, 2-deoxyglucose, sodium azide and apyrase were all purchased from Sigma (Poole, Dorset, U.K.). All other reagents were of the highest quality available. Monoclonal antibody to BiP was a gift from Dr. Linda Hendershot, St. Jude Children's Research Hospital, Memphis, TN 3801, U.S.A. Polyclonal antisera to bovine PDI and to rat P4-H α -subunit were generated as described previously [9,12].

A 2 kb *EcoRI* fragment containing rat P4-H α -subunit sequence (derived from pDJ2) [12] was cloned into the *EcoRI* site of the mammalian expression vector pCMV5 (a gift from D. W. Russell, University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.) to create the vector pCMV5/ α . Similarly, a 2 kb *EcoRI/HindIII* fragment containing the human PDI sequence (derived from pUKC1006) [12] was cloned into *EcoRI/HindIII* endonuclease-treated pCMV5 to create pCMV5/ β . Before use in transfection studies pCMV5/ α and pCMV5/ β were purified from transformed XL-1 Blue cells by conventional alkaline lysis and caesium chloride density-gradient ultracentrifugation [14].

Cell culture, transfection and metabolic labelling

COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with pyruvate (0.11 g/l) supplemented with glutamine (0.3 g/l), penicillin (100 units/ml), streptomycin (100 μ g/ml) and foetal-calf serum (10%, v/v) (complete medium). Cells were grown at 37 °C in a humidified 95% air/5% CO₂ (v/v) atmosphere. Cells were transfected by a modification of the standard diethylaminoethyl-dextran (DEAE-dextran)-mediated method [14]. Briefly, 1×10^6 cells were transfected for 1 h in medium (6 ml) without foetal-calf serum containing DEAE-dextran (0.3 mg/ml) and the relevant DNA constructs (3.3 μ g/ml). Cells were rinsed once in complete medium and incubated for 3 h in complete medium containing chloroquine (0.1 mM). Finally, cells were rinsed three times and incubated overnight in complete medium before trypsinization and splitting into 9-cm-diam. dishes [$\sim (0.3\text{--}0.4) \times 10^6$ cells per dish] followed by a second overnight incubation prior to further procedures.

Prior to metabolic labelling, transfected cells were washed once with PBS, twice with methionine- and cysteine-free medium, and incubated in the latter for 1 h at 37 °C. Cells were labelled (30 min, 37 °C) in prewarmed methionine- and cysteine-free medium containing 29 or 58 μ Ci/ml [³⁵S]methionine and [³⁵S]cysteine mix {either tran[³⁵S]-label (NEN) or PROMix label (Amersham)}. Cells that were to be labelled under reducing conditions were pretreated with DTT (2 mM) added to the starvation medium 5 min prior to labelling and then labelled as above with DTT (2 mM) added to the medium. Chases were initiated by rapidly washing the cells once in chase medium (5 ml) before addition of a further 5 ml of chase medium and incubation of the cells for various periods (up to 2 h) at 37 °C prior to lysis. Chase medium contained cycloheximide (5 mM)

and was either complete medium or ATP-depleting medium [glucose-free DMEM containing glutamine, 2-deoxyglucose (20 mM) and sodium azide (10 mM)]. Prior to lysis, blocking of free sulphhydryls was carried out in intact cells by incubation of the washed cells on ice for 20 min in PBS containing iodoacetamide (20 mM).

Cell lysis and immunoprecipitation

Washed and alkylated cells were disrupted in 0.25 ml of ice-cold lysis buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 2.5 mM EDTA, 1% Triton X-100 containing soybean trypsin inhibitor (0.2 mg/ml) and PMSF (1 mM)]. In addition cell lysis buffer was supplemented, where detailed, with EDTA (2.5 mM), EGTA (5 mM) and apyrase (1 unit/ml) for preservation of putative BiP complexes, or with 2 mM ATP and 2 mM MgCl₂ for disruption of BiP complexes. Cell lysates were incubated on ice for 20 min prior to centrifugation (13000 g, 20 min, 4 °C), the soluble fraction was removed and the pellet rinsed once in lysis buffer (100 μ l). A 1 μ l aliquot of pre-immune serum and 40 μ l of Protein A-Sepharose [10% (v/v) solution in PBS with 0.02% sodium azide] were added to the soluble fraction and the sample incubated with agitation to pre-clear the lysate (30 min, 4 °C). After a brief centrifugation (13000 g, 30 s), the supernatant was recovered and labelled proteins immunoprecipitated by incubation for 2 h or overnight at 4 °C in the presence of antibodies and Protein A-Sepharose (50 μ l). Immune complexes were then retrieved by a brief centrifugation (13000 g, 30 s) and the complexes washed twice in lysis buffer (1 ml), once in lysis buffer containing 0.5 M NaCl (1 ml), and once again in lysis buffer alone (1 ml). Immune complexes that were to be treated with EndoH were washed additionally with 50 mM Tris, pH 7.4 (1 ml).

EndoH treatment

Immune complexes were eluted from Protein A-Sepharose by boiling twice in 20 μ l of elution buffer (50 mM Tris, pH 8.0, 1% SDS). The supernatants were recovered by brief centrifugation and combined before dilution with an equal volume of citrate buffer (150 mM, pH 5.5) containing PMSF (2 mM). The sample was then divided in two and EndoH (2 units) added to one half. Both samples were then incubated at 37 °C for 5 h prior to analysis by SDS/PAGE.

SDS/PAGE, phosphorimage analysis and autoradiography

Samples were prepared for electrophoresis by resuspension in SDS/PAGE sample buffer [60 mM Tris/HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) Bromophenol Blue]. Samples were boiled for 10 min in the presence of DTT (50 mM, reducing conditions) or in the absence of DTT (non-reducing conditions). Samples were then subjected to electrophoresis through 10% polyacrylamide gels in the presence of SDS [15]. Dried gels were subjected to phosphorimage analysis using a Fuji Bas2000 system for quantification purposes and were then autoradiographed at room temperature using Kodak BIOMAX film.

RESULTS

Expression of P4-H α - and β -subunits in COS-1 cells by transient transfection

Previous studies on the expression of human P4-H in insect cells have demonstrated that production of soluble protein requires

Figure 1 Expression of P4-H α - and β -subunits in transfected COS-1 cells

COS-1 cells transiently transfected with pCMV5/ α and pCMV5/ β constructs ($\alpha\beta$ -COS cells) were metabolically labelled with [^{35}S]Met/Cys for 30 min, washed in PBS and lysed. The soluble lysate fraction was then precleared with pre-immune serum before immunoprecipitation with polyclonal antisera raised against either the β -subunit/PDI (lanes 1, 2, 5 and 6) or the α -subunit (lanes 3, 4, 7 and 8). Washed immunoprecipitates were eluted and resuspended for digestion with EndoH, one half of each sample was mock-treated (no enzyme; lanes 1, 3, 5 and 7) and the other half was digested with EndoH (lanes 2, 4, 6 and 8). All samples were then subjected to SDS/PAGE through a 10% gel under reducing (lanes 1–4) or non-reducing conditions (lanes 5–8). The gel was fixed, dried and autoradiographed. Migration positions of the β -subunit (β), unglycosylated/deglycosylated α -subunit (α_0), singly (α) and doubly glycosylated (α') α -subunits and protein standards under reducing conditions are indicated in the Figure.

co-expression of both subunits; expression of α -subunits on their own resulted in the formation of insoluble aggregates which did not assemble with endogenous PDI [16]. Thus, to study the assembly of P4-H in mammalian cells, transfections were performed initially with constructs of both subunits to ensure that sufficient quantities were present for assembly. Levels of radio-labelled α -subunits and β -subunits present in metabolically labelled non-transfected cells were determined to be less than 5% of those present in labelled transfected cells (results not shown). Intact cells were treated with the alkylating reagent iodoacetamide prior to lysis to prevent subsequent disulphide bond formation or rearrangement. It should be noted that under native conditions assembled P4-H can be detected by co-immunoprecipitation of P4-H α -subunits using an antiserum specific for the β -subunit, whereas unassembled α -subunits can be detected using an antiserum raised to a peptide corresponding to the last 15 residues at the α -subunit C-terminus. This antiserum specifically recognizes α -subunits that are partially or fully unfolded and not associated with the β -subunit [9].

When cell lysates from COS-1 cells co-transfected with both α - and β -subunit constructs (from hereon referred to as $\alpha\beta$ -COS cells) were immunoprecipitated with the anti-(β -subunit) serum, two α -subunit glycoforms were co-immunoprecipitated, demonstrating that newly synthesized α -subunits assembled with β -subunits to form the P4-H complex (Figure 1, lane 1). The two glycoforms of the α -subunit (α and α') were identified by digestion of immunoprecipitated material with EndoH which resulted in the production of a single, faster-migrating deglycosylated α -subunit (α_0 ; Figure 1, lane 2). This agrees with previous work [12] which demonstrated that two glycoforms were produced from a single RNA transcript translated in a cell-free system. It should be noted that although the unglycosylated α -subunit was detected in the lysate (see below) only the glycosylated α -subunits appear to be complexed with the β -subunit (compare lanes 1 and 2 in Figure 1). In view of previous results both *in vitro* (results not

shown) and *in vivo* [13] which demonstrated that glycosylation of the α -subunit is not required for α - β subunit assembly, we propose that the absence of unglycosylated α -subunits from α - β complexes observed in this current work is simply due to the rapid co-translational glycosylation of nascent α -chains. Thus glycosylated full-length α -subunit predominates as the form of the α -subunit available for assembly with the β -subunit.

Immunoprecipitations using the anti-(α -subunit) antibody revealed the presence of soluble α -subunits in the cleared lysate (Figure 1, lane 3). All three forms of the α -subunit (α_0 , α and α') were detected, as verified by EndoH treatment which resulted in a single band co-migrating with α_0 (Figure 1, lane 4). It is evident that no β -subunits were present in these α -subunit immunoprecipitates, which indicates that the soluble α -subunits did not originate from assembled P4-H complexes. Thus, we conclude that the non-assembled α -subunits present *in vivo* are at least partially unfolded. The pellet obtained after cell lysis was also analysed for the presence of α -subunits by first solubilizing in SDS and then immunoprecipitating with the anti-(α -subunit) antibody. All three forms of α -subunit (α_0 , α and α') were present with the α -form predominating (results not shown; similar results in Figure 3).

We have shown previously that α -subunits containing intramolecular disulphide bonds have an increased electrophoretic mobility relative to that of reduced subunits. When immunoprecipitates from $\alpha\beta$ -COS cell lysates were analysed by SDS/PAGE without prior reduction a complicated pattern emerged (Figure 1, lanes 5–8). However, it is clear that α -subunits present in β -subunit immunoprecipitates migrated faster under non-reducing conditions (Figure 1, lanes 5 and 6) than under reducing conditions (lanes 1 and 2); the position of the β -subunit remained unchanged. Thus, the assembled α -subunits appear to be intramolecularly disulphide-bonded. Faster-migrating α -subunits were also evident in α -subunit immunoprecipitates (Figure 1, lanes 7 and 8), although there was also an increase in band smearing, possibly due to the presence of many differentially alkylated forms of the α -subunit. Thus, it is also possible that unassembled α -subunits are intramolecularly disulphide-bonded. It should be noted that no significant amount of radiolabelled material was found at the stacking/separating gel interface, indicating that the non-reduced subunits did not form large complexes or aggregates (results not shown). Similar analysis of α -subunits present in the cell pellet showed that these subunits were not intramolecularly disulphide-bonded (results not shown). Thus, co-expression of P4-H α - and β -subunits by transient transfection of COS-1 cells yielded immunoprecipitable P4-H complexes formed from newly synthesized α - and β -subunits. In addition, intramolecular disulphide bonds were detected within the assembled α -subunits and in unassembled, soluble α -subunits. This indicates that following translocation into the ER lumen newly synthesized α -subunits (i) assemble with the β -subunit to form a P4-H complex; or (ii) enter insoluble aggregates; or (iii) remain soluble but are not associated with the β -subunit.

In vivo dissociation of P4-H subunits in $\alpha\beta$ -COS cells

(i) Post-translational DTT treatment: recovery in the presence or absence of ATP

Previous work has shown that P4-H tetramers may be dissociated into subunits by treatment with the reducing agent DTT both *in vivo*, within chick embryo tendon cells [11], and *in vitro* [3], an effect later shown to be due to disruption of α -subunit intramolecular disulphide bonds [9]. We were therefore interested to study the effect of DTT on the stability of the P4-H complex formed within $\alpha\beta$ -COS cells. Thus, P4-H dissociation and

Figure 2 Post-translational DTT treatment and recovery of α - β complex in the presence or absence of ATP

$\alpha\beta$ -COS cells were metabolically labelled with [^{35} S]Met/Cys for 30 min before a short chase (15 min) in complete medium containing cycloheximide either in the absence (lanes 1–5) or the presence of DTT (7.5 mM, lanes 6–10). Cells were then washed and incubated in complete medium (A) or in ATP-depletion medium (B) with cycloheximide for the chase times indicated. Cells were lysed at the time points indicated and the soluble lysate fraction precleared before immunoprecipitation with anti-(β -subunit) serum. Washed immunoprecipitates were analysed under reducing conditions by SDS/PAGE through a 10% acrylamide gel. The gel was then fixed, dried and subjected to phosphorimage analysis. Migration positions of the α - and β -subunits are indicated in the Figure.

reassembly was examined by post-translational treatment of radiolabelled cells with DTT followed by chases of varying duration in the absence of DTT. Cells were metabolically labelled (30 min) and then incubated in complete medium with cycloheximide either in the absence (Figure 2A, lanes 1–5) or presence of DTT (7.5 mM; lanes 6–10) for 15 min. The cells were then incubated further for various times up to 2 h in complete medium containing cycloheximide alone. The concentration of DTT used was determined in preliminary experiments to produce an ~80% reduction in α - β complex compared with non-DTT-treated controls (Figure 2, compare lanes 1 and 6). Table 1 shows the relative yields of α -subunit co-immunoprecipitating with the β -subunit [expressed as a percentage relative to the control time point (see the legend to Table 1)]. It is apparent from these data that on removal of DTT, the levels of immunoprecipitated α -subunit recover to ~60% of the control. Clearly, α -subunits initially present in P4-H complexes become dissociated from the β -subunits on DTT treatment and then reassociate with the β -subunits during the chase. The DTT-induced dissociation of P4-H *in vivo* is thus largely reversible under these conditions, indicating that α -subunits dissociated from β -subunits within the ER of cells remain in a conformation that is still capable of assembling with the β -subunit after removal of DTT. This is in sharp contrast to the *in vitro* dissociation of the purified enzyme, as the α -subunit then forms insoluble aggregates and thus is incapable of reassociating with the β -subunit [6]. This is the first clear demonstration of P4-H dissociation and reassociation from its individual subunits. It is also interesting to note that over the period of the time-course in the control cells (Figure 2, lanes 1–5), the relative proportions of the glycosylated α and α' subunits (α : α') co-precipitating with the β -subunit altered from ~2:1 at 0 min chase, to ~1:1 after 120 min chase. This is probably due to a slow post-translational glycosylation of the singly glycosylated α -subunit.

Table 1 Effect of ATP-depletion on post-translational DTT treatment and recovery of the P4-H complex

Experiments were performed as detailed in the legend to Figure 2 where metabolically labelled $\alpha\beta$ -COS cells were post-translationally treated with or without DTT prior to a chase in complete medium (+ATP conditions, Figure 2A) or in ATP-depletion medium (–ATP conditions, Figure 2B) in the presence of cycloheximide. Cells were lysed after the chase times indicated and the soluble fraction of the cell lysate immunoprecipitated with anti-(β -subunit) serum in order to detect α - β complexes. Samples were subjected to SDS/PAGE under reducing conditions through 10% acrylamide gels and the dried gels were exposed to Fuji phosphorimage plates for the quantification of band intensities using BAS2000 software. A measure of the amount of P4-H complex present was calculated as the ratio (R) of total α -subunit band intensities ($\alpha + \alpha'$) to β -subunit band intensity present in the immunoprecipitates, thus $R = (\alpha + \alpha')/\beta$. For a given recovery condition (+ATP or –ATP), the percentage recovery of P4-H at each time point was then determined by the following equation: % recovery = $100 \times (R_{\text{DTT}}/R_{\text{CON}})$, where R_{DTT} is the ratio R determined after DTT treatment and R_{CON} is that determined under control conditions (no DTT treatment). The means of triplicate experiments are shown.

Chase-DTT (min)	Recovery (%)	
	+ATP	–ATP
0	15	16
10	26	23
30	34	26
60	51	29
120	60	25

The effect of ATP depletion on the post-translational re-assembly process was then studied by a similar experiment to that described above (Figure 2A) except that ATP-depletion medium was used as the final wash and recovery medium (Figure 2B). Recovery of the α - β complex did not occur in the absence of ATP (Figure 2, lanes 6–10). The control, non-DTT-treated cells showed that α - β complexes present at the end of labelling were not adversely affected by up to 2 h incubation in ATP-depletion medium (Figure 2, lanes 1–5). Comparison of relative yields (Table 1) indicated that the maximum recovery of α - β complexes under these conditions was ~25%, well below the 60% recovery observed in the presence of ATP. This indicated that following DTT-induced dissociation the reassembly of P4-H complex was ATP-dependent.

(ii) Co-translational DTT treatment

The previous experiment demonstrated that fully assembled P4-H could be dissociated into individual subunits by the post-translational addition of DTT. We were interested to determine whether DTT present during translation could prevent the assembly of P4-H and whether the α -subunits synthesized under these conditions were also capable of assembly after DTT removal. Preliminary experiments determined that metabolic labelling of cells in the presence of increasing concentrations of DTT (0.1–10 mM) produced a parallel decrease in α - β complex yield and that under these conditions the amount of immunoprecipitated labelled β -subunit remained approximately constant, indicating that protein synthesis was not significantly affected by the treatment (results not shown). However, the concentration of DTT required to produce an 80–90% decrease in α - β complex yield was ~2 mM, approximately threefold less than that required for post-translational DTT treatment (see above). Further experiments to follow the reassociation of α - and β -subunits were thus carried out with 2 mM DTT present during subunit synthesis (see below).

Figure 3 Expression of α -subunit alone: co-translational DTT treatment of α -COS cells and effect of ATP-depletion on recovery

COS-1 cells transiently transfected with pCMV5/ α alone (α -COS cells) were metabolically labelled with [^{35} S]Met/Cys for 30 min in the presence of DTT (2 mM). Cells were then washed and incubated in complete medium (+ATP conditions, lanes 1–5) or ATP-depletion medium (–ATP conditions, lanes 6–10) in the presence of cycloheximide. At the chase times indicated cells were washed and the soluble fraction of the cell lysate immunoprecipitated (i.p.) with antibodies to the β -subunit (A) or the α -subunit (B). In addition, the insoluble pellet fraction of the lysate was resuspended (see the Materials and methods section) and immunoprecipitated with the anti- α -subunit serum (C). Samples were analysed under reducing conditions by SDS/PAGE through 10% polyacrylamide gels. Gels were fixed and dried before phosphorimage analysis and autoradiography. The migration positions of α -subunits (α_0 , α and α'), β -subunit (β) and 78 kDa band (double arrowhead) are indicated in the Figure.

Dissociation of P4-H subunits in α -COS cells

The fact that post-translational dissociation of α – β complexes using DTT was shown to be reversible and that reassembly was ATP-dependent suggested that unfolded α -subunits may bind to chaperone molecules (such as BiP) [17] to prevent self-aggregation prior to refolding and reassembly. However, the presence of BiP in α - or β -subunit immunoprecipitations was not detected under the above experimental conditions using COS-1 cells transfected with both P4-H subunit constructs (see Figure 2). To perturb the expression system further COS-1 cells were transfected with the α -subunit construct alone (α -COS cells) as overexpression of the α -subunit alone was expected to lead to greater transient interactions with potential chaperone molecules. In addition, cells were lysed in the presence of EGTA and apyrase to preserve BiP complexes.

Analysis of P4-H dissociation and reassembly in α -COS cells produced essentially identical results to those observed using $\alpha\beta$ -COS cells, namely, that α -subunits assembled into α – β complexes following both post- (results not shown) and co-translational DTT treatment (Figure 3A). Lysates and cell pellets were also analysed for the presence of α -subunits (Figures 3B and 3C, respectively). Thus, in addition to the chase-dependent increase in the amount of α – β complex, it is clear that there was also a parallel decrease in the amount of α -subunits present in the lysate and an increase in the amount of α -subunits present in the pellet. Unlike the results observed for $\alpha\beta$ -COS cells (Figure 2), both β - and α -subunit immunoprecipitates from α -COS cells possessed an additional protein band of ~ 78 kDa. This additional species was absent when the lysate was denatured with SDS prior to immunoprecipitation (only α - or β -subunits were immuno-

Figure 4 Interaction of α -subunits with 78 kDa protein in α -COS cells: identification of the 78 kDa protein as BiP

α -COS cells were metabolically labelled with [^{35} S]Met/Cys for 30 min in the presence of DTT (2 mM) and were lysed either immediately after labelling (A) or after washing and chasing in either complete medium (+ATP, B) or ATP-depletion medium (–ATP, C) in the presence of cycloheximide. Cells in one of each pair of duplicate plates were lysed in the presence of EDTA, EGTA and apyrase (–ATP in lysis, lanes 1, 3 and 5), or ATP/MgCl₂ (+ATP in lysis, lanes 2, 4 and 6). Samples were analysed under reducing conditions by SDS/PAGE through 10% polyacrylamide gels. Gels were fixed and dried before phosphorimage analysis and autoradiography. The migration positions of α -subunits (α_0 , α and α'), β -subunit (β) and the 78 kDa band (BiP) are indicated in the Figure. The putative untrimmed α -subunit glycoform (see the text) is indicated with an asterisk.

precipitated under these conditions), indicating that this protein was not recognized by the subunit-specific antibodies (results not shown). Thus, the 78 kDa protein was present specifically in native immunoprecipitations non-covalently complexed with either subunit. In addition, the reassembly of P4-H complexes after co-translational DTT treatment was ATP-dependent (compare lanes 1–5 and 6–10, Figure 3A).

Interaction of free α -subunits with BiP

To identify the 78 kDa band as BiP and to determine the role of this protein in P4-H biosynthesis, α -COS cells were treated co-translationally with DTT and analysed by immunoprecipitation immediately after the labelling period (Figure 4A), and after a 120 min chase in either complete (Figure 4B) or ATP-depletion medium (Figure 4C). In addition, cells were lysed in the presence either of EDTA and EGTA (both 5 mM; –ATP lanes) or of ATP and MgCl₂ (both 2 mM; +ATP lanes), conditions which were reported to preserve or dissociate complexes of proteins with BiP, respectively [18]. Several points are clear from these results. First, under conditions where ATP hydrolysis was prevented (–ATP lanes) significant amounts of 78 kDa protein were co-immunoprecipitated with both α - and β -subunit antibodies (lanes 1–4, Figures 4A to 4C) and this 78 kDa band co-migrated with endogenous BiP immunoprecipitated with anti-BiP monoclonal antibody (lanes 5 and 6, Figures 4A to 4C). Conversely, the BiP immunoprecipitates contained α -subunits; both glycoforms α and α' were present (lane 5, Figures 4A to 4C) with the α form predominating. The amount of α -subunits co-

immunoprecipitated with BiP was greatest immediately after the labelling in the presence of DTT (lane 5, Figure 4A) and decreased significantly after chase under both conditions (lane 5, Figures 4B and 4C). An additional band was also present in the BiP immunoprecipitates which migrated slightly slower than the α' form and which was also present in α -subunit immunoprecipitates from cells chased in ATP-depletion medium (lanes 3 and 4, Figures 4B and 4C). It is possible that this protein is an untrimmed α -subunit glycoform; however, more work is necessary to support this conclusion. Furthermore, lysis of cells in the presence of ATP/MgCl₂ (+ATP lanes) led to a reduction in the co-immunoprecipitation of BiP with anti-(α -subunit) serum (compare lanes 3 and 4, Figures 4A and 4B) and in the α -subunits co-immunoprecipitated with anti-BiP serum (compare lanes 5 and 6, Figures 4A to 4C). Thus, the BiP- α -subunit interaction was ATP-sensitive. It should be noted that preservation of BiP complexes is strongly dependent on buffer conditions as previously described [18a], with yields of protein complex reduced in the presence of detergent and moderate salt concentrations, and thus the comparatively low yield of α -subunits co-immunoprecipitating with BiP in our buffer system is an underestimate of such complexes. In addition the release of BiP-bound α -subunits seems to be only partial, which may be a consequence of the release conditions used (2 mM ATP/MgCl₂, 30 min on ice). These conditions were chosen to maximize release from BiP without resulting in significant effects on the relatively unstable α - β complex. Thus, incubation of lysates at 37 °C or lengthy incubations on ice [19] were avoided. In conclusion, these results indicate that a complex was formed between newly synthesized α -subunits and BiP and that release of α -subunits from this complex was dependent upon the presence of ATP.

DISCUSSION

We have studied the synthesis and assembly of the enzyme P4-H by transiently transfecting COS-1 cells with its constituent subunits under various conditions. Expression of the α -subunit in this system resulted in the synthesis of di-, mono- and unglycosylated subunits. Under the appropriate conditions, both α -subunit glycoforms assembled with the β -subunit. All three forms of the α -subunit were detectable in both the soluble fraction and the insoluble (cell pellet) fraction of cell lysates using an antibody which recognizes only unassembled α -subunits. In addition, intramolecular disulphide bonds were detected in the assembled α -subunits and in a proportion of the non-assembled, soluble α -subunits. Thus, after translocation into the ER lumen newly synthesized α -subunits either assembled with the β -subunit to form soluble α - β complex, remained soluble but not β -subunit-associated, or became a component of aggregates.

We have also shown that the assembled α - β complex can be disrupted *in vivo* by post-translational treatment with the reducing agent DTT and that the complex reassembles on removal of DTT. The effect of DTT was essentially reversible with recovery of approx. 60% of α - β complex after 2 h chase in the absence of DTT when compared with control cells. A similar time-dependent appearance of α - β complex was observed after incubating cells in the absence of DTT following co-translational reduction (cells radiolabelled in the presence of DTT). Previous work indicated that no intersubunit (α - β) disulphide bonds are present in the P4-H complex [4,20] (D. C. A. John and N. Bulleid, unpublished work), that β -subunit disulphide bonds are not required for assembly of active P4-H tetramers [8], and that the α -subunit possesses intramolecular disulphide bonds that are required for α - β complex formation [9]. Considering the above

evidence and the demonstration of intramolecular disulphide bonds in the assembled α -subunits shown here, we believe that the DTT-mediated effect observed *in vivo* is not a result of reduction of β -subunit residues. Thus, we conclude that the DTT-induced α - β dissociation is due to the reduction of critical α -subunit intramolecular disulphide bond(s) and that subsequent reassociation of P4-H subunits on removal of DTT occurs as a result of re-oxidation of the critical α -subunit cysteine residues.

It is important to consider the possible roles of PDI in the P4-H complex, although it is difficult to experimentally determine these separate roles independently. However, at least one role for the β -subunit/PDI has been clearly demonstrated, namely that the C-terminal -KDEL sequence of the β -subunit/PDI is required for retention of the P4-H complex within the ER lumen [8]. Given that β -subunit PDI activity is not required for the catalytic activity of P4-H tetramers [8,21] and that, in an insect cell system, expression of α -subunits in the absence of β -subunits led to the formation of insoluble α -subunit aggregates [16], the second role of PDI/ β -subunit could be to provide a structural framework for binding and preserving native α -subunits in a catalytically active, non-aggregated conformation. In addition, there is some evidence to suggest that non-active site sequences of the β -subunit/PDI are involved with the α -subunit in forming parts of the P4-H catalytic sites [1]. As PDI is generally accepted as being the *in vivo* catalyst of disulphide bond formation in proteins destined for export from the ER [2] it is also possible that a further function of β -subunit/PDI may be to catalyse the formation of intramolecular disulphide bonds in the α -subunit of P4-H.

Implicit in the results presented here and our previous finding that intramolecularly disulphide-bonded α -subunits are required for the formation of stable α - β complexes [9], is the fact that the β -subunit does not form stable interactions with unfolded/non-disulphide-bonded α -subunits. Clearly, if PDI does catalyse α -subunit disulphide bond formation then this must occur prior to stable α - β subunit complex formation and accordingly, the α -subunit motifs necessary for stable α - β interactions must only be presented by the fully folded α -subunit. Recognition of these motifs by the β -subunit/PDI may occur immediately after PDI/ β -subunit catalysis of 'native' α -subunit disulphide bond formation or possibly following further maturation of the α -subunit structure. Thus, newly folded α -subunits may bind stably to the same β -subunit/PDI molecule that catalysed their folding or may bind to a different β -subunit/PDI molecule on completion of PDI-mediated intramolecular disulphide bond formation. If PDI does indeed catalyse α -subunit disulphide bond formation then the results [8] showing that PDI-inactive β -subunits co-expressed with α -subunits in insect cells assemble to form functional P4-H complexes strongly suggest that the latter situation occurs. In those studies the presence of a low level of endogenous PDI activity may have been adequate to catalyse α -subunit disulphide bond formation prior to complex formation with the co-expressed (PDI-inactive) β -subunits. Thus, in insect cells at least, an α -subunit molecule is not required to form an obligate complex with the β -subunit/PDI molecule which has catalysed the formation of its intramolecular disulphide bond(s). Clearly, the roles of the β -subunit/PDI in the formation, stability and activity of the P4-H complex are difficult to dissect experimentally, and as yet no direct evidence for the PDI-catalysed formation of α -subunit disulphide bonds has been shown. One implication of this model may be a possible requirement for chaperone molecules to mask the potential β -subunit-interacting surfaces of the α -subunit prior to folding of the α -subunit to form its correct disulphide-bonded conformation.

The participation of *in vivo* factors such as ER chaperones in P4-H biosynthesis was suggested by previous work in a cell-free

translation system in which α -subunits synthesized under conditions that did not allow nascent chain disulphide bond formation and α -subunit folding mutants were detected bound to BiP [9,12]. Interestingly, in preliminary experiments (D. C. A. John and N. J. Bulleid, unpublished work) we detected no significant interaction between the α -subunit and the glycoprotein chaperones calnexin and calreticulin [22]. The discrepancy between our *in vivo* results, which demonstrate post-translational dissociation and subsequent reassembly of P4-H complex, and those of analogous *in vitro* experiments which resulted in loss of α -subunit due to the formation of insoluble aggregates ([6,11]; D. C. A. John and N. J. Bulleid, unpublished work) also suggests the presence of factors assisting α -subunit folding and thus P4-H assembly *in vivo*. Our results now provide several lines of evidence to suggest that a 78 kDa protein participates in the *in vivo* processes resulting in recovery of α - β complexes following co- and post-translational reduction procedures and that this protein is BiP. First, following both co- and post-translational reduction procedures the recovery of α - β complexes was found to be ATP-dependent. Since the binding of unfolded proteins to the chaperone BiP requires ATP and the release of bound protein to refold requires ATP hydrolysis [17], then the observed ATP-dependence of the recovery of α - β complex after co- or post-translational reduction agrees with the suggestion that BiP may interact with unfolded, non- β -subunit-associated α -subunits to preserve them in an assembly-competent state. Secondly, the 78 kDa protein, seen as a minor protein band co-immunoprecipitating from α -COS cell lysates immunoprecipitated with anti- α or anti- β serum, co-migrated with endogenous BiP immunoprecipitated with BiP monoclonal antibody. Thirdly, the presence of the 78 kDa protein in α -subunit immunoprecipitates was sensitive to the addition of ATP/MgCl₂ during cell lysis, conditions that would result in release of BiP-bound polypeptides. Fourthly, α -subunits were detected in BiP immunoprecipitates and this co-immunoprecipitation was sensitive to the addition of ATP/MgCl₂ during lysis. Lastly, the quantity of α -subunits co-immunoprecipitating with BiP decreased after chases performed under both -ATP and +ATP conditions. The above evidence indicates that the 78 kDa protein was indeed BiP. Thus, we consider it likely that after labelling in the presence of DTT and removal of DTT, α -subunits undergo cycles of binding and release with BiP and on attaining native (assembly-competent) structures form complexes with the β -subunit, whereas, under conditions of ATP-depletion, α -subunits may be released from BiP but are unable to re-bind and may thus aggregate and be degraded.

In conclusion, we propose that under normal synthesis conditions the free P4-H α -subunits are bound and released from

BiP until the α -subunit possesses adequate secondary structure to prevent aggregation and/or until intramolecular disulphide bonds are formed. The disulphide-bonded α -subunit then binds to the β -subunit and associates into $\alpha_2\beta_2$ tetramers. Thus, the α -subunit may interact sequentially, first with BiP and then with the β -subunit/PDI.

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REFERENCES

- 1 Kivirikko, K. I., Myllyla, R. and Pihlajaniemi, T. (1992) in *Post Translational Modification of Proteins*, (Harding, J. J. and Crabbe, M. J. C., eds.), pp. 1–51, CRC Press, London
- 2 Freedman, R. B., Hirst, T. R. and Tuite, M. F. (1994) *Trends Biochem. Sci.* **19**, 331–336
- 3 Berg, R. A., Kao, W. W.-Y. and Kedarsha, N. L. (1980) *Biochem. J.* **189**, 491–499
- 4 Nietfeld, J. J., Van der Kraan, J. and Kemp, A. (1981) *Biochim. Biophys. Acta* **661**, 21–27
- 5 Koivu, J. and Myllyla, R. (1986) *Biochemistry* **25**, 5982–5986
- 6 Tuderman, L., Kuuti, E.-R. and Kivirikko, K. I. (1975) *Eur. J. Biochem.* **60**, 399–405
- 7 Wetterau, J. R., Combs, K. A., McLean, L. R., Spinner, S. N. and Aggerbeck, L. P. (1991) *Biochemistry* **30**, 9728–9735
- 8 Vuori, K., Pihlajaniemi, T., Myllyla, R. and Kivirikko, K. I. (1992) *EMBO J.* **11**, 4213–4217
- 9 John, D. C. A. and Bulleid, N. J. (1994) *Biochemistry* **33**, 14018–14025
- 10 Berg, R. A. and Prockop, D. J. (1973) *Biochem. Biophys. Res. Commun.* **52**, 115–120
- 11 Tuderman, L., Oikarinen, A. and Kivirikko, K. I. (1977) *Eur. J. Biochem.* **78**, 547–556
- 12 John, D. C. A., Grant, M. E. and Bulleid, N. J. (1993) *EMBO J.* **12**, 1587–1595
- 13 Lamberg, A., Pihlajaniemi, T. and Kivirikko, K. I. (1995) *J. Biol. Chem.* **270**, 9926–9931
- 14 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning — A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 15 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 16 Vuori, K., Pihlajaniemi, T., Marttila, M. and Kivirikko, K. I. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7467–7470
- 17 Gething, M.-J., McKammon, K. and Sambrook, J. (1986) *Cell* **46**, 939–950
- 18 Kuznetsov, G., Chen, L. B. and Nigam, S. K. (1994) *J. Biol. Chem.* **269**, 22990–22995
- 18a Knittler, M. R. and Haas, I. G. (1992) *EMBO J.* **11**, 1573–1581
- 19 Gaut, J. R. and Hendershot, L. M. (1993) *J. Biol. Chem.* **268**, 7248–7255
- 20 Prockop, D. J., Berg, R. A., Kivirikko, K. I. and Uitto, J. (1976) in *Biochemistry of Collagen* (Ramachandran, G. N. and Reddi, A. H., eds.), pp. 163–273, Plenum Press, London
- 21 Myllyla, R., Kaska, D. D. and Kivirikko, K. I. (1989) *Biochem. J.* **263**, 609–611
- 22 Hammond, C. and Helenius, A. (1993) *Curr. Biol.* **3**, 884–886