Prolyl 4-Hydroxylase: Defective Assembly of α-Subunit Mutants Indicates That Assembled α-Subunits Are Intramolecularly Disulfide Bonded†

David C. A. John and Neil J. Bulleid*

School of Biological Sciences, University of Manchester, 2.205, Stopford Building, Oxford Road, Manchester, U.K. M13 9PT

Received July 26, 1994; Revised Manuscript Received September 1, 1994®

ABSTRACT: The vital hydroxylation of peptidyl proline residues in collagens and proteins with collagen-like amino acid sequences is catalyzed by the tetrameric enzyme prolyl 4-hydroxylase (P4-H). We have previously detailed [John et al. (1993) *EMBO J. 12,* 1587−1595] the redox-dependent assembly of the catalytically important α-subunit (64 kDa) in a cell−free system containing endogenous β-subunits (PDI, 60 kDa). To identify the origin of this redox-dependent assembly, we have now shown directly by an electrophoretic mobility shift assay that the assembled wild-type protein possesses at least one intramolecular disulfide bond. We also analyzed five α-subunit mutants that have single Cys to Ser mutations in one of the five Cys residues present in the wild-type protein and found that (i) subunits mutated at Cys150 or Cys511 formed intramolecular disulfide bonds, whereas subunits mutated at Cys276, Cys293, or Cys486 did not, (ii) mutation of Cys276, Cys293, or Cys486 led to a large reduction in α−β complex formation, (iii) subunits mutated at Cys276, Cys293, Cys486, or Cys511 were recognized by an antiserum raised against an α-subunit C-terminal peptide which failed to recognize the assembled wild-type subunit or the assembled subunit mutated at Cys150, and (iv) the assembled complexes fractionated in a similar position to the purified protein on sucrose gradients whereas the assembly-defective mutants formed higher molecular weight aggregates or complexes with other proteins. On the basis of these results, we propose that P4-H α-subunits possess an intramolecular disulfide bond between Cys276 and Cys293 that is essential for α−β complex formation.

Prolyl 4-hydroxylase (P4-H;[1] EC 1.14.11.2) catalyzes the vital co- and posttranslational hydroxylation of proline residues in collagens and proteins with collagen-like amino acid sequences [reviewed by Kivirikko et al. (1992)]. This modification occurs within the lumen of the endoplasmic reticulum (ER) and is essential for the formation of stable triple-helical molecules from newly-synthesized procollagen monomers. Inhibition of P4-H leads to the accumulation of unhydroxylated chains within the ER which then may be degraded and/or slowly secreted as nonfunctional protein (Prockop et al., 1976). Consequently, P4-H has been investigated as a target for the therapeutic modulation of various fibrotic disorders which are characterized by excessive collagen deposition. Thus, the hydroxylation reaction has been extensively studied (Kivirikko et al., 1992) and effective inhibitors synthesized (Bickel et al., 1991; Baader et al., 1994), but as yet little is known about the structure of the catalytically important α-subunit due to its tendency to aggregate following dissociation of the P4-H tetramer (Tuderman et al., 1975, 1977).

Vertebrate P4-H is an $\alpha_2\beta_2$ tetramer consisting of the glycosylated α-subunit (64 kDa) and the β-subunit (60 kDa) which is the multifunctional enzyme protein disulfide isomerase [PDI; reviewed by Freedman (1990)]. In addition to its proline-rich substrate, P4-H requires Fe(II), O_2, 2-oxoglutarate, and ascorbate for the hydroxylation reaction. The P4-H tetramer appears to assemble from newly-synthesized α-subunits and an endogenous pool of β-subunits present *in vivo* (Berg et al., 1980). Several lines of evidence suggest that the α-subunit is the catalytically more important subunit. First, P4-H is isolated as a 65 kDa monomer from the green algae *Chlamydomonas, Enteromorpha,* and *Volvox* (Kaska et al., 1987), and this 65 kDa protein is antigenically related to the vertebrate α-subunit (Kaska et al., 1988). Second, several photoaffinity-labeling studies have shown that the binding sites of 2-oxoglutarate and the peptide substrate are both located in the α-subunit [reviewed by Kivirikko et al. (1992)]. Third, diethyl pyrocarbonate covalent modification of vertebrate and algal P4-H suggests that α-subunit histidine residues may function as Fe(II)-binding ligands in the P4-H catalytic site (Myllyla et al., 1992). Thus, the bulk of the catalytic site residues are derived from the α-subunit. However, detailed studies on the isolated α-subunit have not been possible due to its tendency to aggregate and become insoluble on dissociation from the β-subunit (Tuderman et al., 1975, 1977).

The α-subunit of P4-H has five cysteine residues and may possess intrachain disulfide bond(s) (Prockop et al., 1976) although direct evidence for this has not yet been shown. Analysis of P4-H synthesized in chick embryo tendon cells indicates that the P4-H tetramer is dissociated by DTT (Tuderman et al., 1977). Studies *in vitro* indicate that purified P4-H may be dissociated to monomers by incubation in DTT (Berg & Prockop, 1973); however, the tetramers may also be dissociated to monomers by urea in the absence of reduction (Nietfeld et al., 1981). Thus, the reduction of

† D.C.A.J. is a research associate funded by the Wellcome Trust (Grant 33035), and N.J.B. is a Royal Society Research Fellow.

* Author to whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* October 15, 1994.

[1] Abbreviations: BiP, immunoglobulin heavy chain binding protein; DTT, dithiothreitol (reduced form); ER, endoplasmic reticulum; GSSG, glutathione disulfide; P4-H, prolyl 4-hydroxylase; PDI, protein disulfide isomerase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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disulfide bonds appears to be nonessential for the conversion of P4-H tetramers to monomers and suggests that disulfide bond(s) in the P4-H tetramer is (are) formed within single subunits and not between subunits.

The β-subunit of P4-H seems to have at least two functions in the tetramer, namely, maintenance of the α-subunit in an active, nonaggregated conformation (Koivu & Myllyla, 1986; John et al., 1993) and retention of the P4-H tetramer within the ER via its carboxyl-terminal KDEL sequence (Vuori et al., 1992). Studies on the activation of stored P4-H preparations by addition of PDI also suggest that PDI catalyzes the rearrangement of non-native disulfides and/or reduction of disulfides in intact enzyme tetramers to produce maximum P4-H activity (Koivu & Myllyla, 1986). Consistent with this are results from a baculovirus expression system which suggest that the PDI activity of β-subunits may not be required for β-subunits to assemble with α-subunits into tetramers and that the PDI activity of the assembled β-subunits is not required for the P4-H activity of the resulting tetramers (Vuori et al., 1992). These results confirmed previous reports that P4-H activity does not necessarily require PDI activity (Myllyla et al., 1989). Thus, the β-subunits are thought to play a mainly structural role in the P4-H tetramer, keeping the α-subunits in an active conformation and retaining the enzyme within the ER, although a possible role of PDI activity in the assembly of the enzyme cannot yet be excluded.

To study the assembly of this enzyme, we translated P4-H α-subunit RNA transcripts in a rabbit reticulocyte lysate system supplemented with canine pancreatic microsomes, which contain PDI (β-subunit). Our previous results indicate that under conditions allowing disulfide bond formation (GSSG oxidant added) translated α-subunits assembled with the pool of β-subunits to form mainly dimers and some tetramers (John et al., 1993). Under conditions where disulfide bond formation was unlikely to occur, the α-subunit did not complex with the β, but became aggregated and was associated with the chaperone BiP. In this present work, we show directly that wild-type α-subunits synthesized in the presence of GSSG possess intramolecular disulfide bonds and have examined the properties of five α-subunit mutants that have single cysteine to serine mutations at one of the five cysteines present in the wild-type α-subunit sequence. Our results suggest that the native P4-H α-subunit possesses at least one intramolecular disulfide bond (between Cys276 and Cys293) which is necessary for the formation of a stable α-β subunit complex. The possible role of the β-subunit (PDI) in the formation of α-subunit disulfide bonds and in P4-H synthesis and assembly is discussed in relation to the results presented here.

EXPERIMENTAL PROCEDURES

Materials. ^{14}C-Labeled SDS−PAGE protein molecular weight standards were prepared by reductive methylation of lysyl residues using the procedure of Dottavio-Martin and Ravel (1978). Microsomal vesicles were prepared from canine pancreas as described previously (Austen et al., 1984). Purified chick P4-H was a gift from Dr. T. J. Franklin, Zeneca Pharmaceuticals, Alderley Park, U.K. The carboxyl-terminal peptide sequence of rat P4-H α-subunit ($_{H_2N}$QEFRRPAbuTLSELE$_{COOH}$; Abu = 2-aminobutyric acid in place of Cys) was conjugated to keyhole limpet hemocyanin via *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester by the method of Kitagawa and Aikawa (1976). This conjugate was used to raise polyclonal anti-P4-H α-subunit sera in rabbits.

Plasmid Constructs, Mutagenesis, and Transcription. Plasmid pDJ2 containing rat P4-H α-subunit sequence was prepared as described previously (John et al., 1993). Uracil−DNA site-directed mutagenesis of rat α-subunit sequence was carried out by the method of Kunkel (Kunkel et al., 1987). Mutagenic oligonucleotides producing Cys to Ser changes in the rat α-subunit sequence were as follows (all sequences 5′-3′): Cys150-Ser150, GCT GAG GAC AGC TTT GAG TTG; Cys276-Ser276, GAA ATG CTG AGC CGT GGG GAG; Cys293-Ser293, AGG CTG TTC AGC CGC TAC CAT; Cys486-Ser486, CAC GCA GCC TCT CCT GTG CTA; Cys511-Ser511; CGA AGG CCG TCT ACC CTG TCA. Plasmids containing either wild-type or mutated α-subunit sequence were linearized with *Sal*I restriction enzyme prior to transcription when full-length transcripts were required. A truncated form of the α-subunit-coding sequence was produced by *Sfu*I digestion of wild-type plasmid DNA in order to generate a truncated RNA transcript, lacking the last 10 amino acid codons of the α-subunit. All linearized plasmids were transcribed using T3 RNA polymerase as described previously (Gurevich et al., 1991).

Cell-Free Translation and Puromycin and Apyrase Treatments. Cell-free translation was carried out in a rabbit reticulocyte lysate system supplemented with canine pancreatic microsomal membranes. In the absence of exogenously added GSSG, the system translated proteins under conditions where the formation of disulfide bonds was unfavorable, whereas addition of GSSG at an optimized concentration (3 mM) enabled disulfide bonds to form in nascent polypeptides. Translation mixtures (25 μL) contained rabbit reticulocyte lysate (Promega, 18 μL), [^{35}S]-methionine (Amersham, 15 μCi, >1000 Ci/mmol), 19 amino acids minus methionine (20 μM each), RNA transcript (0.5 μg), canine pancreatic microsomes (0.12 A_{280} units), and GSSG (3 mM) where appropriate. Translations (60 min, 30 °C) were terminated by incubation on ice, in the presence of 50 mM NEM where indicated, prior to further procedures.

Puromycin (1 mg/mL) was added posttranslationally to translations of truncated RNA transcripts—addition of puromycin ensures release of transcripts containing no stop codons from the translation/translocation complex into the lumen of the microsomes (Connolly & Gilmore, 1986). Further incubation (5 min, 30 °C) was then allowed before other posttranslational procedures.

Posttranslational Isolation of Microsomes, Sodium Carbonate Treatment of Microsomes, and Immunoprecipitation. Microsomal vesicles were isolated from translation mixtures by centrifugation (150000*g*, 10 min 4 °C) through a sucrose cushion (50 mM HEPES−KOH, pH 7.5, 100 mM KCl, and 0.5 M sucrose). The pellet was resuspended in buffer (50 mM HEPES−KOH, pH 7.5, 100 mM KCl, and 0.25 M sucrose), and this sample was spun through a sucrose cushion as above. The final microsome pellet was resuspended in a buffer appropriate to the subsequent procedure.

Sucrose cushion isolated microsomes were subjected to sodium carbonate treatment by a modified method derived from that of Fujiki et al. (1982). Isolated microsomes were resuspended in 300 μL of ice-cold sodium carbonate (0.1

M, pH 11.5) and incubated on ice for 10 min followed by centrifugation (150000*g*, 10 min) to recover pellet and supernatant fractions. This procedure was repeated on the pellet fraction, and the two supernatants of the spins were pooled and precipitated with 10% (w/v) trichloroacetic acid. Pellets and supernatant TCA precipitates were then resuspended in equal volumes of SDS−PAGE loading buffer.

Immunoprecipitation of translation products present in isolated microsomes was carried out at 4 °C in 1 mL of buffer A (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, and 0.02% sodium azide). Samples were precleared for 30 min with 40 μL of protein A−Sepharose (10% v/v suspension) and immunoprecipitated for 2 h with 1 μL of the appropriate polyclonal antiserum. The samples were then incubated for 30 min with 40 μL of protein A−Sepharose suspension before recovery of the immunoprecipitates by centrifugation. Protein A−Sepharose immobilized immunoprecipitates were then washed twice in 1 mL of buffer A, once in 1 mL of buffer A containing 0.5 M NaCl, and finally once in 50 mM Tris, pH 7.5, buffer. Samples were resuspended in SDS−PAGE sample buffer prior to electrophoresis.

Sucrose Gradient Analysis. Microsomes were isolated from translation mixtures by centrifugation through sucrose cushions and resuspended in 0.5 mL of LM buffer (50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 6 mM lauryl maltoside, and 1 mM PMSF; Segal et al., 1992). Following incubation on ice (15 min), this sample was loaded onto a 5−25% sucrose gradient (9 mL) with a base of 50% sucrose (0.5 mL) prepared in LM buffer and subjected to centrifugation for 16 h at 40 000 rpm in a Beckman SW40 rotor at 4 °C. Gradient fractions were collected, precipitated with trichloroacetic acid (12.5%), and analyzed by SDS−PAGE under reducing conditions.

SDS−PAGE. 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, and 0.002% (w/v) bromophenol blue]. For samples to be run under reducing conditions, DTT was added (25 mM), and the samples were boiled for 10 min. Samples to be run under nonreduced conditions were boiled in the absence of DTT. Reduced and nonreduced samples that were to be run in adjacent lanes were boiled as above and treated with *N*-ethylmaleimide (100 mM) after cooling to room temperature. Samples were subjected to electrophoresis through 10% polyacrylamide gels in the presence of SDS by the method of Laemmli (1970). Gels were dried and autoradiographed at room temperature. Quantification of band intensities was carried out by phosphorimage analysis or by scanning densitometry.

RESULTS

Cell-Free Synthesis of P4-H: α-Subunits Are Intramolecularly Disulfide Bonded. In our previous work (John et al., 1993), we have shown that assembly of newly synthesized P4-H α-subunits with preformed β-subunits in a cell-free system occurred only in translations carried out in the presence of oxidized glutathione (GSSG), i.e., under conditions that allow nascent chain disulfide bond formation. To determine whether disulfide bonds were formed during translation, we purified microsomes from translations by centrifugation through sucrose cushions and analyzed the full-length translation products by SDS−PAGE under nonreducing or reducing conditions (Figure 1, lanes A or B, respectively). As shown previously (John et al., 1993), under reducing conditions the newly synthesized wild-type α-subunits appear as three bands of approximately 60−64 kDa (e.g., lane 1B) which were identified as nonglycosylated (60 kDa, lowest band), singly glycosylated (62 kDa, middle band), and doubly glycosylated (64 kDa, upper band) α-subunits. However, wild-type α-subunits synthesized in the presence of GSSG and posttranslationally treated with NEM (to block disulfide bond formation/rearrangement during work-up) migrated as two (or more) diffuse bands (lane 2A) of increased electrophoretic mobility relative to the reduced sample (lane 2B). This band shift indicates that intramolecular disulfide bonds were present in α-subunits synthesized under the above conditions—intramolecularly disulfide-bonded proteins generally have a lower hydrodynamic volume than their reduced form and thus migrate faster in denaturing gels (Goldenberg & Creighton, 1984). Similar analysis of wild-type α-subunits synthesized in the presence of DTT showed no such band shift (compare lanes 1A and 2A), indicating that disulfide bonds were not formed under these conditions.

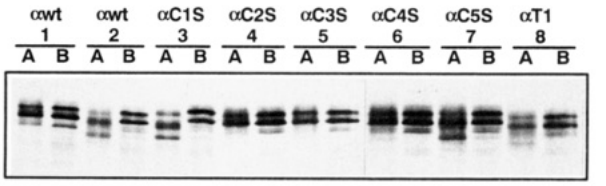

FIGURE 1: Comparison of P4-H α-subunit electrophoretic mobility on SDS−PAGE under reducing and nonreducing conditions. Translation of α-subunit mRNA was carried out using the transcripts as indicated in the figure. All translations were carried out in the presence of GSSG (3 mM) except for translation 1 where DTT (4 mM) was present. Following translation, the samples were placed on ice and split into two equal aliquots (A and B). NEM (50 mM) was added to samples A, and the samples were incubated on ice for 10 min. Both sets of samples were then spun through sucrose cushions twice and the resulting microsome pellets resuspended by heating (10 min, 90 °C) in SDS−PAGE loading buffer in the absence (samples A) or presence of DTT (25 mM, samples B). Samples were then cooled to room temperature, and NEM (100 mM) was added to samples B followed by a further incubation of 15 min. All samples were then subjected to SDS−PAGE through a 10% gel which was fixed, dried, and autoradiographed.

As the P4-H α-subunit contains five cysteine residues, it may possess up to two intramolecular disulfide bonds. To determine the cysteine residues involved in disulfide bond formation, single cysteine to serine mutations were introduced into the wild-type α-subunit sequence. The resulting *in vitro* synthesized α-subunit mutants were designated αC1S to αC5S, representing cysteine to serine mutations in residues 150, 276, 293, 486, and 511, respectively (Figure 2). Each mutant α-subunit RNA transcript was translated in the presence of GSSG and analyzed for disulfide bond formation in the same way as the wild type (see above). It is clear that the α-subunit mutants were all translated and glycosylated in the presence of microsomes, producing two glycosylated forms of similar electrophoretic mobility to the wild type (Figure 1, lanes 3B−7B). Thus, the single cysteine to serine mutations did not alter the pattern of single and double glycosylation as observed for the wild-type α-subunit. However, analysis of the mutant α-subunits under nonreducing conditions revealed that αC1S (lane 3A) and αC5S (lane 7A) alone showed an increase in electrophoretic

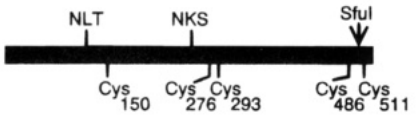

FIGURE 2: Schematic diagram of the mature rat P4-H α-subunit sequence. The mature protein contains 517 amino acids and includes 2 sites for N-linked glycosylation (NLT and NKS). Mutations were introduced individually at each of the five cysteine residues by site-directed mutagenesis. The following notation was used for the mutant α-subunits encoding single Cys to Ser mutations: αC1S, Cys150 to Ser150; αC2S, Cys276 to Ser276; αC3S, Cys293 to Ser293; αC4S, Cys486 to Ser486; αC5S, Cys511 to Ser511. A truncated α-subunit (αT1) was translated from a truncated transcript produced by transcription of coding sequence digested by *Sfu*I—the resulting subunit was truncated at ~Phe507.

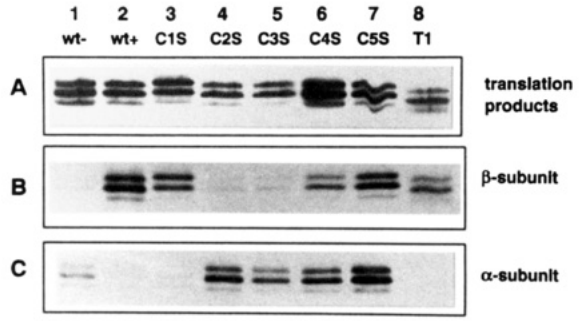

FIGURE 3: Immunoprecipitation analysis of α-subunit mutants. Panels A−C contain samples from translations of α-subunit transcripts as indicated. All translations were carried out in the presence (lanes 2−8) or absence (lane 1) of GSSG. All samples were treated with NEM (50 mM) immediately after termination of translation. Panel A shows the translocated α-subunit bands present in microsomes isolated from translations by centrifugation through sucrose cushions. Three bands of approximately 60−64 kDa were seen: nonglycosylated (60 kDa, lower band), singly glycosylated (62 kDa, middle band), and doubly-glycosylated (64 kDa, upper band) α-subunits. The truncated forms (lane 8) migrate slightly faster than the full-length products. Panel B, samples coimmunoprecipitated with β-subunit antiserum. Panel C, samples immunoprecipitated with α-subunit antiserum. All samples were subjected to SDS−PAGE in 10% gels under reducing conditions. Gels were fixed, dried, and autoradiographed.

mobility, indicating the formation of intramolecular disulfides. A truncated form of the α-subunit (αT1), lacking the last 10 amino acids at the carboxyl terminus (including Cys511; see Figure 2), appeared to form a small proportion of disulfide-bonded subunits (compare lanes 8A and 8B).

Effect of Single Cysteine to Serine Mutations on the Formation of the α-β Complex and α-Subunit Structure. To determine whether mutant α-subunits synthesized in the presence of GSSG assembled with the endogenous β-subunits, translation products were immunoprecipitated using antibodies to the β-subunit; coimmunoprecipitation of α-subunits using this antiserum indicates that α−β complexes have formed. Wild-type (Figure 3B, lane 2), αC1S (lane 3), αC4S (lane 6), αC5S (lane 7), and αT1 (lane 8) were all coimmunoprecipitated with the β-subunit antibody. The yields relative to wild type (set at 100%) were as follows: αC1S, 41%; αC4S, 23%; αC5S, 120% and αT1, 66%. In contrast, a very low yield of coimmunoprecipitation was observed for mutant αC2S (lane 4) and αC3S (lane 5) translations (relative yields 11% and 9%, respectively) and also from wild type translated in the absence of GSSG (lane 1, relative yield 3%). These results clearly indicate that single mutations of α-subunit residues Cys276 and Cys293 drastically affected the ability of the α-subunit to complex with the β-subunit. However, lower than wild-type yields of α−β complex from αC1S, αC4S, and αT1 translations may indicate a lesser but significant effect of these mutations.

In addition, α-subunits were immunoprecipitated using an anti-α-subunit serum raised against the carboxyl-terminal sequence (residues 504−517, see Experimental Procedures). This antiserum recognized both α-subunit glycoforms of chick P4-H on Western blot (results not shown) and could immunoprecipitate both glycosylated and nonglycosylated wild-type α-subunit translated under reducing conditions (Figure 3C, lane 1; relative yield set at 100%). In contrast, α-subunits synthesized in the presence of GSSG were weakly immunoprecipitated by this serum (lane 2; relative yield 23%). Of all the mutant α-subunits synthesized in the presence of GSSG, αC1S alone behaved essentially as the wild type (lane 3; relative yield 17%). However, mutants αC2S, αC3S, αC4S, and αC5S (lanes 4−7; relative yields 950%, 700%, 400%, and 1000%, respectively) were all immunoprecipitated by this serum. Thus, four of the α-subunit mutants displayed the carboxyl-terminal epitope present on wild-type α-subunits synthesized under reducing conditions. However, the large difference in relative yields indicates that the above mutants possess the epitope in a more accessible conformation than the wild type synthesized under reducing conditions. The lack of immunoprecipitate from αT1 translation (relative yield, 15%) was probably due to the fact that 10 of the 13 amino acids of the original antigenic peptide are not present in this truncated subunit.

Note that these immunoprecipitation experiments were carried out following treatment of samples posttranslationally with the alkylating agent NEM; however, identical results were obtained in the absence of this treatment (results not shown).

Redox Analysis of Immunoprecipitates. To determine whether disulfide bonds were present in α-subunits from β- or α-subunit immunoprecipitations (see Figure 3), immunoprecipitation products were also analyzed under reducing and nonreducing conditions by SDS−PAGE (Figure 4). Lanes 1A−5B show the analysis of products immunoprecipitated using antibodies against the β-subunit. Similar band shifts were observed to those shown in Figure 1 which demonstrate that, under oxidizing translation conditions, β-subunit immunoprecipitates of wild-type α-subunit (lanes 1A/B), αC1S (lanes 2A/B), αC5S (lanes 4A/B), and αT1 translations (lanes 5A/B) contained predominantly intramolecularly disulfide-bonded α-subunits. Mutant αC4S, which formed low amounts of α−β complex (see Figure 3B), was predominantly non-disulfide-bonded (lanes 3A/B). Lanes 6A−9B show the analysis of products immunoprecipitated with anti-α-subunit antibodies. Unexpectedly, intramolecular disulfides were also detected in the α-subunit immunoprecipitate of αC5S (lanes 9A/B). As expected, no such band shift was evident for αC2S (lanes 6A/B), αC3S (lanes 7A/B), and αC4S (lanes 8A/B) synthesized under oxidizing conditions nor for wild-type α-subunits synthesized under reducing conditions (result not shown). These results clearly indicate that immunoprecipitated α−β complexes contain intramolecularly disulfide bonded α-subunits.

Microsomal Localization of Translated Mutant α-Subunits. Microsomal membranes may be disrupted by incubation in sodium carbonate, a procedure that converts closed vesicles to open membrane sheets (Fujiki et al., 1982). Subsequent centrifugation yields a supernatant containing lumenal proteins and a pellet containing membrane-bound/associated

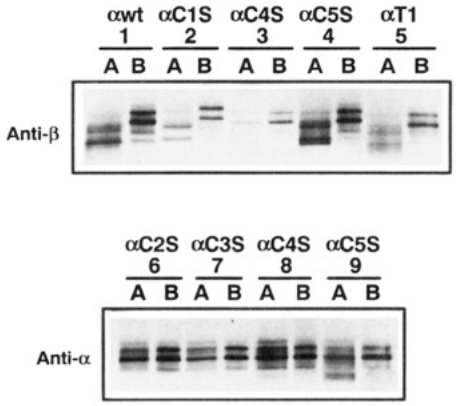

FIGURE 4: Analysis of immunoprecipitates by SDS−PAGE under reducing and nonreducing conditions. Translation of α-subunit mRNA was carried out in the presence of GSSG (3 mM) using the transcripts as indicated in the figure. Following translation, all samples were incubated on ice with NEM (50 mM, 10 min). Samples were then spun through sucrose cushions twice and the resulting microsome pellets resuspended in immunoprecipitation buffer, precleared, and immunoprecipitated with either the β-subunit (lanes 1A−5B) or the α-subunit antibody (lanes 6A−9B) as described under Experimental Procedures. Protein A-bound immunoprecipitates were then heated (10 min, 90 °C) in SDS−PAGE loading buffer and split into two equal aliquots (A and B). DTT (25 mM) was added to samples B, and the samples were reheated (5 min, 90 °C). After cooling to room temperature, NEM (100 mM) was added to samples B, and the samples were incubated for a further 15 min before being loaded onto a 10% SDS−PAGE gel. Following electrophoresis, the gel was fixed, dried and autoradiographed.

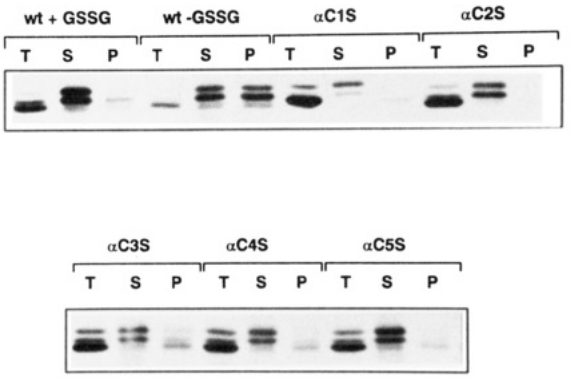

FIGURE 5: Sodium carbonate treatment of microsomes. Translations of α-subunit RNA transcripts were carried out in the presence (wt + GSSG, αC1S, αC2S, αC3S, αC4S, and αC5S) or the absence of GSSG (wt − GSSG). Microsomes were isolated by centrifugation through sucrose cushions and were subjected to sodium carbonate treatment (see Experimental Procedures) to yield supernatant (S) and pellet (P) fractions. Translation aliquots (T) were taken prior to microsome isolation and analyzed with supernatant and pellet fractions by SDS−PAGE through a 10% gel under reducing conditions. The gel was fixed, dried, and autoradiographed. The distribution of recovered α-subunit in the supernatant or pellet was calculated as $S/(S + P)$ or $P/(S) + (P)$, respectively, and expressed as a percentage.

proteins and protein aggregates. Previous work showed that α-subunit synthesized under oxidizing conditions appeared predominantly in the supernatant fraction, whereas α-subunit synthesized under reducing conditions appeared predominantly in the pellet fraction (John et al., 1993). This result suggested that under reducing conditions the α-subunit may be unfolded and consequently become aggregated, leading to its isolation in the pellet fraction. Similar analysis of the mutant α-subunits synthesized under oxidizing conditions is shown in Figure 5 together with the analysis of wild-type subunits synthesized in the presence and absence of GSSG. Wild type synthesized in the absence of GSSG was found equally in both fractions (~49% in supernatant), whereas wild type synthesized in the presence of GSSG was found predominantly (>70%) in the supernatant. Interestingly, all of the mutant α-subunits were also found predominantly (>80%) in the supernatant fraction. This indicated that the mutant α-subunits were in a soluble (lumenal) form and not in a highly aggregated or membrane-bound form.

Sucrose Gradient Analysis of Translation Products. Translation products, purified by centrifugation through sucrose cushions, were fractionated by centrifugation through 5−25% sucrose gradients containing the nonionic detergent lauryl maltoside (Segal et al., 1992). Gradients of purified chick P4-H incubated in the absence (panel A) or presence of DTT (50 mM, panel B) were used as controls for intact and dissociated $\alpha-\beta$ complexes respectively. Figure 6 shows that intact P4-H sedimented toward the middle of the gradient (panel A, lanes 7, 8, and 9), where the single β- and two α-subunits cofractionate. In contrast, DTT treatment of P4-H, which dissociates P4-H into its component subunits (Berg & Prockop, 1973), results in recovery of α-subunits predominantly in an SDS-soluble pellet recovered from the sample tube after gradient removal (panel B, lane P), whereas β-subunits sedimented over fractions 5−9. A sedimentation pattern similar to intact chick P4-H was seen for wild-type α-subunit (panel C), αC1S (panel E), and αT1 (panel K) translation products. A mixed pattern of sedimentation was seen for αC4S (panel H) and αC5S (panel J)—translation products sedimented both in the middle (fractions 7 and 8) and at the bottom of the gradient (fractions 1 and 2). Sedimentation of αC2S (panel F) and αC3S (panel G) occurred from the base to near the middle of the gradient (fractions 1−7). Wild-type α-subunit synthesized under reducing conditions sedimented predominantly into an SDS-soluble pellet recovered after removal of the gradient from the sample tube (panel D). Immunoprecipitation of gradient fractions with β-subunit antiserum demonstrated that α-subunits were immunoprecipitated from fractions 6−8 for wild type (synthesized in the presence of GSSG), αC1S, αC4S, αC5S, and αT1 gradient samples (results not shown). On this basis, it was concluded that the translation products and standard P4-H sedimenting at fractions 6−8 were an $\alpha-\beta$ complex and thus that α-subunit aggregates and/or complexes of the α-subunit with other unidentified proteins sedimented between fractions 1 and 6.

DISCUSSION

To facilitate the study of interactions between the subunits of P4-H, we have used a cell-free system to synthesize the α-subunit (and α-subunit mutants) under redox conditions that we can readily control. By addition of microsomal vesicles, this system synthesizes α-subunits in the presence of a pool of β-subuntis, conditions analogous to those thought to occur *in vivo* during P4-H assembly (Berg et al., 1980). Our previous work established that in this system P4-H could be assembled under conditions that enabled disulfide bonds to form in the newly-synthesized polypeptides (John et al., 1993). This result suggested that the P4-H α-subunit possesses intramolecular disulfide bond(s), a result consistent with data on P4-H dissociation in vitro (Berg & Prockop, 1973; Nietfeld et al., 1981) which suggested the presence

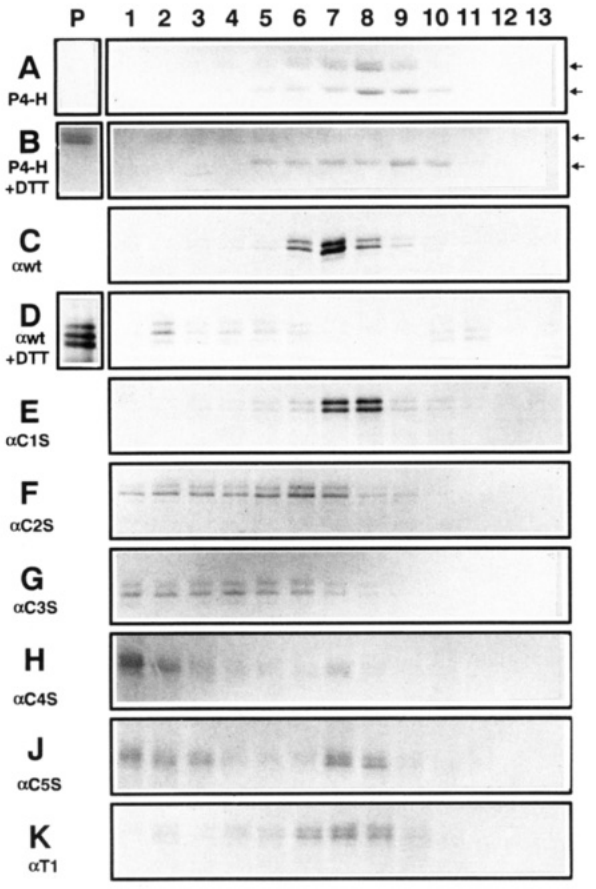

FIGURE 6: Sucrose gradient size fractionation of translation products. Microsomes were isolated from translations of the appropriate α-subunit RNA carried out in the presence of GSSG (panels C and E−K) or the presence of DTT (panel D) prior to fractionation through 5−25% sucrose gradients. As controls, purified P4-H (15 μg) was incubated on ice for 30 min in the absence (panel A) or the presence of DTT (50 mM, panel B) prior to fractionation as above. Collected fractions were precipitated with trichloroacetic acid and analyzed by SDS−PAGE through 10% gels under reducing conditions. Gels were fixed and stained with Coomassie blue (panels A and B) or fixed, dried, and autoradiographed (panels C−K). Fractions 1 and 13 represent the bottom and the top of the gradients, respectively. Fraction P was insoluble material present at the bottom of the centrifuge tube following centrifugation of the gradient and was recovered by resuspension in SDS−PAGE sample buffer following gradient removal. Panels A and B show both the β-subunit (PDI, lower arrow) and the two α-subunit glycoforms (upper arrow), whereas panels C−K show radiolabeled α-subunits alone.

of intrasubunit disulfide bonds. However, until now there has been no evidence to confirm the presence of disulfides in the α-subunit. Using our cell-free system, we have now shown that under folding conditions synthesis of wild-type α-subunits produces intramolecularly disulfide-bonded α-subunits.

In order to determine which of the five cysteine residues present in the α-subunit participate in intramolecular disulfide bond formation, we separately mutated each cysteine to serine and analyzed the properties of these mutants as compared to the wild type. When synthesized under conditions optimized for disulfide bond formation (GSSG present), only two of the mutants (αC1S and αC5S) behaved as the wild type and formed intramolecular disulfide bonds, whereas the remaining mutants did not and thus resembled wild-type α-subunit synthesized under reducing conditions. The truncated α-subunit (αT1) also formed intramolecular disulfides. Thus, αC2S, αC3S, and αC4S were defective for intramolecular disulfide bond formation.

These results correlate with immunoprecipitation results determining the presence of assembled α−β complexes containing mutant subunits synthesized under oxidizing conditions. In this instance, mutants αC1S, αC5S, and αT1 formed α−β complexes essentially at levels comparable to the wild type, but the remaining mutants αC2S, αC3S, and αC4S yielded very low amounts of complex and thus resembled wild-type α-subunits synthesized under reducing conditions. The lowest yields of complex found with mutants αC2S and αC3S indicated that of all the mutations the single mutations at Cys276 and Cys293, respectively, disrupted the α-subunit structure to an extent where complex formation was ~10% of the wild type. This strongly suggests that an intramolecular disulfide bond between Cys276 and Cys293 exists in native α-subunits and implies that this disulfide is essential for α−β subunit complex formation. In support of this conclusion, we have also shown that the α-subunits present in assembled α−β complexes, formed by wild-type α-subunit, αC1S, αC5S, and αT1 subunits, all possess intramolecular disulfide bonds.

The formation of complexes as determined by sucrose density size fractionation indicated that α-β complexes were formed under oxidizing conditions in translations of wild-type, αC1S, αC5S, and αT1 subunits. Mutant αC4S formed complexes detectable by this method even though a very low yield of complex was observed in immunoprecipitations (~25%). The reason for the low immunoprecipitation yield may be due to the mutation causing an alteration in the stability of the α-β complex. Similar results were obtained using native gel electrophoresis (results not shown); wild-type α-subunits, mutants αC1S, αC4S, αC5S, and the truncation αT1 (synthesized in the presence of GSSG) all formed complexes that migrated at similar positions to purified chick P4-H.

The effect of these mutations on the α-subunit structure was also monitored by immunoprecipitation of mutants using antibody against the α-subunit carboxyl-terminal peptide. Under oxidizing synthesis conditions, αC2S, αC3S, αC4S, and αC5S were all immunoprecipitated by this serum and thus exhibited properties similar to the wild-type α-subunit synthesized under reducing conditions (non-disulfide-bonded subunit). Howver, the above mutants were all immunoprecipitated at levels 4−10 times that of the wild-type α-subunit synthesized under reducing conditions. Therefore, these mutants possessed a structure in which the carboxyl-terminal epitope was more accessible to antibody binding than in the non-disulfide-bonded wild-type molecule. This enhanced accessibility may originate from large-scale perturbations to the subunit conformation caused by disruption of an essential disulfide bond interaction (e.g., mutants αC2S and αC3S) or by a local change in structure caused by a mutation in the epitope (mutant αC5S) or in sequences close to the epitope (mutant αC4S) which may associate with the epitope. As there is one cysteine (Cys511) in the epitope sequence, it is possible that this residue forms a disulfide with another cysteine in the correctly folded subunit, effectively removing the epitope from the molecule. This may possibly be a second intramolecular disulfide required for P4-H activity but not required for complex assembly. The dual reactivity of αC5S is consistent with this idea in that it is assembly-competent but also displays a non-native α-subunit epitope

which arises as a consequence of the Cys to Ser mutation of Cys511. As mutant αC1S was not immunoprecipitated by this antibody, it thus appeared to have similar properties as the wild-type, native subunit formed under oxidizing conditions.

The combined results of these experiments strongly suggest that native P4-H α-subunits possess at least one intramolecular disulfide bond which is required for α−β complex formation and that this bond is formed between Cys276 and Cys293. However, it is possible that a second disulfide, nonessential for complex formation, is formed, perhaps involving Cys511. The residues involved in this disulfide and the role of this second disulfide remain to be identified. It is noted that the identification of up to two disulfide bonds in the α-subunit indicates that cysteine residues are probably not involved in Fe(II) chelation [postulated by Kivirikko et al. (1990)] and thus the suggestion that conserved His residues are involved in this process (Myllyla et al., 1992) is considered more likely.

Unexpected results were obtained using the sodium carbonate extraction technique. In our previous work, we showed that wild-type α-subunit synthesized under reducing conditions (i.e., no α−β complex formation) was present predominantly in the pellet fraction and was thus probably in an aggregated state. It was expected that α-subunit mutants that failed to assemble would similarly be found predominantly in the pellet fraction upon sodium carbonate treatment. However, this was not the case as all the mutant α-subunits were found predominantly in the supernatant (soluble protein) fraction upon sodium carbonate treatment. Thus, in the case of αC2S and αC3S, although these subunits did not assemble with the β-subunit, they unexpectedly remained largely soluble and did not form high molecular weight aggregates. This difference in behavior between non-native wild-type and mutant subunits may be due to the different synthesis conditions, e.g., the absence or presence of GSSG, respectively. Thus, mutants allowed to form disulfide(s) may form structures with partial native character, whereas synthesis of wild type under reducing conditions precludes this. One possible explanation of the apparently conflicting results may be that there are accessory proteins, present within the microsomes, which are capable of binding non-native α-subunits to maintain them in a soluble state but that these proteins do not function efficiently under reducing conditions. The sucrose gradient fractionation profiles of αC2S and αC3S also showed that high molecular weight complexes or aggregates were formed by these α-subunits which may perhaps involve BiP or other as yet unidentified proteins. Such an involvement of a molecular chaperone in α-subunit synthesis/folding and thus P4-H tetramer assembly may explain the difficulties experienced *in vitro* with aggregation and insolubility of isolated α-subunits (Tuderman et al., 1975, 1977).

In this present work, we have not considered the role of PDI and PDI activity in the assembly of P4-H. PDI itself has six cysteine residues (Edman et al., 1985), four of which form the two active-site disulfides (Hawkins & Freedman, 1991). We consider that it is unlikely that these active-site cysteines form structural disulfides required for the stability of P4-H tetramers because the PDI activity of β-subunits (i) is not required for tetramer formation (Vuori et al., 1992) and (ii) is not required for P4-H activity (Myllyla et al., 1989; Vuori et al., 1992). Although the role of the two remaining cysteine residues of PDI was not addressed in the baculovirus work of Vuori et al. (1992), we conclude that in view of the above work and our results it is extremely unlikely that the reducing agent-induced dissociation of P4-H (Berg & Prockop, 1973) results from reduction of β-subunit disulfides. Thus, we propose that it is the formation of disulfide bonds within P4-H α-subunits that leads to stable α-β subunit interaction and tetramer assembly and that reduction of these bonds leads to tetramer dissociation.

However, the precise role(s) of PDI in P4-H tetramer formation/stability remain(s) to be clarified. At least two roles may be envisaged: a catalytic role, i.e., in the formation/rearrangement of α-subunit disulfides, and/or a structural role with the β-subunit acting as a chaperone in assisting α-subunit folding by preventing unfavorable aggregation interactions. The role of PDI in the reduction/rearrangement of disulfides in P4-H tetramers has been postulated on the basis of the reactivation of stored P4-H by the addition of PDI (Koivu & Myllyla, 1986)—the authors concluded that in the case of P4-H PDI catalyzes the isomerization and not the formation of disulfides. Our work identifies these disulfides as being present in the α-subunits. Thus, free PDI may preserve the catalytically active P4-H structure by maintenance of the correct disulfide/thiol structures in the α-subunit, and, consequently, a mechanism for regulation of P4-H activity in vivo may be via disassembly of the complex by reduction of these disulfide(s). The results of the baculovirus coexpression studies (Vuori et al., 1992) which clearly showed that active P4-H tetramers were formed by assembly of wild-type α-subunits with PDI-inactive β-subunits (both active-site sequences -Cys-Gly-His-Cys-mutated to -Ser-Gly-His-Cys-) suggest that PDI does not utilize its disulfide isomerase activity as a β-subunit within the P4-H tetramer and thus, in this respect, plays only a structural role. It was also apparent in this study that the involvement of endogenous PDI activity in subunit assembly could not be excluded. Thus, endogenous PDI activity may have catalyzed α-subunit disulfide bond formation without consequent formation of stable α−β complexes—this is possible as the endogenous (insect) PDI may be (a) significantly dissimilar to the wild-type (human) β-subunit and/or (b) present at a substoichiometric level compared to α-subunits expressed in this system. The function of PDI as a specific chaperone in preventing nascent α-subunit aggregations during folding may be proposed on the basis of analogous roles for PDI as a component of the microsomal triglyceride transfer protein complex (Wetterau et al., 1991) and as the putative essential function of PDI in yeast (La Mantia & Lennarz, 1993). The possible role of other chaperones, e.g., BiP, in P4-H assembly remains to be proven.

In conclusion, we have now shown that the P4-H α-subunit is intramoleuclarly disulfide bonded and that up to two disulfides may be formed. The first, Cys276-Cys293, is essential for α−β complex formation, whereas a second, involving Cys511, appears to be nonessential. The precise role of the β-subunit (PDI) in this complex formation and the events involved in tetramer assembly remain unclear. Elucidation of the α-β subunit binding interactions, in particular the presence of binding motifs on the β-subunit that may be involved in preventing α-subunit aggregation, may lead to the successful design of pharmacologically active compounds to specifically inhibit P4-H by either prevention

of assembly or dissociation of the tetramer. These and other aspects of P4-H subunit folding and assembly both *in vitro* and *in vivo* are currently under active investigation in our laboratory.

ACKNOWLEDGMENT

We thank Trevor Franklin for the purified chick P4-H.

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