Comparing the Refolding and Reoxidation of Recombinant Porcine Growth Hormone from a Urea Denatured State and from *Escherichia coli* Inclusion Bodies†

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ABSTRACT: Overexpression of cloned genes in bacteria often leads to insoluble refractile body formation requiring solubilization and refolding to obtain biologically active proteins. A refolding pathway was established for a model protein, porcine growth hormone (PGH), yielding an appreciably high recovery of 85%. The conditions include the dilution of a urea, β-mercaptoethanol (β-ME) denatured PGH solution in a refolding environment containing 3.5 M urea and 10 mM β-ME/HED at a 10:1 ratio at pH 9.1 and 0.5 mg/mL PGH. The intrinsic fluorescence-detected transition of PGH in urea gives 3.8 kcal/mol for the free energy of denaturation (ΔG_{H_2O}) of PGH. The native-like conformation of PGH is dependent on disulfide bonds because reduced and carboxymethylated PGH is devoid of tertiary structure as assessed by intrinsic tryptophan fluorescence. Physical analysis of C-terminally truncated recombinant PGH indicated no significant difference in the free energy of denaturation of P-band in urea as full-length PGH. This suggests that the first disulfide, forming the large loop domain of PGH, provides a significantly greater contribution to the conformational stability of PGH than the second disulfide, which forms the carboxy-terminal small loop domain. The rate of formation of native structure during refolding was biphasic, with native structure identified by intrinsic fluorescence and hydrophobicity spectroscopy prior to disulfide bond formation. Thus "framework" intermediates are prerequisites for correct disulfide formation and tertiary folding of PGH. This study shows how a protein refolds, forms disulfides, and self-associates, which may be useful for examining the refolding of other recombinant proteins.

Recombinant DNA technology allows the use of prokaryotic and eukaryotic microorganisms or tissue culture cells to express heterologous proteins from other prokaryotic, viral, or eukaryotic organisms. The most extensively used host for expression (production) of foreign proteins has been the Gram-negative microorganism *Escherichia coli (E. coli)*[1] (Marston, 1986). This is useful in medicine, for example, in the synthesis of recombinant proteins, hormones, and enzymes as therapeutics in the treatment of metabolic disease states, and in academic laboratories where the function and structure of uncharacterized proteins are explored. Once an organism has been genetically engineered to produce a specific protein, it is a major challenge to isolate the protein and purify it cost-effectively with a high yield. For example, high level expression of recombinant proteins in *E. coli* may lead to the formation of intracellular protein aggregates called inclusion bodies (IBs).[1] Expression of proteins as IBs can have advantages for the large-scale purification of biologically active protein from IBs even though the protein is initially present in an inactive, denatured, and aggregated state (Kane & Hartley, 1988). Proteins have an intrinsic property of being able to attain a conformation in an autonomous way, based on the information contained in the sequential amino acid sequence or primary structure of the polypeptide chain (Anfinsen, 1973). The reason why the purification and renaturation of the recombinant proteins is so complicated is because very little is known regarding their *in vivo* folding. The purpose of this paper is to show, in detail, how the refolding of a model protein can be optimized during its purification from an inactivated state. The approach involves understanding the thermodynamics and kinetics of the individual steps along the folding pathway during the *in vitro* refolding of the protein and should be applicable to a variety of refolding problems including the reactivation of denatured protein.

One of the current models for the folding of a single polypeptide includes the framework model (Kim & Baldwin, 1990). The framework model suggests that folding follows a hierarchical process, in which simple structures are formed first, giving rise to a stable framework of secondary structures (Kim & Baldwin, 1990). The tertiary structure is then assembled by eliminating water from the hydrophobic core, as a consequence of salt linkages occurring between acidic and basic residues, hydrophobic aggregation of the aliphatic

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[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; BGH, bovine growth hormone; β-ME, β-mercaptoethanol; CTAC, cetyltrimethylammonium chloride; CD, circular dichroism; DEAE, (diethylamino)ethyl; DTE, dithioerythritol; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; *E. coli, Escherichia coli*; ΔG_{H_2O}, free energy change of denaturation; GuHCl, guanidine hydrochloride; HED, β-hydroxyethyl disulfide; HRP, horseradish peroxidase; IgG, immunoglobulin; RP-HPLC, reversed phase high performance liquid chromatography; IBs, inclusion bodies; NATA, *N*-acetyltryptophanamide; PAGE, polyacrylamide gel electrophoresis; PGH porcine growth hormone; PMSF, phenylmethanesulfonyl fluoride; RCAM, reduced and carboxymethylated protein; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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and aromatic amino acids, and hydrogen bonding between polar and charged amino acids (Kim & Baldwin, 1990). The framework model suggests that as folding proceeds, a stable backbone is formed by the formation of fluctuating secondary structure in the unfolded intermediates which then associate by hydrophobic interactions into an intermediate with globular structure that forms the hydrophobic core of the native protein. Subsequently, alterations of this intermediate structure proceed to the final native, folded state (Kim & Baldwin, 1990).

A number of methods have been reported for solubilizing and refolding recombinant proteins from IBs (Marston, 1986). Denaturants, such as urea and guanidine hydrochloride (GuHCl), and the use of highly alkaline pHs (pH $\geq$10) have been claimed to solubilize a significant percentage of the insoluble proteins found in IBs (Marston, 1986). However, there is often poor recovery of biologically active protein, and the procedure is still very much misunderstood. Some reports state that GuHCl effectively solubilizes all the protein present in inclusion bodies, but only a small portion may be converted into a biologically active form even after refolding the protein at very dilute protein concentrations (Emtage, 1985).

Strong denaturants such as urea, GuHCl, or SDS are often used in concert with strong thiol-containing reductants such as dithiothreitol (DTT) or dithioerythritol (DTE) or β-mercaptoethanol (β-ME) to disrupt any intermolecular disulfide bonds in IBs (Jaenicke & Rudolph, 1990). Chelating reagents such as ethylenediaminetetraacetic acid (EDTA) are also added to remove trace metals capable of catalyzing oxidation. Typical concentrations that have been reported in the literature are 3−6.5 M GuHCl, 5−10 M urea, 10−100 mM DTT (and DTE), 1−5 mM EDTA, and 0.1−0.3 M for β-ME (Jaenicke & Rudolph, 1990). Wetzel (1986) has reported the use of sulfitolysis during the solubilization of IBs, which has the effect of converting disulfides into sulfonate groups. Refolding occurs by reconverting the sulfonated groups into free thiols, which in turn are allowed to form disulfides in the presence of a weak oxidizing agent (Wetzel, 1986).

Many of the reports that describe the methods used to renature recombinant proteins *in vitro* to achieve biologically active products are to be found in the patent literature. In most cases, the process begins by the gradual removal of the denaturant used to solubilize IBs (Builder & Ogez, 1985; Rausch, 1987). Dilution is the simplest method to initiate refolding in bench-scale experiments (Hagar & Burgess, 1980; Jaenicke & Rudolph, 1990). However, on a large scale, the volumes and cost become significant; therefore, other diffusion methods such as diafiltration (Wetzel, 1986), dialysis, and more recently electrodialysis have been used for desalting and buffer exchange of charged species as well as for concentrating the purified recombinant protein (Reed, 1984). The major disadvantage of diffusion-based techniques is that they are slow. However, many proteins have been successfully refolded using dialysis, including bovine growth hormone (Olson, 1985; Wetzel, 1986), *E. coli* tryptophanase (London *et al.*, 1974), and urokinase (Langley *et al.*, 1987).

The degree of *in vitro* aggregation during refolding, which dictates the final yield of native protein, is generally accepted to be dependent on many factors including the denaturant and protein concentration, pH, ionic strength, temperature, the presence of folding catalysts, thiol−disulfide agents, and stabilizing agents (Creighton *et al.*, 1980; Jaenicke & Rudolph, 1986, 1990; De Bernardez-Clark & Georgiou, 1991). Refolding yields have been claimed to be increased at intermediate denaturant concentrations, for example, 2−5 M urea and 1−2 M GuHCl (Tsuji *et al.*, 1987; Sarmientos *et al.*, 1989). Temperature must also be controlled as elevated temperatures greater than 25 °C have a pronounced effect on a protein's propensity to aggregate (Goerlich & Holler, 1984; Buchner & Rudolph, 1991).

Refolding yields have been reported to be much higher when carried out with pure protein solutions, since the presence of contaminants such as the components of the bacterial cell walls can interfere with protein folding (Ogez *et al.*, 1990). The phenomenon of intermolecular aggregation is a major competing reaction that causes very high losses during *in vitro* and sometimes *in vivo* folding of proteins. The conditions that minimize aggregation during refolding must be empirically determined for each protein (Jaenicke & Rudolph, 1990); for example, aggregation may be controlled by using a pH that is far away from the isoelectric point (p*I*) of the protein (Jaenicke & Rudolph, 1990).

Air oxidation is the oldest available technique to renature proteins containing disulfide bonds (Jaenicke & Rudolph, 1990). The process is usually catalyzed by trace metal ions such as Cu^{2+} and Co^{2+}. The rate and yield of this process are reported to be very low (Pigiet & Schuster, 1986b). Jaenicke and Rudolph (1990) have reviewed alternative approaches for promoting the thiol-exchange reactions. Optimum conditions for disulfide bonding are usually obtained when the concentration of thiol reagent is 10-fold greater than the molar concentration of cysteine in the protein (Jaenicke & Rudolph, 1990). Refolding and concomitant reshuffling of mispaired disulfides in RNase have been reported to be facilitated by using combinations of thioredoxin and DTT (Pigiet & Schuster, 1986a), and foldases such as an *E. coli* derived thioredoxin reductase have also been reported to refold proteins containing incorrect disulfide cross-links (Pigiet *et al.*, 1987). However, the presence of disulfide bonds needs not necessarily constitute a difficult refolding reaction. For example, tissue plasminogen activator (TPA) has 17 disulfides and has been successfully refolded without the presence of additives that promote thiol−disulfide exchange (Sarmientos *et al.*, 1989).

Tandon and Horowitz (1986) have reported that rhodanase may be successfully refolded after exposure to GuHCl treatment using the neutral (nondenaturing) detergent lauryl maltoside. The detergent is believed to bind to the exposed hydrophobic surfaces of the intermediate folded forms of the protein, thereby preventing their aggregation. Cleland and Wang (1990) reported the use of poly(ethylene glycol) that prevents the aggregation of carbonic anhydrase in a similar manner. The cosolvent binds to the nonpolar regions of an early intermediate that is therefore excluded from forming inappropriate intermediates which lead to aggregate formation (Cleland & Wang, 1990).

The formation of IBs is often considered undesirable since the deposited protein can only be solubilized using strong denaturants such as 6 M GuHCl or 7.5 M urea and other harsh conditions of pH or temperature (Sharma, 1986; Schein, 1990; Marston & Hartley, 1990; Jaenicke & Rudolph, 1990). The use of high concentrations of urea and/or GuHCl results in the loss of secondary structure, and the solubilized

proteins exist mostly in the so-called "random coil" formation (Dill & Shortle, 1991).

The denaturation of recombinant proteins during solubilization from IBs requires *in vitro* "refolding" to regain native conformation. For intrachain disulfide-linked proteins such as recombinant porcine growth hormone (PGH), the *in vitro* formation of undesirable high molecular weight aggregates, due to aberrant interchain disulfide bonding and nonspecific interactions between the hydrophobic segments of unfolded proteins, is a major problem limiting recovery of native (i.e., monomeric 22K) protein. We have previously shown that aberrant aggregation during refolding of recombinant proteins expressed as inclusion bodies (recombinant PGH, IL-1β, and IGF-2) can be circumvented if a cationic surfactant is used to solubilize the protein from insoluble IBs (Puri et al., 1992). Such a detergent, CTAC, is a far superior solubilizing agent compared with more traditional denaturants because it preserves secondary structure of the solubilized protein (Puri & Cardamone, 1993; Cardamone *et al.*, 1994).

In this study, optimal solution conditions were sought which would maximize the yield of soluble monomeric and correctly refolded Met(1-190)PGH from CTAC solubilized IBs. The refolding of soluble purified PGH was characterized in the following manner. The protein was first unfolded using very high concentrations of urea ($\geq$7.5 M urea). The *in vitro* renaturation of urea unfolded PGH was then monitored under different solution conditions by varying pH, urea, protein, and reducing agent concentration. The likely unique set of solution conditions which optimized the yield of renaturation and oxidization of purified PGH from its chaotroph denatured form would be classified as the "model" refolding pathway. The model refolding conditions were then used to renature recombinant PGH from IBs *in vitro*. Intrinsic protein fluorescence was used in these studies to investigate the rate and thermodynamics of folding of two different preparations of recombinant PGH. The fluorophore 8-anilino-1-naphthalenesulfonate (ANS) was also used to investigate the hydrophobicity of different folded states of PGH along the model refolding pathway (Cardamone & Puri, 1992). Finally, Ellman titration (Ellman, 1959) and nonreducing SDS−PAGE was used to measure the rate of disulfide bond formation in the model refolding pathway of recombinant PGH.

EXPERIMENTAL PROCEDURES

Materials

Unless otherwise stated, all laboratory reagents were of analytical grade and were supplied by Ajax Chemicals, BDH, Boehringer Mannheim or Sigma Chemicals. All solutions were prepared with MilliQ (MQ) water. Analytical grade *n*-cetyltrimethylammonium chloride (CTAC) was purchased from ICI (Australia) as a 30% (w/v) stock solution in 0.1% (v/v) ethanol. Purified proteins in lyophilized form and all other analytical grade reagents used were obtained from the Sigma Chemical Co. (Poole, U.K.), and HPLC solvents were from Mallinckrodt (U.S.A.). Electrophoresis grade reagents, MiniProtean gel electrophoresis equipment, and Mini Trans-Blot electrophoretic transfer units were obtained from Bio-Rad Laboratories, Inc. (North Ryde, N.S.W., Australia), and Novex (North Ryde, N.S.W., Australia). TMS-TSK reversed phase HPLC columns and Superose 12 HPLC gel permeation columns were purchased from Pharmacia (Melbourne, Australia). The HPLC system consisted of two Beckman Model 114M solvent delivery modules, a Beckman Model 340 organizer containing injection valve and gradient mixing chamber, a Beckman Model 421 HPLC controller, and a Model 165 variable wavelength detector equipped with an analytical cuvette of 10 mm path length (Beckman Instruments, Melbourne, Australia) and a deuterium lamp (Photron Instruments, Melbourne, Australia). Data analysis was performed using Varian software (Varian Instruments, Melbourne, Australia).

Methods

(a) Purification of Recombinant Porcine Growth Hormone. The recombinant porcine growth hormone used was the 191 amino acid protein methionyl(1-190 amino acid)PGH (abbreviated: Met(1-190)PGH) obtained using a bacterial expression system described in Puri *et al.* (1992). The PGH was expressed in *E. coli* as IBs. IBs were isolated by cell disruption using a French press and harvested by differential centrifugation, and the pellets were stored at 4 °C until required. Approximately 100 mg of total protein (dry weight) was obtained from 3.72 g wet weight *E. coli* cells. The pellet containing the IBs was resuspended in 1 L of 20 mM Tris-HCl, pH 8.0, containing 5 mM EDTA, 0.02% (w/v) lysozyme, 75 μg/mL PMSF, 75 μg/mL leupeptin, and 75 μg/mL benzamidine, and incubated with stirring at room temperature for 1 h. Then 250 mL of a 10% (w/v) sodium deoxycholate solution was added to give a final concentration of 2% (w/v). After incubating with stirring at room temperature for another hour, the solution was centrifuged at 25 °C for 30 min at 17000*g*. The pellet fraction containing the "detergent washed" IBs was finally washed with a solution of 5 M urea, 1% (v/v) Triton X-100, 5 mM EDTA, 75 μg/mL PMSF, 75 μg/mL leupeptin, and 75 μg/mL benzamidine in 0.1 M Tris-HCl buffer, pH 7.0. Washed IBs were further purified by sucrose density gradient centrifugation for spectroscopic studies. Briefly, the IBs were resuspended in 10 mM Tris-HCl, pH 8.0, containing 0.25 M sucrose and 1 mM EDTA and layered onto a stepwise gradient consisting of equal volumes of 67%, 53%, and 40% (w/v) sucrose in 1 mM Tris-HCl and 1 mM EDTA, pH 8.0. The final gradient was established by centrifugation at 108000*g* for 2 h. The IBs appeared as a white band at the 53−67% sucrose interface and were collected with a Pasteur pipet and washed three times with MQ water. The final protein pellet, equivalent to approximately 45 mg dry weight, was resuspended in 4.5 mL of solubilization buffer. IBs were solubilized essentially as described in Puri *et al.* (1992), using a 5% (w/v) solution of CTAC in 0.1 M Tris-HCl, pH 9.1, containing 0.25M β-ME, 75 μg/mL PMSF, 75 μg/mL leupeptin, and 75 μg/mL benzamidine at 50 °C for 1 h, or with solubilization buffer containing 6.4 M GuHCl or 8.0 M urea in 50 mM glycine, 0.25M β-ME, 75 μg/mL PMSF, 75 μg/mL leupeptin, and 75 μg/mL benzamidine, pH 9.1. IBs were solubilized to protein concentrations of 40 mg/mL. The solubilized IBs were clarified by centrifugation (10000*g*; 10 min), and the supernatant was retained for analysis. Under these conditions approximately 85−90% of the recombinant PGH was solubilized. The soluble material was clarified by centrifugation (12000*g*) and the recombinant PGH concentration in the supernatant determined by reversed phase high performance liquid chromatography (RP-HPLC)

as described below. Where required, CTAC, free in solution or bound to protein, was removed by cation exchange chromatography. Solubilized recombinant PGH in CTAC was mixed with DOWEX 50W X 4 cation exchange resin (Dow Chemical Corp., U.S.A.) equilibrated in 0.1 M glycine hydrochloride and 5 M urea, pH 9.1. The surfactant free recombinant PGH was isolated in the supernatant after centrifugation of the protein/DOWEX mixture at 1900*g* for 5 min. Briefly, PGH (30−40 mg/mL in 5% CTAC) was diluted 1:2 with 8 M urea and 0.1 M glycine hydrochloride, pH 9.1, and the solubilization mixture was added to the equilibrated DOWEX resin in the following ratio: 1 mL of 5% CTAC (w/v) to 4 mL of settled DOWEX pre-equilibrated with urea/glycine buffer, pH 9.1. Refolding was first initiated by buffer exchange using G-25 Sephadex gel filtration columns (PD-10 desalting columns, Pharmacia-LKB) equilibrated in the refolding buffer. Surfactant free Met(1-190)PGH at 0.5−5 mg/mL was subsequently renatured by dialyzing against the refolding buffer (1 mM EDTA, 3.5 M urea, 10−100 mM β-ME in 20 mM Tris-HCl, pH 9.1) at 4 °C overnight, with gentle agitation to promote oxidation. The refolded protein preparation was purified from aggregated forms and other contaminants by batch ion-exchange chromatography using Whatman DEAE-52 ion-exchange resin (Whatman Biosystems Ltd., Maidstone, Kent, U.K.) pre-equilibrated in 20 mM Tris-HCl, pH 9.1 at 4 °C. Prior to batch chromatography, the protein solution was dialyzed against DEAE-52 chromatography buffer (20 mM Tris-HCl, pH 9.1) at 4 °C. Approximately 1 mL of equilibrated DEAE resin was used for 20 mg of protein. Monomeric PGH was preferentially eluted from the resin using 30 mM NaCl in 20 mM Tris-HCl, pH 9.1. The yield of purified monomeric PGH was approximately 75% of the material bound to the DEAE-52 column. The DEAE-52 purified monomeric Met(1-190)PGH was rechromatographed on a FPLC MonoQ column equilibrated in buffer A (10 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.01% (v/v) Tween-20) attached to the Beckman HPLC system with specialized fittings (Activon, Melbourne, Australia). The flow rate was 0.5 mL/min, and a gradient of increasing buffer B (buffer A containing 2.0 M NaCl) was used to elute the protein from the column. Protein was monitored at 280 nm because of the presence of the 0.01% Tween-20 which interferes with detection at 220 nm. Purified monomeric Met(1-190)PGH was dialyzed exhaustively against 5 mM ammonium carbonate buffer, pH 8.0, for 16 h, and the solution was lyophilized by freeze-drying, or smaller volumes were dried using a vacuum centrifuge (Dynavac, Melbourne, Australia) linked to a high vacuum pump (Javac, Melbourne, Australia).

(b) Establishing a "Model" for in Vitro Refolding of Recombinant PGH. Unfolding was carried out by incubation of soluble recombinant Met(1-190)PGH in 9.0 M urea, 0.25 M β-mercaptoethanol (β-ME), and 50 mM Tris-HCl, pH 9.1, at a final protein concentration of 10 mg/mL for 1 h at 50 °C. The reducing agent was then removed by size exclusion chromatography using G-25 Sephadex gel filtration columns (PD-10, desalting columns; Pharmacia-LKB) and refolding initiated by dilution into refolding buffer. Renaturation was achieved by dialyzing against the refolding buffer at 4 °C overnight, with gentle agitation to promote aeration. Refolding efficiency was estimated using reversed phase HPLC (RP-HPLC) and electrophoretic methods described in detail below.

(c) Quantitation of Recombinant PGH and Yield of Monomer by Reversed Phase High Performance Liquid Chromatography (RP-HPLC) Analyses. All samples were centrifuged at 14000*g* prior to HPLC analysis. The amount of recombinant PGH obtained after CTAC solubilization was determined by RP-HPLC. Solubilized protein was separated from surfactant protein mixtures using a C_1-alkyl-bonded silica column (LKB Ultropac; TSK TMS-250) with dimensions of 4.6 mm × 75 mm, 10 μm particle size, and 25 nm mean pore diameter. Solubilized protein was loaded on to the columns in 15% acetonitrile/0.1% trifluoroacetic acid and eluted 3 min after injection with a linear gradient of 30−70% (v/v) acetonitrile over 15 min. Detection was at 220 nm. Briefly, 50 μL aliquots of the supernatant were analyzed by HPLC, and the PGH concentration was estimated using a standard curve of peak area plotted against concentration of PGH in mg/mL. Samples were analyzed in duplicate. Proteins were also separated using a C-8 alkyl bonded silica column (Beckman Ultrasphere octyl column) with dimensions of 5 μm particle size, 30 nm mean pore diameter and 4.6 mm × 250 mm. Proteins were loaded onto the column equilibrated with 0% acetonitrile/0.1% TFA and eluted using a linear gradient of 0−100% acetonitrile over 20 min.

(d) Sodium Dodecyl Sulphate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE in the presence or absence of 5% (v/v) β-ME was conducted in polyacrylamide gels (9 cm wide, 6 cm high, and 1 mm thick) according to the method of Laemmli (1970). The stacking gels contained 4% (w/v) acrylamide and separating gels 15% (w/v) acrylamide. Samples in 2% (w/v) SDS, 10% (w/v) glycerol, and 62.5 mM Tris-HCl, pH 6.8, were immersed in boiling water for 5 min prior to loading into gel wells. The gels were stained for proteins with 0.12% Coomassie brilliant blue R250 and/or silver-stained. The gels were calibrated with phosphorylase B (106 000 Da), bovine serum albumin (66 300 Da), ovalbumin (49 500 Da), carbonic anhydrase (37 500 Da), soybean trypsin inhibitor (27 500 Da), lysozyme (18 500 Da), and aprotinin (6500 Da). Densitometric scanning of stained gels was carried out using a Zeineh Model SLR-TRFF soft laser scanning densitometer (Biomed Instruments Inc., Fullerton, CA, U.S.A.), and integration was accomplished using one-dimensional scanning software (Biomed Instruments, Fullerton, CA, U.S.A.).

(e) Western Blotting. Proteins were transferred onto 0.45 μm poly(vinylidene difluoride) membranes (Immobolin P; Millipore Pty. Ltd., Melbourne, Australia) at 100 V·h using a Mini Trans-Blot electrophoretic transfer apparatus (Bio-Rad). After electrophoretic transfer, the membrane was blocked with 1% Tween-80 in wash buffer (20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.05% (v/v) Tween-80) for 1 h at room temperature. The membrane was then washed three times for 5 min in wash buffer. The membrane was then incubated for 1 h at room temperature in murine monoclonal anti-PGH antibody (clone 21.51, isotype IgG_1, Centre for Animal Biotechnology, The University of Melbourne) diluted 1:1000. The membrane was further given three washes for 5 min with wash buffer, followed by incubation in a 1:400 dilution of rat anti-mouse horseradish peroxidase (HRP)-conjugated antibody (DAKO-Immunoglobulins, Glostrup, Denmark) for 1 h at room temperature. Following a further wash step, the membrane was developed

with HRP substrate made up by dissolving 6.0 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, U.S.A.) in 10 mL of 10 mM citrate buffer, pH 5.0, and finally adding 10 μL of H_2O_2. Once the bands were visible, the membrane was then washed thoroughly with MQ H_2O. Pituitary-derived PGH was used as a positive control, and a murine monoclonal anti-ovine CD1 antibody (clone 20.27, isotype IgG_1, Centre for Animal Biotechnology, The University of Melbourne) was used as a negative control antibody.

(f) Spectroscopy. UV absorbance spectral measurements were performed using a Varian 240 spectrophotometer using a matched pair of 1 cm quartz cells. The PGH concentration was determined using an extinction coefficient of 15714 M^{-1} cm^{-1}. Fluorescence spectroscopy was measured using a Hitachi Model F-2000 fluorescence spectrophotometer. The samples were temperature equilibrated using a turretted four-cell thermostatically controlled holder connected to a circulating water bath, and fluorescence measurements were conducted in quartz optical cells of 10 mm path length. Intrinsic fluorescence was measured and quantum yields were calculated using the method of Parker and Rees (1962) relative to β-carboline as standard (Ghiggino *et al.*, 1985). The tryptophan residues of recombinant PGH were excited at a wavelength of 295 nm and emission spectra monitored between 300 and 350nm.

(g) Preparation of IB Material for Analyses of in Vitro Refolding Efficiency of Recombinant PGH. Methionyl(1-190)PGH was expressed in *E. coli* and IBs purified as described previously in Puri *et al.* (1992). The IBs from fermentation batch F135 (dry weight 90 mg/mL) were solubilized in 100 mM Tris-HCl, pH 9.1, containing 1 mM EDTA, 2% (v/v) β-ME, and either 8 M urea or 5% (w/v) CTAC for 1−2 h at ambient temperature or 55 °C as described previously in Puri *et al.* (1992). Approximately 50 mg dry weight of IBs was solubilized in 5 mL of solubilization buffer, and 1 mL aliquots of each of the solubilized recombinant PGH preparations were subsequently refolded by dialysis at a concentration of 0.5 mg/mL against 100 mM Tris-HCl, pH 9.1, 1 mM EDTA, and 10 mM β-ME with 2.5−4.5 M urea. Refolding was for 48−72 h at 4 °C with shaking and aeration. As studies in Puri *et al.* (1992) indicated that the IBs were contaminated with *E. coli* associated proteins, it was necessary to represent the refolding results as the percentage of reoxidized PGH after 16 h refolding as a proportion of the percentage reduced PGH that was present initially; this ratio ($\%\ P_{ox,t=16h}/\%\ P_{red,t=0h}$) is termed the refolding efficiency (Puri *et al.*, 1992).

(h) Preparation of IB Material for Fluorometric Analyses. IBs were solubilized using a final concentration of 5% (w/v) CTAC in 0.1 M Tris-HCl, pH 9.1, containing 0.25 M β-ME and 1 mM EDTA at 55 °C for 1 h or with solubilization buffer containing 6.4 M GuHCl or 8.0 M Urea in 0.1 M Tris-HCl, pH 9.1, containing 0.25 M β-ME and 1 mM EDTA. IBs were solubilized at a protein concentration of 40−50 mg/mL. The solubilized IBs were clarified by centrifugation (10000*g*; 10 min), and the supernatant was retained for analysis. CTAC, free in solution or bound to protein, was removed by cation-exchange chromatography using DOWEX (Puri *et al.*, 1992) equilibrated in 0.1 M glycine hydrochloride and 5 M urea, pH 10. Briefly 20 mg/mL protein in 5% CTAC was added to the equilibrated DOWEX resin in the following ratio: 100 μL of 5% CTAC (w/v) to 400 μL of settled DOWEX pre-equilibrated with urea/glycine, pH 10 buffer. The surfactant free recombinant PGH was isolated in the supernatant and a sample of this solution used for intrinsic fluorescence measurements.

(i) Thiol Analyses. The number of free thiols available in a protein sample was assessed by a modified Ellman assay (Ellman, 1959). All work was carried out under nonoxidizing conditions using N_2 to purge all buffers. The Ellman reagent 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was used to establish a standard curve of absorbance versus amount of thiols using β-ME or L-cysteine as primary standards in thiol assay buffer (6.4 M GuHCl, 1 mM EDTA in 0.1 M phosphate, pH 7.5). Each protein sample was then assayed in triplicate for thiol groups and for protein content using the Lowry assay (Lowry *et al.*, 1951) and by RP-HPLC to ensure that the sample was monomeric. A ratio was calculated of moles of SH/moles of monomeric protein. In order to obtain reduced proteins, the protein sample was first treated with nitrogen purged reducing buffer containing 6.4 M GuHCl and 100 mM DTT in 0.1 M Tris-HCl, pH 8.8, for 1 h at 50 °C. The reducing agent in the sample was then removed using G-25 Sephadex gel filtration (PD-10 desalting columns; Pharmacia-LKB) equilibrated in N_2 purged thiol assay buffer.

(j) Preparation of Reduced and Carboxymethylated (RCAM) PGH. Approximately 50 nmol of Met(1-190)PGH was dissolved at 37 °C in 9 M urea and 100 mM Tris-HCl, pH 8.8. The disulfides in the proteins were then reduced with the addition of DTT to a final concentration of 100 mM, and the mixture was incubated at 37 °C for 16 h. Then, 200 μL of a 1.4 M iodoacetamide solution in 0.1 M KOH was added to the reduced protein and the mixture incubated further for 30 min in the absence of light as iodoacetamide is known for light catalyzed modification of proteins. The reaction was subsequently quenched by acidifying with trifluoroacetic acid (TFA) to a final concentration of 1% (v/v). The sample was then desalted by size exclusion chromatography using G-25 Sephadex gel filtration columns incubated in a nitrogen purged 0.1 M phosphate buffer at pH 8.0 in order to remove excess iodoacetamide. Carboxymethylation of thiols was judged as complete using the Ellman assay for the presence of free thiols and by SDS−PAGE analysis of the RCAM protein. As carboxymethylation of proteins is usually associated with a loss in solubility, it was essential that the desalting buffer was purged with an inert gas and a low concentration of chaotrophic reagent (i.e., 1−2 M urea) be included to optimize the recovery of RCAM PGH after chromatography.

(k) Monitoring the Refolding of Soluble Recombinant PGH by Intrinsic Fluorescence Spectroscopy. The rate of refolding and the conformational stability of PGH were analyzed by monitoring changes in intrinsic fluorescence intensity using an interfaced Hitachi F-2000 spectrofluorimeter and SpectraCalc Software. The temperature of the samples during the analyses was maintained at 24 °C. Fluorescence intensity was measured at 350 nm with excitation at 295 nm and a band-pass of 10 nm. Conformational stability curves were obtained from fluorescence data using the method of Pace (1986). Briefly, the change of fluorescence was used to determine conformational stability of the protein, assuming a two state unfolding mechanism for the major unfolding transition of PGH in urea. The equilibrium constant (K_D) and the free energy of unfolding of PGH in

urea (ΔG_D) were calculated from:

$$K_D = e^{-\Delta G_D/RT} = \frac{f_D}{f_N} = \frac{y - y_N}{y_D - y} \quad (1)$$

where y_N is the intrinsic fluorescence of the native state, y_D is the intrinsic fluorescence of the denatured state, and f_N and f_D represent, respectively, the fraction of the protein present in the native and denatured states (Pace, 1986). The dependencies of the fluorescence intensities of the native and denatured states were taken as the linear extrapolation of points on either side of the main transition. A plot of ΔG_D on urea concentration and linear extrapolation to zero urea concentration provide a value for the free energy of unfolding in urea, ΔG_{H_2O}, which is a measure of the conformational stability of PGH.

Kinetic refolding experiments were performed by preincubating 1 mL of 50 mM Tris-HCl and 1 mM EDTA, pH 8.0, containing the desired refolding buffer in a 1 × 1 cm quartz cuvette at the desired temperature for approximately 10 min. Renaturation of unfolded Met(1-190)PGH was initiated by immediate dilution into the relevant equilibrated refolding buffer at 24 °C. As the G-25 chromatography step was omitted in the fluorescence studies, refolding occurred in a buffer containing at least 0.05 M urea and 1.5 mM β-ME. The fluorescence data were fitted to a curve using the following equation:

$$F = A_0 + A_1t^1 + A_2t^2 + A_3t^3 + A_4t^4 + A_5t^5 + A_6t^6 \quad (2)$$

where F is the emission fluoresence intensity measured at 350 nm at an excitation wavelength of 295 nm and t represents time in seconds. The rate of folding was measured as the change in fluorescence over the change in time as $\delta F/\delta t$ and was determined by differentiating eq 2 to give:

$$\frac{\delta F}{\delta t} = A_1 + A_2t^1 + A_3t^2 + A_4t^3 + A_5t^4 + A_6t^5 \quad (3)$$

The rates were approximated to the first fitted coefficient, A_1, because the fitted values of A_2, A_3, A_4, A_5, and A_6 decay since they are multiplied by a time rate to the power "n" and were $\leq 10^{-5}$ in magnitude.

(l) Monitoring the Disulfide Formation of Soluble Recombinant PGH by Thiol Chemistry. The formation of disulfide bonds was monitored during the reoxidation of PGH in "model" refolding conditions of 3.5 M urea and 50 mM Tris-HCl buffer, pH 9.1, at 20 °C, by nonreducing SDS−PAGE as described in Laemmli (1970). At various time points, a sample was withdrawn and mixed with 100 mM iodoacetamide, 2% (w/v) SDS, 10% (w/v) glycerol, and 62.5 mM Tris-HCl, pH 6.8, and incubated in darkness for 10 min at 50 °C. Samples were stored at −20 °C until all samples were collected and then analyzed by nonreducing SDS−PAGE. Time-dependent changes in free sulfhydryl content, during the reoxidation of PGH in "model" refolding conditions of 3.5 M urea and 50 mM Tris-HCl buffer, pH 9.1, at 20 °C were determined using 4.6, 9.1, 18.3, 36.6, 73, and 136 μM reduced PGH. At various time points, a sample was withdrawn and mixed with 0.5 mM 5,5′-dithiobis(2-nitrobenzoic acid) DTNB in buffered 5 M urea and allowed to stand for 25−30 min before measurement of free thionitrobenzoate anion versus a protein-free buffer blank for the same period of time (Ellman, 1959). The first-order rate law for the disappearance of a reactant, A, is given by:

$$\text{rate} = d[A]/dt = k[A] \quad (4)$$

since initially (at $t = 0$) the concentration of A is $[A]_0$, and at a later time t it is $[A]_t$; therefore:

$$\int_{[A]_0}^{[A]_t} \frac{d[A]}{[A]} = \int_0^t k\, dt \quad (5)$$

or

$$\ln [A]_t - \ln [A]_0 = kt \quad (6)$$

which can be expressed in two useful forms:

$$\ln([A]_t/[A]_0) = -kt \quad (7)$$

and

$$[A]_t = [A]_0 \exp(-k_it) \quad (8)$$

Thus, in a first-order reaction, the reactant concentration decreases exponentially with time, with a rate determined by k such that if $\ln([A]_t/[A]_0)$ is plotted against t, then a first-order reaction will give a straight line (Atkins, 1986; Jaenicke & Rudolph, 1989). If the reaction is first order, the value of k may be obtained from the slope of the straight line (the slope is $-k$). Data were plotted as ln (% thiols remaining) versus time at which the samples were taken according to the method of Holzman *et al.* (1986). Rate constants were calculated from the kinetic data using:

$$\text{rate} = \sum_{i=1}^{n} A_i \exp(-k_it) \quad (9)$$

and values using $i = 1$ and 2 exponentials were routinely examined (Holzman *et al.*, 1986). Values of the rate constants (k_i) were determined from the averages of three measurements at each protein concentration.

(m) Investigation of the Model Folding Pathway Using ANS Fluorescence. The binding of ANS to native PGH was studied in oxidizing buffer consisting of air-saturated 0.1 M Tris-HCl, pH 8.8, containing 0.1 M NaCl, and ANS binding to the denatured and reduced state of PGH was studied by incubating PGH and ANS in reducing buffer consisting of nitrogen purged 9.0 M urea, 0.25 M β-ME, 0.1 M NaCl, and 0.1 M Tris-HCl, pH 8.8. Quantum yields of ANS fluorescence and equilibrium binding data were evaluated using the methods described in Cardamone and Puri (1992). To investigate the time-dependent changes in the hydrophobicity of PGH during the optimized refolding pathway from the denatured and reduced state, an aliquot of the refolding mixture (1.0 mL) was removed at each time point (including that from a protein-free starting solution incubated for the same period of time) and incubated with ANS (final concentration of 0.110 mM) at 26 °C for 30 min. The ANS emission of the samples was measured by scanning between 400 and 600 nm using an excitation wavelength of 350 nm, at a band-pass of 10 nm, for both excitation and emission slits, and a photomultiplier voltage of 400 V. The scans were then integrated and estimates of ANS quantum yields obtained using SpectraCalc software, as a percentage of the ANS quantum yield measured at zero time point, which was

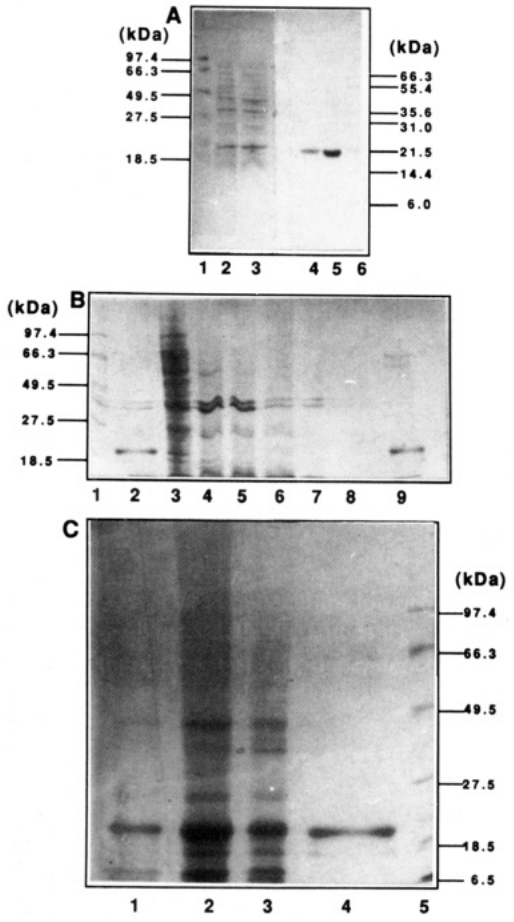

FIGURE 1: Effect of various isolation procedures on the purity of recombinant PGH from *E. coli* IB392 cells. (A) Nonreducing SDS−PAGE analysis of the expression of recombinant PGH in *E. coli*. Lane 1: MW markers (Bio-Rad). Lane 2: SDS extracts of whole *E. coli* IB392 cells. Lane 3: SDS extracts of whole *E. coli* IB392 cells incubated with 100 mM iodoacetate. Lane 4: Purified sample of recombinant PGH. Lane 5: Pituitary-derived PGH standard. Lane 6: MW markers (Novex). (B) Reducing SDS−PAGE analysis of the lysozyme, deoxycholate, and water washes of *E. coli* IBs. Lane 1: MW markers. Lane 2: Purified Met(1-190)PGH. Lane 3: Supernatant from the first centrifugation step (17000*g*, 30 min). Lane 4: Soluble proteins after washing IBs with 0.02% (w/v) lysozyme/10% deoxycholate. Lane 5: Soluble proteins after subsequently washing IBs with MQ water. Lane 6: Soluble proteins after washing with 5 M urea, 1% (v/v) Triton X-100, 5 mM EDTA, 75 μg/mL PMSF, 75 μg/mL leupeptin, and 75 μg/mL benzamidine in 0.1 M Tris-HCl buffer, pH 7.0, wash. Lane 7: Final MQ water wash step. Lane 8: Supernatant after sucrose-density ultracentrifugation. Lane 9: Standard PGH. (C) Reducing SDS−PAGE analysis of the insoluble fractions after various washing treatments. Lane 1: Insoluble fraction after sucrose density ultracentrifugation. Lane 2: Insoluble fraction after the first spin at (17000*g*, 30 min; see Experimental Procedures). Lane 3: Insoluble fraction after cumulative washes with deoxycholate, lysozyme, urea, and Triton X-100. Lane 4: Purified recombinant PGH. Lane 5: MW markers.

taken as the 100% fully unfolded state. The final data were plotted as ln (% relative ANS quantum yield) versus time.

RESULTS

(a) Expression of Recombinant PGH and Extraction from CTAC Solubilized IBs. Recombinant *E. coli* cells were grown, harvested, and disrupted, and the IB concentrate was obtained as described in Puri *et al.* (1992). Densitometric scanning of the SDS−PAGE gel lanes representing total solubilized protein (Figure 1A, lanes 2 and 3) showed that the recombinant PGH band (MW 22K) accounted for about 20% of the observed proteins. The pellet obtained after low speed centrifugation of the cell lysates contained IBs highly enriched for the aggregated recombinant protein. However, this low speed pellet also contained other cellular components (Figure 1B, lane 3) that may be either integral parts of the inclusion body or nonspecifically absorbed on the surface of the IBs following cell lysis or cosedimented with the IBs during centrifugation. The IBs were further purified by a series of washing steps using nonionic detergent (deoxycholate), chelators (EDTA, which binds divalent cations), and lysozyme (which degrades peptidoglycans) which are known to be effective in disrupting the *E. coli* envelope structure (Bowden *et al.*, 1991). The mild solubilizing conditions using deoxycholate, EDTA, and lysozyme do not appear to solubilize the hormone from the IBs as the recombinant PGH remains in the insoluble fraction during the extraction (Figure 1B). Subsequently, sucrose density ultracentrifugation improved the preparation more noticeably than the detergent and enzyme wash (Figure 1C). This was also observed macroscopically as the yellow pigment associated with the IB preparation was removed by the ultracentrifugation step and the IBs appeared as a white band at the 53−67% sucrose interface. The use of sucrose density gradient centrifugation for the purification of IBs is not essential and is not commonly used for commercial application and industrial downstream processing of recombinant PGH. Ultracentrifugation was used to derive material that was subject to high resolution spectroscopic analyses as described in later sections.

The yield of recombinant PGH after key steps in the overall purification process is summarized in Table 1. The data were drawn from several runs. The fractionation was followed by SDS−PAGE (Figure 2) which served as a practical and efficient means of evaluating the purity of the product at each step. Three hundred ninety milligrams of highly purified recombinant PGH (purity ≥99%; Figure 1A, lane 4) was obtained from 456 g (wet weight) of *E. coli* cells containing 2.46 g of recombinant PGH in a total of 12.3 g (dry weight) of protein; the overall recovery of the process was therefore 16% (Table 1).

Solubilization was best achieved using a 5% (w/v) or a 1:1 (w/w) ratio of CTAC to dry weight of inclusion bodies at pH 10 and at 50 °C for 30−60 min, at a protein concentration of 20 mg/mL. These conditions resulted in maximal solubilization of IBs, estimated at 85−90% of dry weight. The soluble material was also analyzed by RP-HPLC (Figure 3). On the basis of RP-HPLC estimation of PGH, approximately 50% of the total protein expressed in the IBs was recombinant PGH (Figure 3A,E).

The recombinant PGH solubilized from IBs using CTAC was isolated in a reduced (non-disulfide bonded) and therefore biologically inactive state (Schleyer *et al.*, 1983). In order to effect renaturation, the reduced recombinant PGH was chromatographed at pH 9.1 on a cation-exchange resin to remove bound and free CTAC. Under these conditions the recombinant protein carried a net negative charge (p*I* of recombinant PGH is pH 6.1) and did not adhere significantly to the resin. During the removal of the solubilization agent, there was, however, a small loss (3%, Table 1) of the recombinant protein. The use of 4−5 M urea in the column buffer was to maintain maximum solubility of the protein, particularly at high concentrations of the protein (30−40 mg/mL). The use of urea increases the recovery of recombinant

Table 1: Recovery of Recombinant PGH from *E. coli* IBs

purification step	vol (mL)	total protein[b] (g)	yield of recombinant PGH[c] (g)	recovery of recombinant PGH[d] (%)
(1) broken cells[a]	500	12.3	2.46	(100)
(2) broken cell pellet	150	7.3		
(3) pellet washed with EDTA, lysozyme, and deoxycholate[e]	1250	6.5	2.19	89
(4) pellet after sucrose ultracentrifugation	50	5.0		
(5) CTAC solubilization[f]	200	4.0	2.03	83
(6) removal of CTAC by DOWEX cation exchange	900	3.83	1.63	66
(7) refolding of PGH	1800	1.93	0.48	19.5
(8) DEAE anion-exchange purification	180	0.49	0.47	19
(9) MonoQ purification	32	0.40	0.39	16

[a] The starting amount of cells for the preparation was 456 g (wet weight) which is equivalent to 12.3 g of total protein (dry weight) in 500 mL total volume. [b] Protein was determined by the modified Lowry method relative to pituitary-derived PGH as a standard (Lowry *et al.*, 1951). [c] Yield of recombinant PGH was determined by densitometric scanning and integration of the appropriate reduced PGH band areas from the SDS−PAGE gel lanes in Figures 1 and 2, to give percentage of total protein represented by the recombinant protein. [d] Recovery of recombinant PGH was calculated relative to 2.46 g of recombinant Met(1-190)PGH in the starting material. [e] Suspended in 20 mM Tris-HCl, 5 mM EDTA, 0.016% (w/v) lysozyme, 2% (w/v) sodium deoxycholate, 75 μg/mL benzamidine, 75 μg/mL leupeptin, 75 μg/mL PMSF, pH 8.0. [f] Solubilization of purified IBs was conducted in 5% (w/v) CTAC, 0.25 M β-ME, 75 μg/mL benzamidine, 75 μg/mL leupeptin, 75 μg/mL PMSF, 0.1 M Tris-HCl, pH 9.1 at 50 °C.

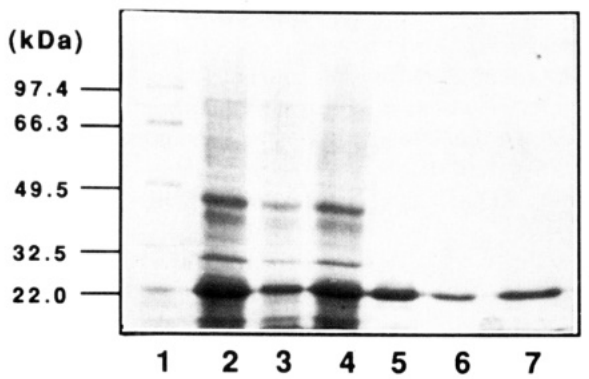

FIGURE 2: SDS−PAGE analysis of major intermediate recombinant PGH fractions during the solubilization and purification from *E. coli* IBs. Lane 1: MW markers. Lane 2: Insoluble fraction after differential centrifugation of *E. coli* IBs. Lane 3: SDS-solubilized IBs after sucrose centrifugation. Lane 4: Material after DOWEX CTAC removal of CTAC solubilized IBs. Lane 5: Refolded met-(1-190)PGH. Lane 6: DE-52 purified met(1-190)PGH. Lane 7: Pituitary-derived PGH.

PGH during the DOWEX process although it is not essential (Puri *et al.*, 1992).

(b) Renaturation of Recombinant PGH from CTAC Solubilized IBs. Recombinant PGH can be refolded *in vitro* to reform intrachain disulfide bonds, by dialyzing at 4 °C overnight against an aqueous buffer containing 1 mM EDTA, 3.5 M urea, 55 mM β-ME, and 50 mM Tris-HCl, pH 9.1, at a protein concentration of 0.5 mg/mL PGH. These conditions are believed to facilitate slow oxidation and renaturation kinetics and prevent aggregation (Jaenicke & Rudolph, 1990). Air oxidation of recombinant PGH was complete after 16 h as monitored by RP-HPLC. Integration of the relevant peak areas from RP-HPLC analysis indicated that approximately 25% of the refolded PGH was obtained as the monomeric 22 kDa form of PGH as judged previously by HPLC and SDS−PAGE (compare panels B and F of Figure 3). The remainder was a polydispersed population of high molecular weight material. The greatest loss during the purification was incurred at refolding (60% loss; Table 1). Procedures that improve the recovery during refolding would therefore significantly influence the final yield of recombinant PGH.

The refolded material was subjected to two anion-exchange chromatography steps. The first purification step involved batch ion-exchange chromatography using (diethylamino)-ethyl-cellulose (Whatman DEAE-52; Whatman, England) with the monomeric form of the protein eluted using 30 mM NaCl (Figure 1C). Higher concentrations of NaCl elute the higher molecular weight aggregated forms and non-PGH-related, IB-associated proteins. Approximately 75% of the recombinant PGH material loaded onto the DEAE was recovered. However, some protein may have been lost due to the selective precipitation of aggregated PGH during exchanging from the refolding buffer which contained urea, to the DEAE-52 chromatography buffer which does not contain it. Monomeric, oxidized met(1-190)PGH maintained its solubility during the buffer exchange step to the DEAE buffer system. The DEAE anion-exchange chromatography also resulted in a 10-fold concentration of the material (Table 1). The second anion-exchange step utilized a MonoQ column which was adapted to a HPLC system. Prior to this step, material was dialyzed exhaustively against pre-equilibration buffer (10 mM Tris-HCl, pH 8.8, containing 0.01% Tween-20). Three peaks were obtained in the MonoQ separation (Figure 4A), and the peak eluting at 150 mM NaCl was the oxidized monomeric form of recombinant PGH (Figure 4B,C). The material was then subjected to detailed biophysical and biochemical characterization.

(c) Truncated Recombinant PGH. During the development of the surfactant solubilization procedure for recombinant PGH, significant proportions of a lower molecular weight variant of recombinant PGH (Figure 5, observed MW of 20K) was observed on SDS−PAGE relative to pituitary-derived PGH (Figure 5C). The formation of this truncated form, designated P-band, was found to occur *in vitro* during the solubilization of IBs by CTAC (Puri *et al.* 1993) and was dependent on pH (pH $\leq$9.0) and the temperature of solubilization ($\leq$37 °C), but was not due directly to the use of CTAC, as purified soluble full-length PGH could not be converted to P-band by exposure to CTAC alone (Puri *et al.*, 1993). Bacterial proteolysis was suspected as being responsible for the *in vitro* formation of P-band. However, a cocktail of known protease inhibitors (including 2 μg/mL aprotinin, 2 mM EDTA, 1 μg/mL pepstatin, and 2 μg/mL leupeptin) did not inhibit its formation (Puri *et al.*, 1993). The formation of P-band could be inhibited by alkaline pH and high temperatures in the presence of CTAC, and by the solubilization of IBs in 5% SDS, 6.4 M GuHCl, or 7.5 M

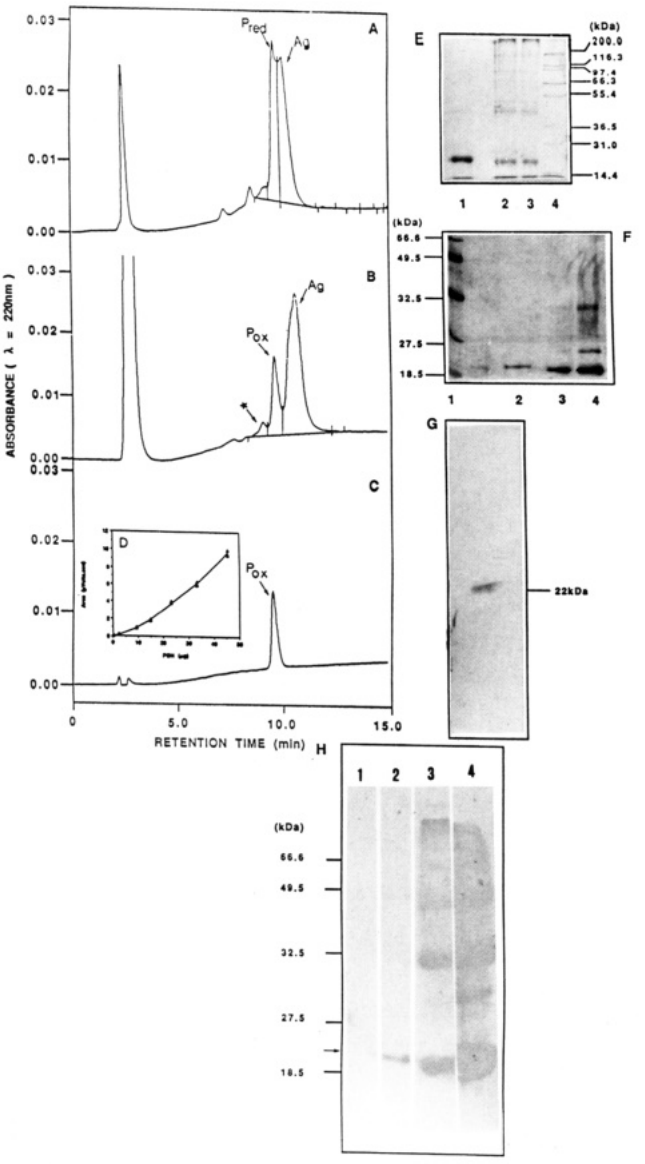

FIGURE 3: Isolation of oxidized monomeric PGH from *E coli* IBs analyzed by RP-HPLC, SDS−PAGE and Western analyses. (A) RP-HPLC analysis of Met(1-190)PGH IBs post-solubilization with 5% CTAC (w/v) in 0.25 M β-ME and 0.1 M Tris-HCl, pH 9.1 at 50 °C, for 1 h on a C_1-column. Solubilized protein was loaded onto the column in acetonitrile/TFA/H_2O (150:1:849) isocratically for 3 min and eluted with a linear gradient of 30−70% (v/v) acetonitrile over 15 min. (B) RP-HPLC analysis of recombinant Met(1-190)PGH after 16 h refolding by dialyzing against an aqueous buffer containing 1 mM EDTA, 3.5 M urea, and 55 mM β-ME in 20 mM Tris-HCl, pH 9.1 at 4 °C, with gentle agitation to promote oxidation. The peak labeled "Ag" constitutes aggregated forms and PGH-related proteins. The protein eluting ahead of oxidized monomer (indicated by the *) has not been characterized. (C) RP-HPLC analysis of monomeric and oxidized recombinant Met(1-190)PGH batch eluted from DEAE-52 ion-exchange chromatography with 30 mM NaCl at pH 9.1. (D) Calibration curve for the estimation of recombinant PGH using RP-HPLC analysis. Standard Met(1-190)PGH solutions were made using 0.1 M Tris-HCl, pH 8.8, 20 μL aliquots of these were loaded onto the C_1-silica column, and Varian software evaluated the total area under the peak corresponding to Met(1-190)PGH. Aliquots of the solubilization supernatants could then be analyzed by HPLC and the amount of PGH estimated from the standard curve. (E) SDS−PAGE analysis of the RP-HPLC fractions of plate A. Lane 1: Peak with a retention centered at 10.5 min (P_{red}). Lanes 2 and 3: 1:1 and 1:2 dilutions of the peak centered at 11 min in plate A. Lane 4: MW markers. (F) SDS−PAGE analysis of the RP-HPLC fractions of plate B. Lane 1: MW markers. Lane 2: Standard reduced PGH. Lane 3: Peak with retention centered at 9.5 min (P_{ox}). Lane 4: Peak centered at 11 min in plate B (Ag). (G) Expressed Met-(1-190)PGH isolated from *E. coli* IBs, refolded and purified on DEAE-52 anion exchange. It has been overloaded on the silver-stained gel (15% SDS−PAGE) to show the purity of the recovered PGH. (H) Western blot analysis of the fractions in plate A using murine monoclonal anti PGH 21.51. Lane 1: Negative control (membrane probed with murine monoclonal anti-ovine CD1 IgG_1 (20.27)). Lane 2: P_{red}. Lanes 3 and 4: 1:1 and 1:2 dilutions of Ag.

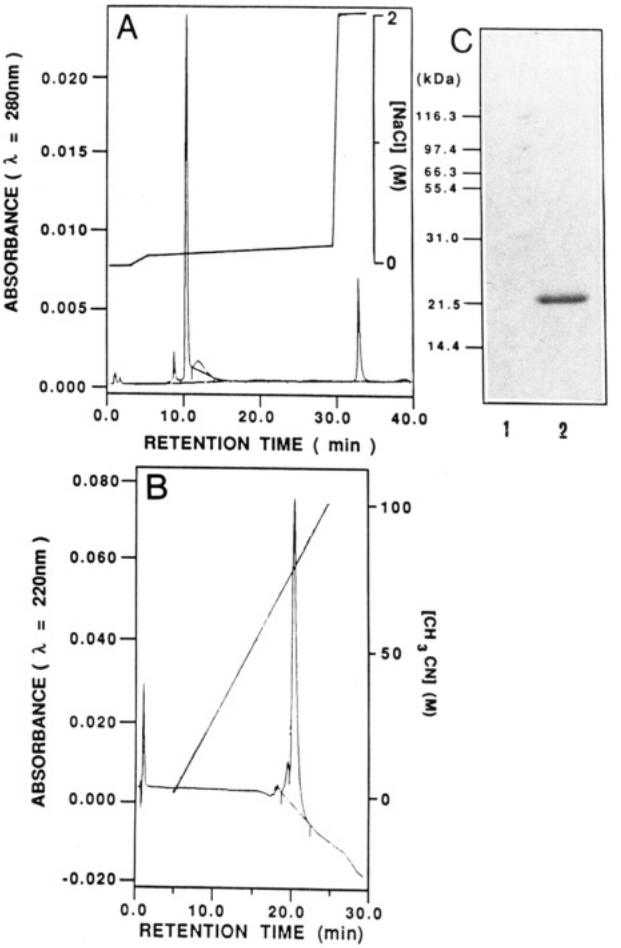

FIGURE 4: The elution profile of recombinant PGH from MonoQ anion exchange chromatography and RP-HPLC analysis of met-(1-190)PGH eluted from MonoQ chromatography. (A) Met(1-190)-PGH was rechromatographed on a FPLC MonoQ column equilibrated in buffer A (10 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.01% (v/v) Tween-20). The flow rate was 0.5 mL/min, and an increasing gradient of buffer B (buffer A containing 2.0 M NaCl) was used to elute the protein from the column. The column effluent was monitored at 280 nm. (B) The protein eluting at approximately 0.15 M NaCl was analyzed using a C-8 alkyl bonded silica column (Beckman Ultrasphere octyl column) with dimensions of 5 μm particle size, 30 nm mean pore diameter, and 4.6 mm × 250 mm. Proteins were loaded onto a column equilibrated with 0.1% (v/v) TFA and eluted using a linear gradient of 0−100% (999:1 (v/v) acetonitrile/TFA) over 20 min. (C) Lane 1 shows MW markers, and lane 2 shows expressed met(1-190)PGH purified on MonoQ anion exchange analyzed by reducing SDS−PAGE (15% (w/v) acrylamide) and silver-stained to show the homogeneity of the sample.

urea regardless of the pH and temperature used (Puri *et al.*, 1993).

(d) Chromatographic Analysis of Recombinant Met(1-190)PGH and P-Band. The *E. coli* synthesized 22 kDa PGH and pituitary-derived PGH displayed generally indistinguishable mobilities and chomatographic retention times in most

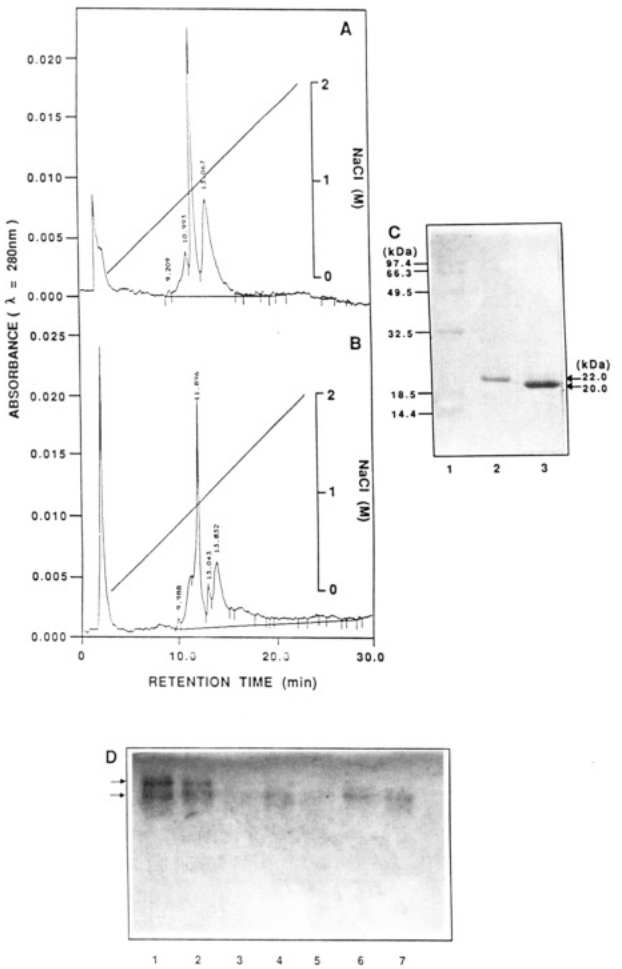

FIGURE 5: Characterization of a truncated form of recombinant met-(1-190)PGH generated *in vitro* during solubilization of *E. coli* IBs. (A) The elution profile of Met(1-190)PGH and (B) P-band from MonoQ anion-exchange chromatography. Proteins were chromatographed on a MonoQ anion-exchange column equilibrated in buffer A (10 mM Tris-HCl, pH 8.5, 1 mM EDTA, and 0.01% (v/v) Tween-20). The flow rate was 0.5 mL/min, and a gradient of increasing buffer B (buffer A containing 1.0 M NaCl) was used to elute the protein from the column. The column effluent was monitored at 280nm. (C) Reducing SDS−PAGE analysis of MonoQ purified met(1-190)PGH (lane 2) and P-band (lane 3) with apparent molecular masses of 22 000 and 20 000 kDa, respectively. Lane 1 shows the MW markers. (D) High pH native PAGE analysis of MonoQ purified Met(1-190)PGH and P-band fractions. Lane 1: DEAE-52 purified recombinant met(1-190)PGH. Lane 2: DEAE-52 purified P-band before MonoQ anion exchange. Lane 3: Met-(1-190)PGH fraction with retention time of 10.9 min. Lane 4: Major Met(1-190)PGH fraction eluting at 12 min. Lane 5: fraction eluting at 13 min. Lane 6: major P-band fraction eluting at 11.8 min. Lane 7: P-band fraction centered at 13.8 min.

of the analytical systems used. However, P-band demonstrated some interesting differences in its physical and biochemical properties from the full-length forms. Recombinant Met(1-190)PGH elutes at approximately 30% and 60% acetonitrile (CH_3CN) on trimethylsilane and C-8 reversed phase columns, respectively (Figures 3 and 4). Figure 3D shows an example of a calibration curve used in estimating the amount of recombinant PGH in a sample by RP-HPLC analysis. Under the chromatographic conditions used for RP-HPLC, the oxidized forms, P_{ox} (with respect to disulfide formation), of Met(1-190)PGH and P-band samples coelute at 9.5 min. Reduced froms of the protein, P_{red}, elute later at 10.0 min. There is no indication that there are two distinct

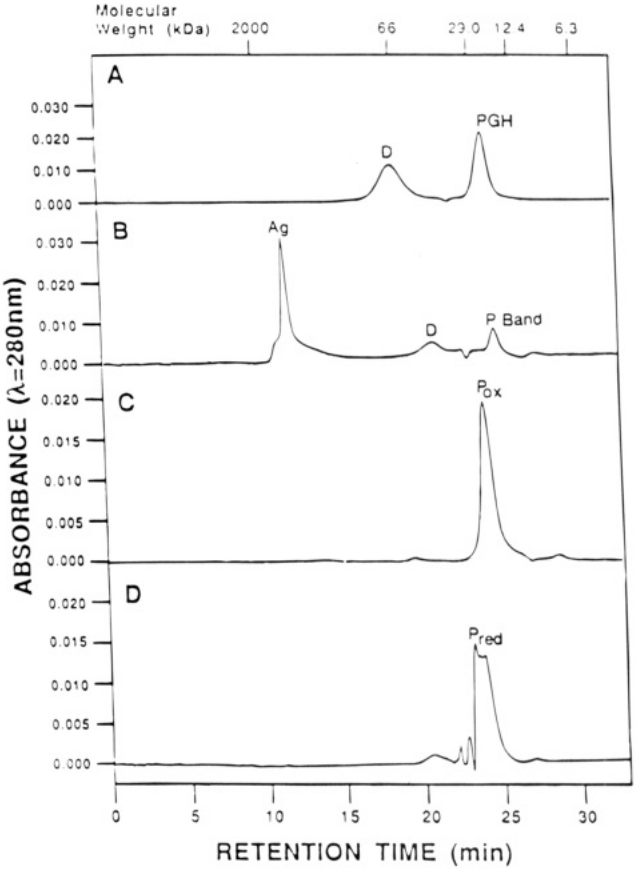

FIGURE 6: Analytical high performance gel permeation chromatography of (A) pituitary PGH, (B) P-band, (C) the oxidized native form (P_{ox}) of recombinant Met(1-190)PGH, and (D) the reduced denatured form (P_{red}) of Met(1-190)PGH using TSK G3000SW. The column effluent was monitored at 280 nm, and the running buffer was 50 mM phosphate buffer, pH 7.5. The labels "Ag" and "D" represent aggregated and dimerized forms of PGH, respectively.

species, which are often seen in SDS−PAGE (Figure 1C), present in the RP-HPLC analyses of met(1-190)PGH (Figure 3C). This behavior suggests that the recombinant PGH is fully and correctly oxidized with respect to the disulfides. The peak eluting ahead of the major PGH peak in Figure 3B (indicated by the *) has not been identified. However, the major peak following the PGH monomer is very broad and has been identified as encompassing all the aggregated and related forms of PGH (Figure 3B, peak "Ag") on the basis of a Western blot analysis of the peaks (Figure 3H). The mature form and P-band could be barely distinguished on the basis of their elution on reversed phase and ion-exchange chromatography (Figure 5).

To characterize the nature of the recombinant proteins in a physiologically relevant environment, high performance gel permeation chromatography (HPGPC) was carried out in 50 mM phosphate buffer, pH 7.5 (Figure 6). The Met-(1-190)PGH preparation appears to be homogeneous by size exclusion criteria, as a single symmetrical peak. Based on a plot of log(MW) versus K_{av} for standard proteins, where $K_{av} = (v_e - v_o)/(V_T - v_o)$ and V_e, V_o, and V_T represent elution volume of the protein, column void volume, and total column volume, respectively, the Met(1-190)PGH (Figure 6C) and P-band (Figure 6B) were estimated to have apparent molecular masses of 25 and 20kDa, respectively. The reduced preparation of Met(1-190)PGH elutes earlier than the oxidized form (Figure 6D), indicating that the reduced form

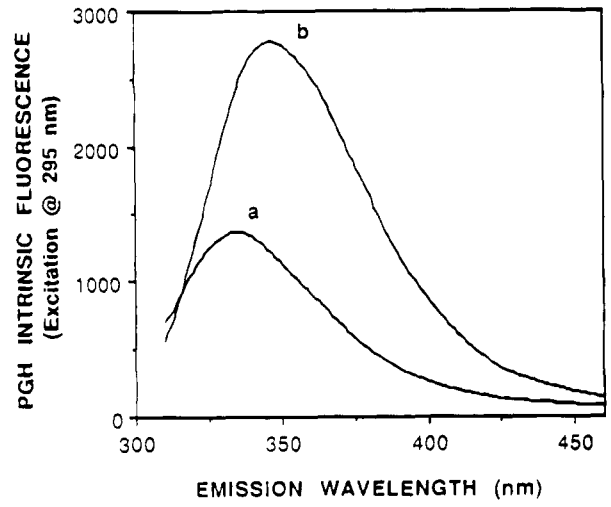

FIGURE 7: The effect of urea on the intrinsic fluorescence of spectra of PGH. The fluorescence emission spectra of (a) 4.6 μM native PGH in 50 mM Tris-HCl, pH 8.0, and (b) urea denatured PGH in the same buffer containing 9 M urea were measured between 300 and 450 nm with excitation at 295 nm at 25 °C. The photomultiplier voltage used was 400 V. Note the increase in the quantum yield of intrinsic fluorescence emitted by PGH in the presence of 9 M urea. Fluorescence intensity is shown in arbitrary units adjusted for the contribution of the Raman spectra of the buffer. The quantum yield of the Met(1-190)PGH 22 kDa form was 0.132 relative to the quantum yield of *N*-acetyltryptophanamide (NATA) at pH 8 of 0.14 (Kauffman *et al.*, 1989) with a maximum emission at 336 nm.

has a greater hydrodynamic radius, with an apparent molecular mass of 35 kDa, than the mature and folded monomeric PGH. This confirms that reduced Met(1-190)-PGH is also denatured. The reduced material in Figure 6D shows a broad elution band with multiple peaks. This is indicative of stable intermediates of folding in the reduced form. This represents a physical manifestation of the refolding of the protein within the time frame of the chromatography such that, as the protein is separated from the reducing and chaotrophic agents, the sample begins to refold. This is represented by the broad, heterogeneous peak in Figure 6D which corresponds to a mixture of unfolded and folded forms and possibly even self-associated forms of recombinant PGH. Consequently, it was decided to alkylate the protein with iodoacetamide in order to maintain the protein in a fully reduced state. High molecular weight aggregates, resulting either from noncovalent interactions and/or from disulfide interactions, tend to be present in the P-band and pituitary-derived forms (Figure 6A,B). Under these conditions of pH and ionic strength, Met(1-190)PGH is a monomer. Pituitary-derived PGH behaves as a dimeric form (Figure 6A, label D) under physiological conditions of pH and ionic strength, and this has also been reported previously (Becker *et al.*, 1987; Baumann, 1991). The results in Figure 6B also show that P-band has a greater propensity than pituitary-derived PGH and recombinant PGH to aggregate under the chromatography conditions used.

(e) The Conformational Stability of Native Met(1-190)-PGH, Reduced and Carboxymethylated Met(1-190)PGH, and P-Band. The unfolding and refolding of PGH can also be monitored by measuring changes in its circular dichroism or fluorescence emission spectra. High concentrations of urea increase the intrinsic fluorescence of PGH especially at concentrations greater than 5 M urea (Figure 7) and shift the wavelength of maximum emission from 341 to 348 nm. The increase in fluorescence was found to be sigmoidal with a linear change over the 5−8 M range at pH 8.0 (Figure 8). Measuring the increase in intrinsic fluorescence emission at 350 nm using an excitation wavelength of 295 nm was chosen for these studies because the largest change in the emission spectrum of PGH occurred at an emission wavelength of 350 nm during unfolding with urea (Figure 7). The fluorescence emission yield of PGH increased as the protein structure changed from a native state to a urea denatured state (Figure 7), allowing folding to be conveniently followed by the loss in fluorescence intensity at 350 nm when the tryptophan residue of PGH was selectively excited at 295 nm. The quantum yields of the Met(1-190)PGH 22 kDa form and of the truncated recombinant product, P-band, were recorded as 0.132 and 0.077, respectively, relative to the quantum yield of *N*-acetyltryptophanamide (NATA) at pH 8 of 0.14 (Kauffman *et al.*, 1989), and fluorescence emissions were maximum at 336 and 333 nm, respectively (Puri *et al.*, 1993). Whereas the quantum yield of intrinsic fluorescence of Met(1-190)PGH and P-band is dissimilar (Puri *et al.*, 1993), the urea denaturation of PGH and of P-band follow a very similar relationship with urea concentration, with the greatest degree of denaturation occurring between 5 and 9 M urea having midpoints at approximately 7.1 M and 6.8 M urea for Met(1-190)PGH and P-band, respectively (Figure 8, Table 2). The similar denaturation behavior of Met(1-190)PGH and P-band indicates that the conformational stability of these proteins are not significantly different, with a free energy of denaturation, ΔG_{H_2O}, of 3.8 ± 0.2 kcal/mol (Table 2). We observed that the fluorescence emission yield of reduced and carboxymethylated PGH (RCAM-PGH) rises less appreciably with urea concentration than both the intact and C-terminally cleaved proteins (Figure 8). The conformational stability of the protein was compromized by reducing and blocking the four thiols by carboxymethylation with iodoacetamide. The free energy of unfolding for the fully reduced and alkylated form cannot be estimated because there is not sufficient structure in the fully alkylated species to assess its free energy of unfolding. The observation that the reduced and carboxymethylated lacks sufficient structure as measured by fluorescence spectroscopy indicates that the native-like conformation of PGH is dependent on the integrity of the large loop domain defined by the disulfide bond between cysteine residues 53 and 164 because the complete removal of the disulfide bonds in the reduced and carboxymethylated PGH (RCAM) sample is associated with a severe loss in native-like structure of the protein whereas the P-band sample (which has only one disulfide lacking only the C-terminal disulfide between cysteine residues 181 and 189) did retain native-like structure.

(f) Establishing a "Model" Refolding Pathway for PGH Using Chaotroph Denatured PGH. Different solution conditions were investigated in order to optimize the oxidation and renaturation of Met(1-190)PGH from a urea denatured and reduced state. Denaturation of the protein with 9 M urea and then reducing the protein and blocking the resultant thiols with iodoacetamide totally remove the known secondary structure elements, as assessed by circular dichroism (data not shown). Curve-fitting the spectra indicates that the protein is essentially denatured with 0% α-helicity.

A typical nonreducing SDS−PAGE profile of refolded recombinant PGH is shown in Figure 9 with differences in the electrophoretic mobility between the monomeric (native)

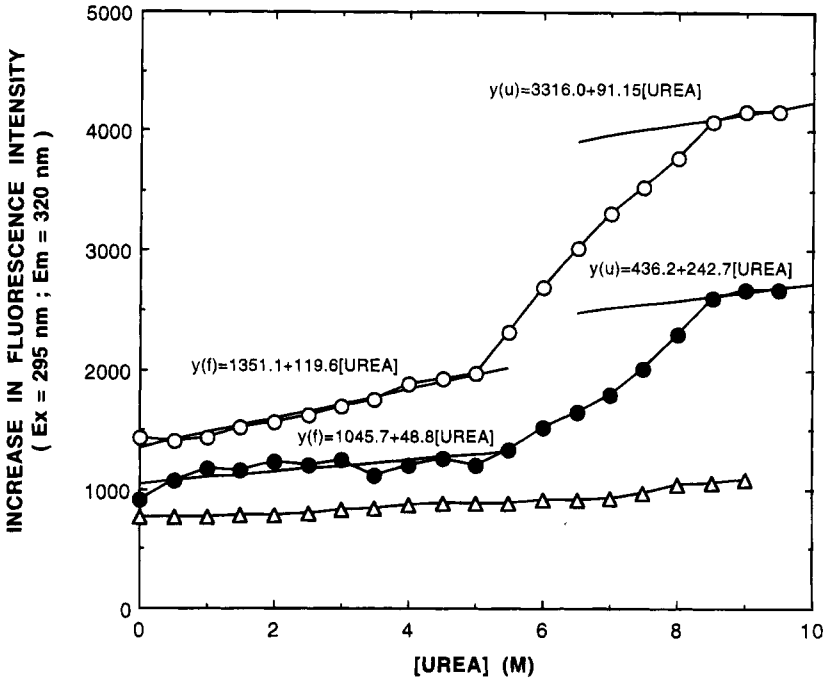

FIGURE 8: The equilibrium denaturation of recombinant PGH as a function of urea concentration at 26 °C. The denaturation curves of P-band (○), Met(1-190)PGH (●), and fully reduced and iodoacetamide carboxymethylated Met(1-190)PGH (RCAM) (△) were obtained from fluorescence emission intensity measurements at 350 nm (Em = 350 nm) after exciting at 295 nm (Ex = 295 nm) at 26 °C in 50 mM Tris-HCl buffer, pH 8.0. The equations for $y(f)$ and $y(u)$ give the value of the increase in fluorescence for the folded and unfolded states, respectively, where [urea] is the molar concentration of urea. The transition midpoints and the values for the apparent free energy change of denaturation (ΔG_{H_2O}) of PGH in urea are summarized in Table 2.

Table 2: Estimated Urea Denaturation Parameters of PGH

protein	transition midpoint[a] (M urea)	ΔG_{H_2O}[b] (kJ/mol)	ΔG_{H_2O}[b] (kcal/mol)	$\Delta\Delta G_{H_2O}$[c] (kcal/mol)
Met(1-190)PGH[d]	7.1	16.0 ± 0.7	3.8 ± 0.2	0
P-band[e]	6.8	15 ± 2	3.6 ± 0.4	−0.2 ± 0.6

[a] Transition midpoints were obtained from fluorescence emission data in Figure 8 and the apparent free energy of unfolding (ΔG_{H_2O})[b] using the denaturation curve analysis described by Pace (1986). The errors represent the 95% confidence limits. [c] The difference in the apparent free energy of unfolding of native Met(1-190)PGH and the modified forms ($\Delta\Delta G_{H_2O}$). This value represents the loss in conformational stability associated with the derivatization of the protein. [d] Met(1-190)PGH is the full-length PGH with 2 intact disulfides. [e] P-band is the 20K variant of PGH lacking the carboxy-terminal disulfide loop (Puri *et al.*, 1993).

form (P_{ox}), the reduced form (P_{red}), the dimerized form (P_{dimer}), and aggregated recombinant PGH (Ag) (Figure 9A). During the model refolding of PGH, there appeared to be a gradual decrease in the proportion of the reduced protein band and a simultaneous increase in the proportion of folded native PGH and also some increase in the aggregated protein peak (Figure 9B). Three solution parameters investigated were observed to influence monomer yield during the air oxidation of denatured Met(1-190)PGH: pH, and urea and protein concentration (Figure 10). Solution pH was investigated between pH 1 and pH 11 at a set protein concentration of 0.5 mg/mL (Figure 10A). Optimal refolding yields were obtained at a pH greater than 9 (Figure 10A).

The refolding of the protein was strongly dependent on the concentration of the Met(1-190)PGH during the renaturation. Figure 10B shows renaturation yields between 0.1 and 10 mg/mL, with the latter concentrations being more typical of those used during large-scale industrial processes. Maximum monomer yields of greater than 90% were obtained at protein concentrations of 0.1 mg/mL, with a declining yield found with increasing protein concentration. The effect of varying urea concentration over the range between 1 and 9 M on renaturation yields at a concentration of 0.5 mg/mL Met(1-190)PGH, pH 9.1, is shown in Figure 10C. A maximal yield of 89% was obtained at a urea concentration of 3.5 M urea.

The effect of the ratio and concentration of reducing equivalents added to the renaturing solution was also examined (Table 3). The concentration and ratio of β-ME to β-hydroxyethyl disulfide (HED), the oxidized redox conjugate of β-ME, were found to have a dramatic effect on the aggregation level. However, this decrease was at the expense of slowing down the rate of oxidation of Met(1-190)PGH, as indicated by the high proportion of reduced protein (40−68% P_{red}) after 16 h refolding with 55 mM thiol/disulfide pair (Table 3). Increasing the concentration of β-ME/HED reagents had the effect of increasing the concentration of dimerized PGH (3% P_{dimer} at 0.5 mM β-ME/HED, 7% at 5 mM β-ME/HED, and 21% at 55 mM β-ME/HED).

Therefore, the conditions for maximal Met(1-190)PGH renaturation from the urea denatured state were found to be optimal at a urea concentration of 3.5 M, a protein concentration at 0.5 mg/mL, and a pH of 9.1 as well as the addition of 10 mM β-ME/HED at a ratio of 10:1 (Table 3). The overall yield from the optimized refolding protocol comprising 3.5 M urea, 10 mM β-ME, 1 mM EDTA, and 50 mM Tris-HCl, pH 9.0, was approximately 85% as assessed by nonreducing SDS−PAGE. RP-HPLC analysis of the same sample indicated that reoxidation resulted in the formation of 89% monomer and 10% high molecular weight forms. This set of optimized refolding conditions was, for the purpose of this study, the "model" refolding pathway.

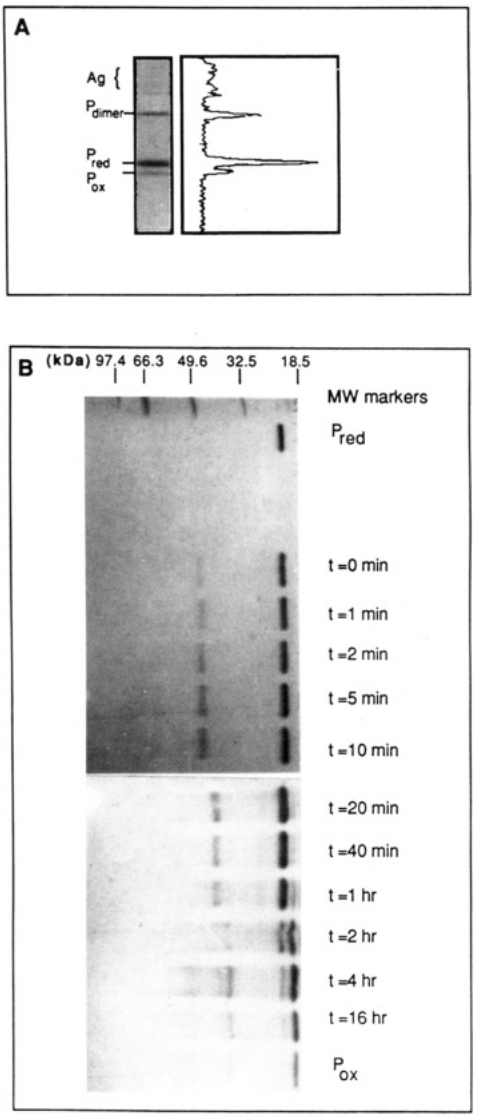

FIGURE 9: Nonreducing SDS−PAGE analysis of *in vitro* reoxidation of recombinant PGH. (A) The monomeric (P_{ox}) oxidized Met-(1-190)PGH band is clearly resolvable from dimeric PGH (P_{dimer}), from reduced PGH (P_{red}), and from polydisperse "aggregated" PGH (Ag) on nonreducing SDS−PAGE. Gels such as these were silver-stained and densitometrically scanned to obtain % P_{ox}. (B) The time course of reoxidation of reduced Met(1-190)PGH in 3.5 M urea, 10 mM β-ME, 1 mM EDTA, and 0.1 M Tris-HCl, pH 9.0, as assessed by nonreducing SDS−PAGE. Samples were quenched with 100 mM iodoacetate 2% (w/v) SDS, 10% glycerol, and 62.5 mM Tris-HCl, pH 6.8, and incubated at 50 °C for 10 min in darkness and then stored at −20 °C prior to nonreducing SDS−PAGE analysis.

(g) Refolding of Urea, and CTAC Solubilized Recombinant PGH from IBs. The effect of urea on the renaturation of met(1-190)PGH from CTAC and urea solubilized IBs using the model refolding conditions was also studied. It was found that there was a 48% refolding efficiency if Met(1-190)PGH was solubilized with CTAC and refolded under the "model" refolding conditions of 3.5 M urea, 10 mM β-ME, 1 mM EDTA, and 50 mM Tris-HCl, pH 9.0, whereas the refolding efficiency was only 24% if urea was used as the solubilizing agent (Table 4). The refolding efficiency of urea solubilized IBs was optimal by refolding at low urea concentrations (compare 26% efficiency at 3.0 M urea and 24% at 3.5 M urea, Table 4). The percent (%) oxidized monomeric PGH using CTAC solubilized IBs was clearly

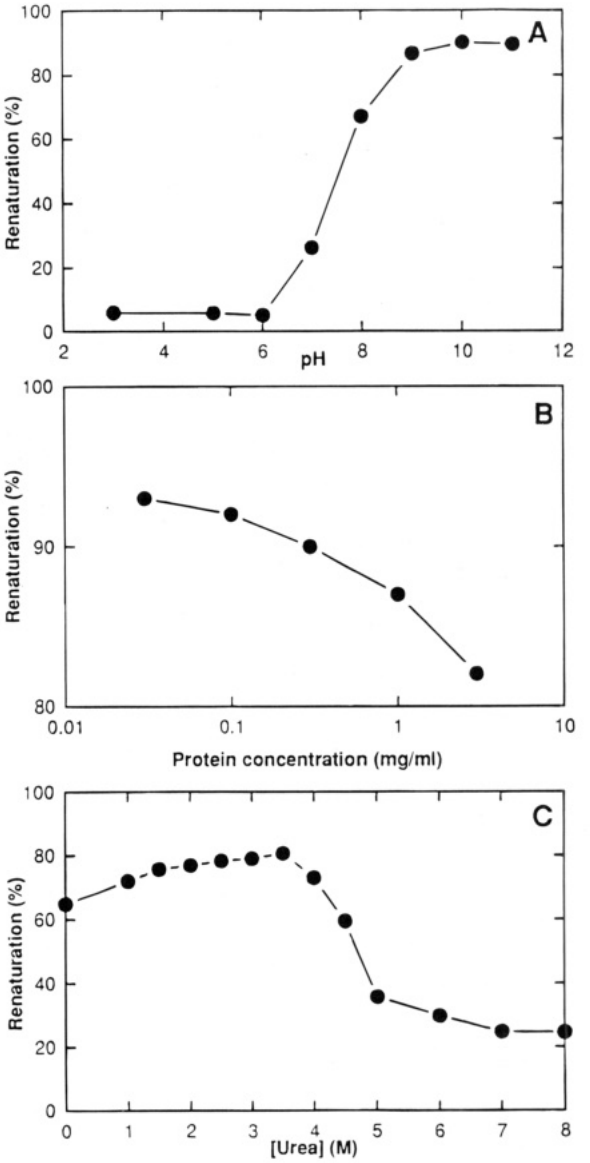

FIGURE 10: Effect of (A) pH, (B) protein, and (C) urea concentration on the renaturation of Met(1-190)PGH. Renaturation was calculated following densitometic scanning of silver-stained nonreducing SDS−PAGE analysis of various refolding samples. For the determination of the pH dependence of refolding, the urea denatured and reduced Met(1-190)PGH was diluted into a series of refolding buffers including 50 mM citrate, pH 3.0; 50 mM acetate, pH 5; 50 mM acetate, pH 6.0; 50 mM phosphate, pH 7.0; 50 mM Tris-HCl, pH 8.0; 50 mM glycine−NaOH, pH 9.0; 50 mM ethanolamine hydrochloride, pH 10; and 50 mM ethanolamine hydrochloride, pH 11, containing protein at a concentration of 0.5 mg/mL. For determination of the protein concentration dependence of refolding, unfolded protein was buffer exchanged into 50 mM glycine−NaOH, pH 9.0, containing 1 mM EDTA and 3.5 M urea. For the determination of the urea concentration dependence of refolding, the final protein concentration was kept at 0.5 mg/mL and the pH was kept constant at pH 9.1 using 50 mM Tris-HCl buffer, pH 9.1, containing 1 mM EDTA. The urea concentration was varied from 0 to 8 M.

2-fold greater than that with urea solubilized IBs, irrespective of urea concentration (Table 4).

(h) The Kinetics of Tryptophan Burial during Refolding of Met(1-190)PGH. Time-dependent changes in the intrinsic fluorescence of PGH during reoxidation and refolding in different solution conditions were measured. Refolding was initiated by dilution of Met(1-190)PGH in 9 M urea into refolding buffer, and refolding was allowed to proceed in

Table 3: Effect of [β-Hydroxyethyl Disulfide]/[β-Mercaptoethanol] Ratio on the Renaturation of Met(1-190)PGH[a]

concn[b] (mM)	[β-ME]/[HED])[c]	% P_{ox}[d]	% P_{red}[e]	% P_{dimer}[f]	% aggregate[g]
55	1:10	12 ± 2	40 ± 8	21 ± 1	25 ± 6
	1:1	15 ± 1	68 ± 2	16 ± 2	0
	10:1	21 ± 1	68 ± 3	10 ± 4	0
5	1:10	12 ± 2	8 ± 2	7 ± 2	70 ± 6
	1:1	13 ± 6	6 ± 2	5 ± 2	74 ± 8
	10:1	9 ± 3	7 ± 2	6 ± 2	79 ± 2
0.5	1:10	13 ± 3	1 ± 2	3 ± 1	81 ± 3
	1:1	11 ± 1	0	1 ± 2	87 ± 3
	10:1	11 ± 1	0	2 ± 2	86 ± 4
0	0	13 ± 2	0	1 ± 1	85 ± 9

[a] Unfolding was carried out by incubation of recombinant protein in 9.0 M urea, 0.25 M β-ME, 50 mM Tris-HCl, pH 9.1 at 50 °C, and a final protein concentration of 10 mg/mL (457 μM) for at least 1 h. The reducing agent was then removed by G-25 size chromatography. Refolding was initiated by dilution into refolding buffer (50 mM Tris-HCl, pH 9.1) at 22.7 μM PGH containing 1 mM EDTA and the thiol−disulfide pair. Renaturation proceeded at 4 °C overnight, with gentle agitation. [b] Final total concentration of the thiol−disulfide pair in the refolding buffer. [c] The ratio of the thiol−disulfide pair in the refolding buffer. [d] The proportions of oxidized PGH (P_{ox}), reduced PGH (P_{red}),[e] dimerized PGH (P_{dimer}),[f] and aggregated PGH-derived protein[g] were estimated by densitometric scanning of silver-stained nonreducing SDS−PAGE gels of refolding samples. Data represent the mean ±SD of triplicate measurements.

≤0.1 M urea. It was found that the refolding from a urea denatured state was associated with a decrease in intrinsic fluorescence, up to an equilibrium value which was generally reached 5−8 min after dilution into the refolding buffer (Figure 11). The time-dependent increase in fluorescence associated with PGH folding was assumed to follow first-order refolding kinetics, i.e.:

$$\text{rate} = \delta F/\delta t = k[\text{PGH}]\ (\text{s}^{-1}) \qquad (10)$$

where $\delta F/\delta t$ represents the the rate of fluorescence change over time, k represents the rate constant, and [PGH] represents the concentration of unfolded PGH because the rate of folding was observed to be linearly dependent with protein concentration (Figure 11) in accordance with eq 10. In a first-order reaction, the reactant concentration (in this case, unfolded PGH) decreases exponentially with time, with a rate determined by k. The presence of EDTA had the effect of slowing the rate of refolding (Figure 12) due most likely to the ability of EDTA to remove divalent ions, which are known to increase the kinetics of reoxidation and disulfide/thiol exchange (Jaenicke & Rudolph, 1990). It appears that

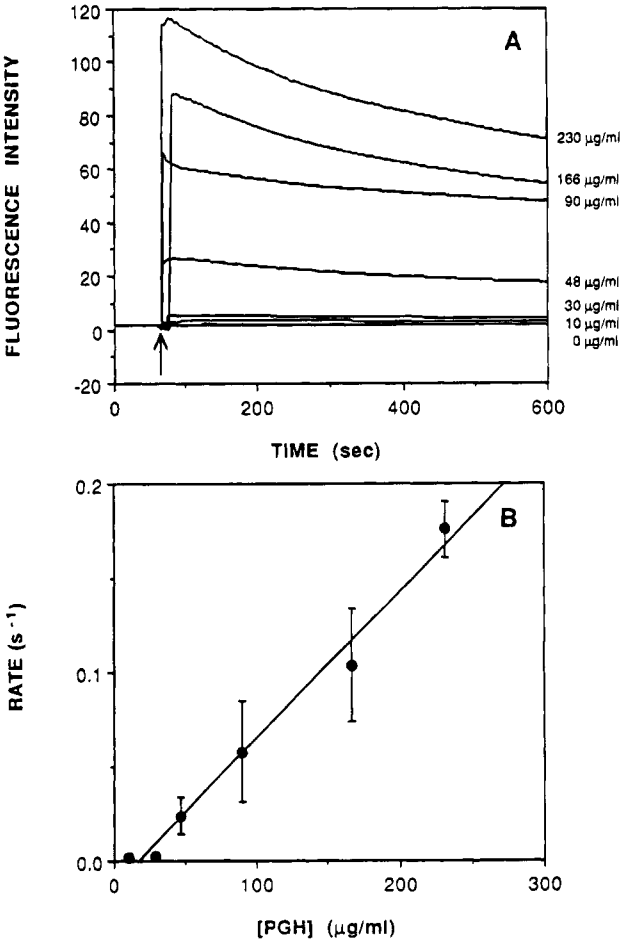

FIGURE 11: Refolding of recombinant PGH as a function of protein concentration at 26 °C. (A) The renaturation of PGH was monitored by the decrease in fluorescence at 350 nm (295 nm excitation) following a 1:21 dilution of a 45.4 μM Met(1-190)PGH solution containing 9 M urea and 100 mM Tris-HCl, pH 8.8, into refolding buffer (50 mM Tris-HCl, pH 8.8). The arrow marks the point at which protein was added to the refolding solution at final concentrations of 0, 10, 30, 48, 90, 166, and 230 μg/mL. (B) The fluorescence data were fitted to give estimated rates of folding. The kinetic rates of refolding as monitored by fluorescence increased linearly with protein concentration and therefore followed first-order kinetics. A rate constant of $k = 7.8 \times 10^{-4}$ s^{-1} (μg/mL)$^{-1}$ with a correlation coefficient (r^2) of 0.986 was obtained. Vertical bars denote the SD of the mean for three determinations.

Table 4: Effect of Urea on the Renaturation of Met(1-190)PGH from Urea and CTAC Solubilized Inclusion Bodies in 10 mM β-Mercaptoethanol, 1 mM EDTA, and 0.1 M Tris-HCl, pH 9.1

	CTAC solubilized IBs[a]			urea solubilized IBs[b]		
[urea] (M)	% P_{ox}[c,d]	% aggregate[d]	% refolding efficiency[e]	% P_{ox}[c,d]	% aggregate[d]	% refolding efficiency[e]
2.5	17 ± 1	83 ± 2	34 ± 2	11.6 ± 0.8	88.4 ± 0.9	21 ± 2
3.0	22 ± 2	78 ± 4	44 ± 4	13.2 ± 0.6	86.8 ± 0.8	26 ± 1
3.5	24 ± 4	76 ± 5	48 ± 3	12 ± 1	87.7 ± 0.9	24 ± 6
4.5	20 ± 3	80 ± 8	42 ± 2	11.3 ± 0.7	88.7 ± 0.7	22 ± 4

[a] Solubilization was performed using 5% (w/v) CTAC in 0.1 M Tris-HCl, pH 9.1, containing 0.25 M β-ME and 1 mM EDTA at 50 °C for 1 h at a recombinant PGH concentration of approximately 10 mg/mL. The mixture was composed of 50% reduced PGH as analyzed by RP-HPLC. [b] Solubilization was performed in 8 M urea in 0.1 M Tris-HCl, pH 9.1, containing 0.25 M β-ME and 1 mM EDTA, at 50 °C for 1 h at a recombinant PGH concentration of approximately 10 mg/mL. The mixture was composed of 50% reduced PGH as analyzed by RP-HPLC. [c] Refolding was performed in 10 mM β-mercaptoethanol, 1 mM EDTA, and 0.1 M Tris-HCl, pH 9.1, at a recombinant PGH concentration of approximately 0.5 mg/mL for 48 h at 4 °C with aeration. [d] % monomeric reoxidized PGH (% P_{ox}) and % aggregated PGH-derived protein were estimated by RP-HPLC as validated and described previously in Puri *et al.* (1992). [e] Refolding efficiency was calculated as the % monomeric oxidized PGH yields shown in % P_{ox} divided by the percentage of reduced PGH at the start of *in vitro* refolding, normally approximately 50% of the solubilized IBs mixture (Puri *et al.*, 1992). Data represent the mean ±SD of triplicate measurements.

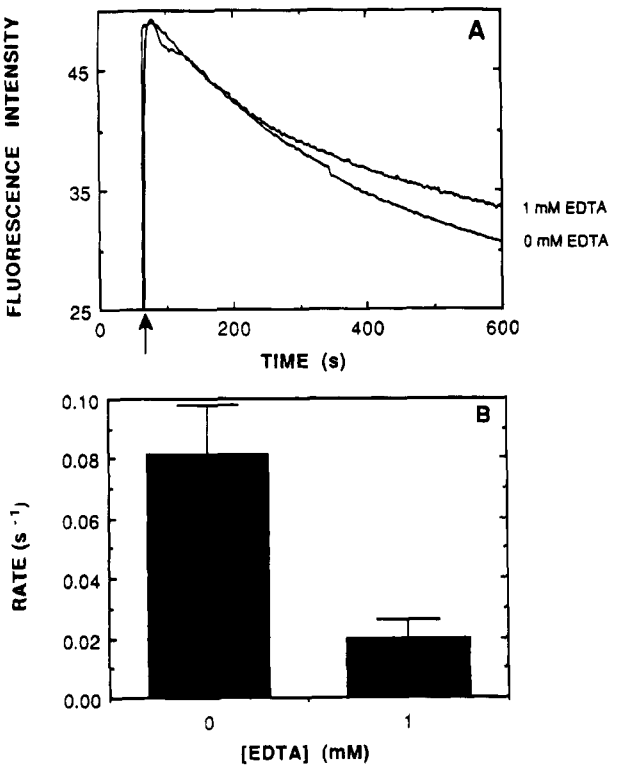

FIGURE 12: Refolding kinetics of recombinant PGH in the presence and absence of 1 mM EDTA at 26 °C. (A) The renaturation of PGH was monitored by the decrease in fluorescence at 350 nm (295 nm excitation) following a 1:21 dilution of a 45.4 μM Met-(1-190)PGH solution containing 9 M urea and 100 mM Tris-HCl, pH 8.8, into refolding buffer (50 mM Tris-HCl, pH 8.8 in the presence and absence of 1 mM EDTA). The refolding solution was monitored for any change in fluorescence before the addition of protein. The arrow marks the point at which the refolded protein was added to the refolding solution. (B) Data in the absence of EDTA were fitted to give a rate of 0.08 $\pm$ 0.02 s^{-1}, and the fluorescence data in the presence of 1 mM EDTA were fitted to give a rate of 0.020 $\pm$ 0.005 s^{-1}.

the refolding of Met(1-190)PGH is promoted by the presence of trace heavy metals in solution and that the presence of EDTA causes an inhibition in the rate of folding (Figure 12).

The effects of pH and urea and β-ME concentration in the refolding buffer on the rate of folding were also investigated (Figure 13). A linear increase in the rate of folding with pH was found, with the rate of folding reaching a plateau at alkaline pHs, between 8 and 10 (Figure 13A). Conversely, the rate of folding was markedly inhibited by urea concentrations greater than 4 M (Figure 13C). The reducing agent, β-ME, generally decreased the rate of folding as assessed by intrinsic fluorescence (Figure 13B).

(i) The Kinetics of Sulfhydryl Reoxidation of Met(1-190)-PGH. The above results suggest that the formation of tertiary structure, as assessed by intrinsic fluorescence, was essentially complete after 10 min. However, the reoxidation of PGH to its intradisulfide linked form was much slower, taking at least 1 h as measured by nonreducing SDS−PAGE analysis (Figure 9).

Detailed studies were undertaken to investigate the rate of formation of disulfides. The rates of disulfide formation in the "model" refolding pathway were monitored using the conventional Ellman assay, which measures the changes in free sulfhydryl content by DTNB titration (Ellman, 1959). The reoxidation process was not log-linear in that it has an initial slow phase and a faster secondary phase (Figure 14A). This has also been observed for BGH (Holzman *et al.*, 1986).

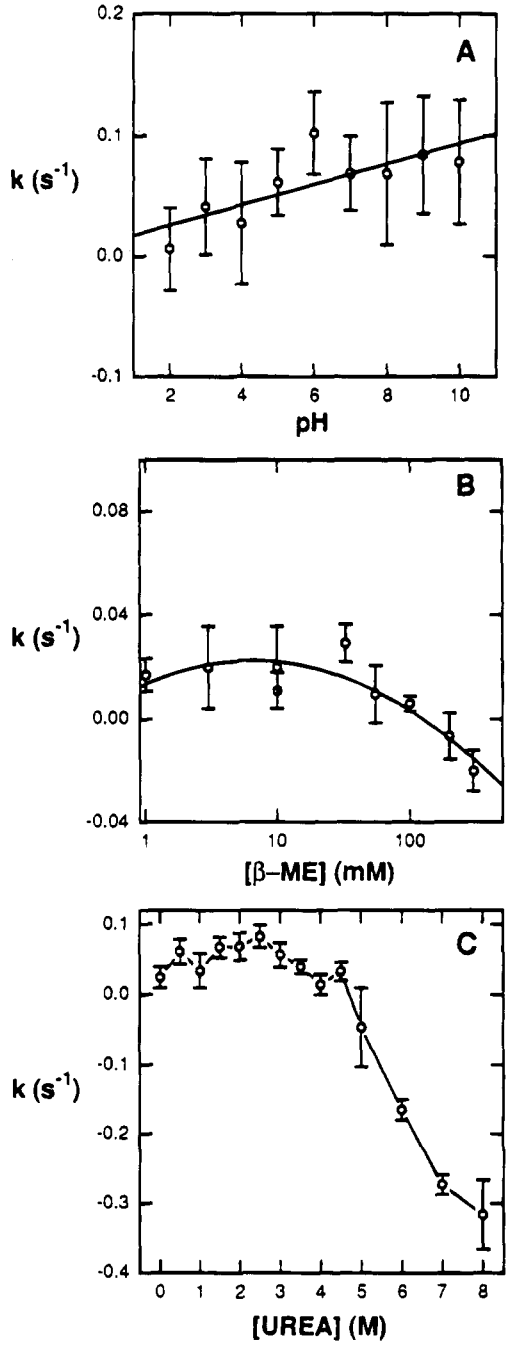

FIGURE 13: Effect of pH, β-ME, and urea on the rate of refolding of recombinant PGH. The rate of renaturation was monitored by the decrease in fluorescence emission at 350 nm (295nm excitation) following a 1:21 dilution of a 45.4 μM Met(1-190)PGH solution containing 9 M urea, 0.25 M β-ME, and 100 mM Tris-HCl, pH 8.8, into one of the following: (A) refolding buffer of varying pH (50 mM citrate buffer, pH 3.0; 50 mM citrate buffer, pH 4.0; 50 mM acetate buffer, pH 5.0; 50 mM acetate buffer, pH 6.0; 50 mM phosphate buffer, pH 7.0; 50 mM Tris-HCl buffer, pH 8.0; 50 mM Tris-HCl buffer, pH 9.0; and 50 mM ethanolamine hydrochloride buffer, pH 10.0) containing 1 mM EDTA; (B) refolding buffer of varying β-ME concentration (1−200 mM) in air-saturated 50 mM Tris-HCl, pH 8.8, containing 1 mM EDTA (a negative rate indicates that there was an increase in fluorescence, suggesting further unfolding of the protein); (C) refolding buffer of varying concentrations of urea (0−8 M) in air-saturated 50 mM Tris-HCl, pH 8.8, containing 1 mM EDTA. Vertical bars represent SD of triplicate measurements.

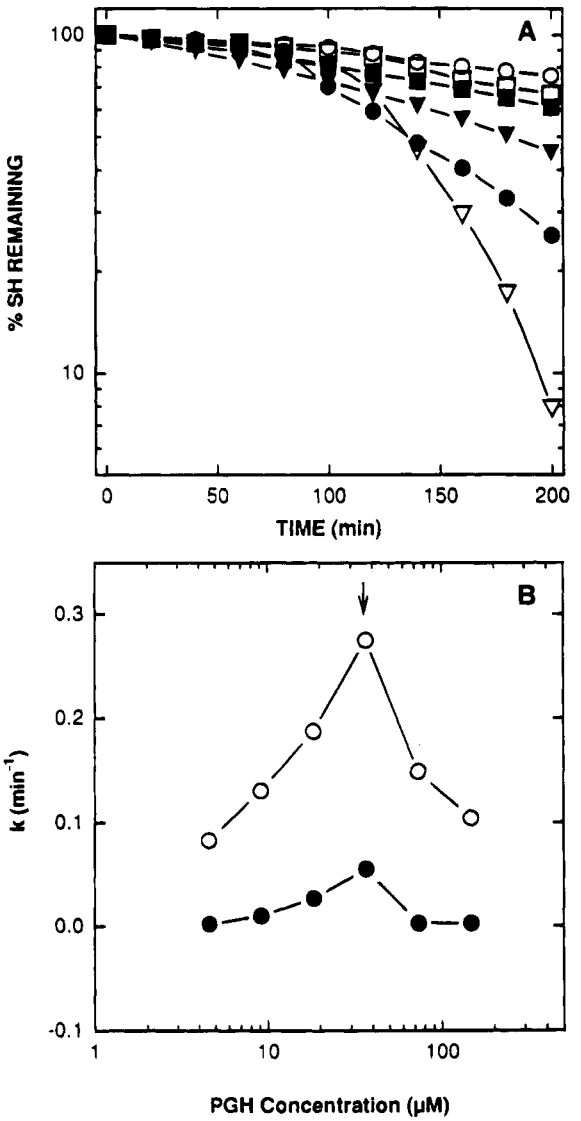

FIGURE 14: Effect of protein concentration on the time-dependent changes in the free sulfhydryl content during the reoxidation of recombinant PGH in air-saturated 3.5 M urea and 50 mM Tris-HCl, pH 9.1, containing 1 mM EDTA. (A) Changes in free SH groups were determined by using reduced protein at 4.5 μM (○), reduced protein at 9.1 μM (▼), reduced protein at 18.2 μM (●), reduced protein at 36.4 μM (▽), reduced protein at 72.7 μM (■), and reduced protein at 145 μM (□). At each time point, an aliquot was withdrawn and mixed with a final concentration of 0.5 mM DTNB in 5 M urea and allowed to stand for 30 min before measurement of the free thionitrobenzoate anion versus a protein-free control for the same period of time. (B) The curve for the rate of reoxidation shows an initial slow phase and a faster secondary phase, and the plot can be curve fitted from the sum of two exponentials to give two rate constants for the slow phase (●) and for the faster secondary phase (○) at each protein concentration. The rate constants increase linearly with protein concentration until 36.4 μM (marked by the arrow) and then rapidly fall. disulfide-linked aggregation is believed to be responsible for this behavior at the higher protein concentrations.

Investigation of the effects of protein concentration on the kinetics of disulfide formation (Figure 14B) indicated that the reaction also increased linearly with protein concentration between 4.5 μM (0.1 mg/mL) and 36.4 μM (0.8 mg/mL) such that:

$$\text{rate} = \delta F/\delta t = k[\text{PGH}]\ (\text{s}^{-1}) \qquad (11)$$

where k is the rate constant governing the rate of disulfide formation. Above 0.8 mg/mL protein, there was a rapid decrease in the rate of disulfide formation with protein concentration (Figure 14B).

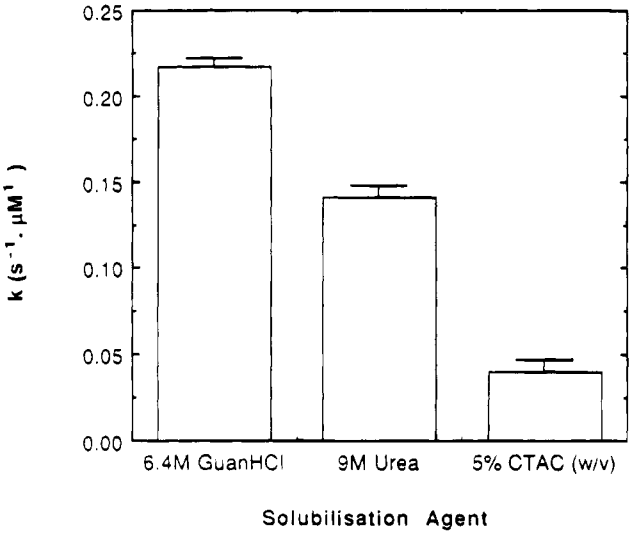

FIGURE 15: Effect of various solubilization agents on the rate of refolding of recombinant Met(1-190)PGH inclusion bodies in air-saturated 50 mM Tris-HCl, pH 8.8, containing 3.5 M urea, 10 mM β-ME, and 1 mM EDTA. The rate of refolding was monitored by the decrease in fluorescence at 350 nm (295 nm excitation) following a 1:21 dilution of a 20 mg/mL IB solution that was solubilized with either 9 M urea, 6.4 M GuHCl, or 5% (w/v) CTAC solutions containing 0.25 M β-ME and 100 mM Tris-HCl, pH 8.8, directly into the refolding buffer (3.5 M urea, 50 mM Tris-HCl, pH 8.8, containing 1 mM EDTA). Vertical bars represent SD of the mean of triplicate experiments.

(j) The Rate of Folding of Solubilized IB-Derived Recombinant PGH. Intrinsic fluorescence was used to follow the folding of recombinant PGH from solubilized preparations of IBs. The intrinsic fluorescence of reduced recombinant PGH solubilized from IBs was examined to assess the effect of different solubilizing agents on the rate of folding of urea, GuHCl, and CTAC solubilized proteins (Figure 15). The IBs were solubilized under the three different solubilization protocols, and the sample was then refolded by dilution into "model" refolding buffer conditions (3.5 M urea, 33 mM β-ME, and 1 mM EDTA in 0.1 M Tris-HCl, pH 9.1). The rate of folding was monitored by fluorescence (excitation at 295 nm and emission at 350 nm). The rates were divided by the concentration of protein for each sample to give a rate constant in units of $\text{s}^{-1}\ \mu\text{M}^{-1}$. The rate constant of 0.22 $\text{s}^{-1}\ \mu\text{M}^{-1}$ was obtained using GuHCl, 0.14 $\text{s}^{-1}\ \mu\text{M}^{-1}$ using 9 M urea to solubilize IBs, and 0.04 $\text{s}^{-1}\ \mu\text{M}^{-1}$ using CTAC solubilized IBs. GuHCl and urea solubilized IBs were observed to have a significantly greater rate of folding over 10 min compared to CTAC treated IBs as assessed by monitoring rate constants of folding using PGH intrinsic fluorescence.

(k) The Equilibrium Binding of ANS to the Urea Unfolded State of Recombinant PGH. The temperature denaturation of PGH was followed by ANS fluorescence as described in Cardamone and Puri (1992). The number of ANS binding sites per PGH molecule was increased from 3 to 8 sites as temperature was raised from 4 to 50 °C, indicating that the surface hydrophobicity of PGH is increased with increased temperature (Table 5). The association constant also increases with temperature, and therefore, these results obey the vanHoft relationship (Atkins, 1986) and were used to

Table 5: Effect of Temperature on the ANS Equilibrium Binding Capacity of Recombinant PGH

temp (°C)	n[a]	K_A[b] (M^{-1})
4	2.5	7.20×10^4
25	3.0	1.78×10^5
50	8.0	4.2×10^5

[a] Total number of ANS binding sites per protein molecule. [b] Association constant derived from least squares regression.

derive an reaction enthalpy of 28.5 ± 0.2 kJ/mol ($r^2 \geq 0.999$) from the slope of a vanHoft plot (data not shown) of ln K versus $1/T$ (Atkins, 1986) for the interaction of ANS with PGH between 4 and 50 °C (Table 5).

The urea denatured state of PGH was also analyzed by the ANS method (Figure 16). The binding of ANS to full-length (22K) recombinant PGH is associated with a significant increase in the quantum yield of fluorescence. The quantum yield of 75 μM ANS increased from 0.014 to 0.056 following the binding of 4.5 μM denatured recombinant PGH, with an associated blue shift in the fluorescence spectrum (Figure 16A, curve b, and Table 6). There were notable (3-fold) differences in the quantum yield of the ANS-unfolded PGH reaction (value of 0.160; Table 6) relative to that recorded for ANS-native PGH (value of 0.055). Given that the urea unfolded and reduced state was characterized by a significantly increased ANS fluorescence relative to the native protein, the number of binding sites and the respective ANS equilibrium binding constants (average K_A values) were determined for both states of the protein (Figure 16B and Table 6). The binding data show an increase in the number of ANS binding sites on PGH with unfolding. The unfolded and reduced PGH displayed 18 major ANS binding sites compared with only 2 ANS binding sites on native PGH. This number is greater than that observed following thermal denaturation at 50 °C of oxidized PGH with intact disulfide bonds. Here, PGH was observed to have 8 ANS binding sites (Table 6). There was also an approximate 4-fold difference in the affinity of binding of ANS to the unfolded form relative to that of ANS and native PGH. Given that the correlation between average K_A constants and the number of ANS binding sites with the "average" surface hydrophobicity was established in Cardamone and Puri (1992), the average surface hydrophobicity of the reduced and urea denatured state was clearly different to that of native recombinant PGH.

(l) An Examination of the Average Hydrophobicity of Intermediates in the "Model" Refolding of PGH. The time course profile following the folding during the "model" refolding pathway of PGH was assessed by ANS fluorescence. The folding was clearly not log-linear (Figure 17), being a similar dual state mechanism as observed during disulfide formation in PGH (compare Figure 9). Interestingly, it appears that the early intermediates of folding (occuring within the first 10 min of refolding) were characterized by consistently higher hydrophobicity as they consistently tended to have comparable ANS quantum yields to the unfolded protein. The initial plateau phase was followed by a gradual decrease in ANS fluorescence as the protein approached its native folded state (Figure 17). Investigation of the effects of protein concentration (from 4.54 to 18 μM protein) on ANS binding resulted in little apparent change, with a 6.5% reduction in the apparent

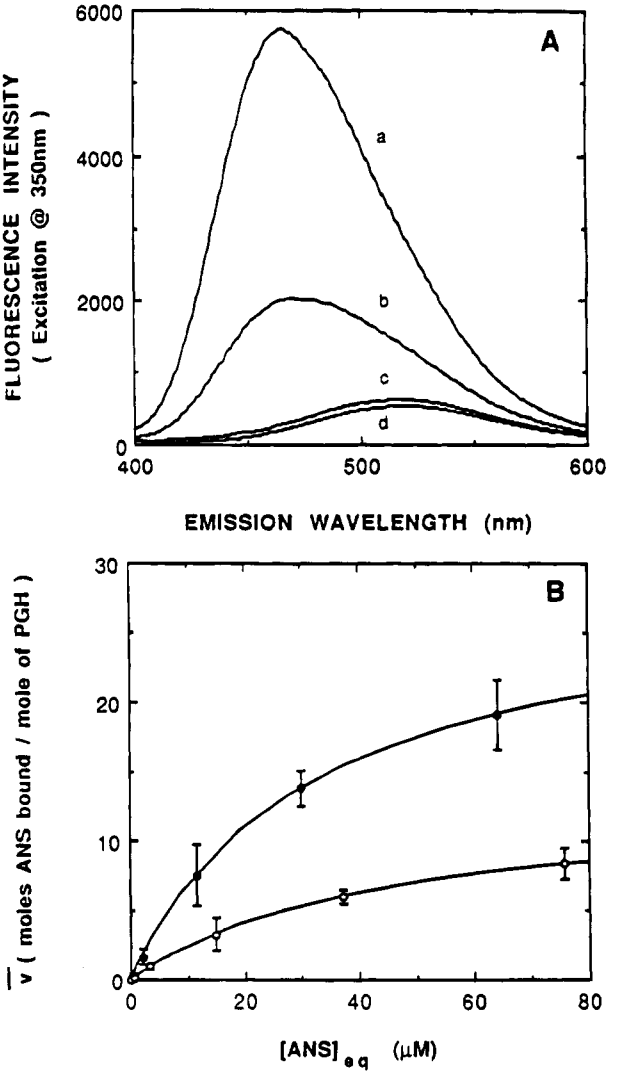

FIGURE 16: ANS-based assessment of the surface hydrophobicity of the urea denatured and reduced soluble PGH. (A) The fluorescence emission spectra of (a) 75 μM ANS added to 4.54 μM reduced and denatured PGH, (b) 75 μM ANS added to 4.54 μM recombinant PGH, (c) 75 μM ANS alone, in nitrogen purged 9 M urea, 0.25 M β-ME, 0.1 M Tris-HCl, and 0.1 M NaCl, pH 8.8, and (d) 75 μM ANS in air-saturated 0.1 M Tris-HCl and 0.1 M NaCl, pH 8.8. The ANS was excited at 350 nm. ANS alone had a quantum yield of 0.017 and 0.014 in reducing and nonreducing buffers, respectively, with an emission maximum at 520 nm. The emission maximum of ANS was blue shifted to 470 nm, and the quantum yield of fluorescence increased to 0.16 following the binding to reduced, denatured PGH. The photomultipler voltage was 400 V. (B) Binding data for ANS binding to urea denatured and unfolded PGH (●) and native PGH at 25 °C (○). Least squares fit analysis of the binding data gave a total of 2 ANS binding sites for the oxidized proteins; however, reduced, denatured PGH had 18 ANS binding sites with an average K_A of 8.0×10^5 M^{-1}, while for oxidized PGH, the average K_A was 2.0×10^5 M^{-1}. This represents approximately a 4-fold difference in the average affinity for ANS (Table 6). Vertical bars represent SE of triplicate measurements.

reaction rates during the first phase and an approximate 5.5% reduction in the apparent reaction rates during the second phase. There was no significant change in the order of the slow and fast reaction phases based on the shape of the curves. The time course of the second phase of folding, as assessed by ANS hydrophobicity, was typically much slower than the rate of folding assessed by disulfide bond formation. Using ANS fluorescence, the rate constant of the first phase of folding of PGH along the model refolding pathway was

Table 6: Extrinsic ANS Fluorescence Properties of Oxidized and Reduced, Denatured Met(1-190)PGH

ANS fluorescence	PGH in oxidizing buffer[e]	oxidizing buffer alone[e]	PGH in reducing buffer[f]	reducing buffer alone[f]
quantum yield[a]	0.056	0.014	0.160	0.017
emission maximum (nm)[b]	480	520	470	520
K_A[c] (M^{-1})	$(2.0 \pm 0.1) \times 10^5$		$(8.0 \pm 0.1) \times 10^5$	
n[d]	2		18	

[a] Quantum yield of of the fluorescence emission spectra of 75 μM ANS and 4.54 μM PGH (Figure 16A), relative to the standard β-carboline (Ghiggino *et al.*, 1985). [b] Emission maximum of the fluorescence emission spectra of 75 μM ANS and 4.54 μM PGH (Figure 16A). [c] Average association constant derived from least squares fit of the ANS binding plots (Figure 16B). [d] Total number of ANS binding sites per protein molecule based on ANS binding analyses (Figure 16B). [e] The oxidizing buffer was air-saturated 0.1 M NaCl and 0.1 M TrisHCl, pH 8.8. [f] The reducing buffer was 9 M urea, 0.25 M β-ME, 0.1 M NaCl, and 0.1 M TrisHCl, pH 8.8.

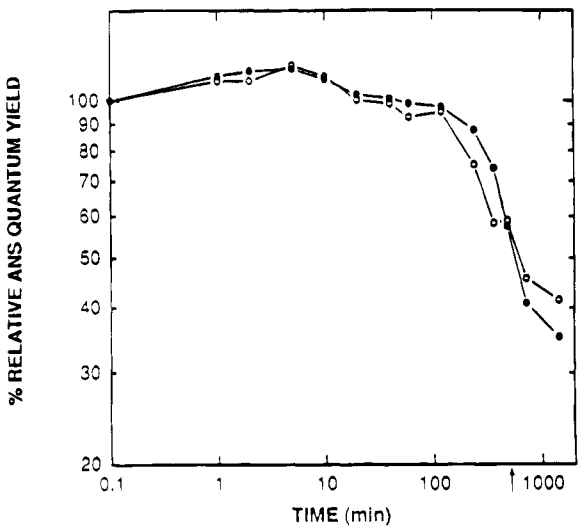

FIGURE 17: Time-dependent changes in ANS fluorescence during the "model" refolding pathway of PGH. Changes in ANS fluorescence were determined by using urea denatured and reduced PGH at 4.5 μM (○) and at 18 μM (●). At each time point, a 1 mL aliquot was withdrawn from refolding PGH solutions containing 3.5 M urea, 10 mM β-ME, and 50 mM Tris-HCl, pH 8.8, and mixed with a final concentration of 111 μM ANS. This binding reaction was continued for 30 min before measurement of the ANS fluorescence of the sample and a protein-free control incubated for the same period of time. The arrow indicates the time at which reoxidation was complete, which was assessed by nonreducing SDS−PAGE analysis (Figure 9).

measured to be 0.08 ± 0.01 min^{-1}, and the rate constant of the second phase of folding as 0.38 ± 0.01 min^{-1}. The very slow kinetics of the first phase of folding ($\leq$10 min) suggests that there is a rate limiting step which must occur to achieve the native-like state.

DISCUSSION

The formation of IBs is a phenomenon commonly encountered in the production of recombinant proteins (Marston, 1985). While there are many advantages to expressing recombinant proteins as IBs, especially in large-scale downstream processing (Marston, 1985, 1986; Rausch, 1987; Hartley & Kane, 1988; Kane & Hartley, 1988; Marston & Hartley, 1990; De Bernardez-Clark & Georgiou, 1991; Bowden *et al.*, 1991), significant problems exist in solubilizing IBs and refolding the protein correctly to obtain bioactive material. The successful use of IBs has been limited by low yields obtained at the refolding step, as the factors that determine *in vitro* protein folding are not completely known, and there are no general rules or procedures established for the recovery of IB proteins in high yield. Techniques were developed to enable the solubilization from IBs and the refolding of recombinant PGH so as to recover high levels of biologically active protein.

Recombinant proteins solubilized from IBs using chaotrophic agents in a "strongly" denaturing environment (7.5 M urea or 6 M GuHCl) are commonly renatured *in vitro* in "weakly" denaturing environments using 0.5−2 M GuHCl or 1−4 M urea (Sharma, 1986; Bentle *et al.*, 1987; Jaenicke & Rudolph, 1990; Marston & Hartley, 1990; Schein, 1990). In this study appropriate solution conditons for the reoxidation and refolding of PGH from an unfolded state were studied using a purified source of the hormone in order to find "model" solution conditions which would refold the secondary and tertiary structure of the unfolded protein to the native state. The results showed that the yield and rate of refolding of PGH depended upon the solvent conditions in which the refolding occurred. The pH dependence of refolding reflects the decreased solubility of the reduced protein and that the alkaline-induced ionization of the side chains on the protein renders it more soluble. Also, exposing the protein to acidic conditions ensures that the thiol groups are protonated and prevents the formation of the thiolate radical which is the reactive species for the formation of disulfides (Weissman & Kim, 1991). Therefore, folding is "quenched" by lowering the pH of the solution. The "model" pathway established for PGH had a relatively high recovery of 85% of monomeric, correctly refolded PGH, as assessed by RP-HPLC, in a refolding environment containing critical concentrations of urea (3.5 M) and reducing agent (10 mM β-ME), and a pH of 9.1 at a protein concentration of 0.5 mg/mL. The observed increase in the proportion of dimerized PGH, at the expense of aggregated forms of PGH, with concentration of β-ME/HED reagents suggests that β-ME/HED slows down (or may even prevent) the formation of intermediates which are committed toward the aggregation pathway. Similar observations by Creighton (1978), working with BPTI, have identified appropriate solution conditions with mixtures of reducing and oxidizing agents to optimize kinetic pathways for sulfhydryl shuffling in which the unfolded molecules eventually attain disulfides which were identical to the native state.

The "model" pathway did not result in the same yield of renaturation (85%) of recombinant PGH from CTAC solubilized IBs. The renaturation conditions were clearly not as successful as with the "model" refolding of a previously purified, soluble source of recombinant PGH. It has been suggested that there may be morphopoietic factors intrinsic to the IB material which prevent proteins derived from IBs following the same refolding pathway that the previously purified and "artificially" unfolded protein follows in a

"model" pathway (Darby & Creighton, 1991). In fact, Darby and Creighton (1991) report that optimal yields of refolding were obtained when the IB-derived proteins were purified away from the intrinsic factors associated with IBs prior to refolding during downstream processing. It is also possible that the method of solubilization determines the subsequent extent of renaturation of the protein to its native state (Tandon & Horowitz, 1986).

The equilibrium transition between the native and the urea denatured state of PGH was also studied here. The transition was cooperative, as indicated by the sigmoidal rise in fluorescence after incubation of the protein in solutions containing urea (≥5 M), and the transition followed a two-state behavior, consistent with intermediate folding states in urea being unstable and not highly populated (Creighton, 1985; Pace, 1990). The urea denaturation of BGH has been previously studied by Edelhoch and Burger (1966), who observed that there was a strong dependence between the intrinsic fluorescence of BGH and urea concentration above 6 M urea. However, below 6 M urea the dependence was not so notable. Edelhoch and Burger (1966) also observed that complete denaturation of the secondary structure of BGH did not occur even at very high concentrations of urea based on optical rotatory dispersion (ORD). These results are likely to be directly applicable to PGH as the sequence homology between PGH and BGH is extremely high (>85%) (Abdel-Mequid *et al.*, 1987). In contrast, GuHCl denaturation of PGH destroyed all secondary structure (Bastiras & Wallace, 1992). The results presented in this study showed that urea increased the quantum yield of PGH intrinsic fluorescence (Figure 7), and the effect was significant above 5 M urea (Figure 8). The free energy of GuHCl denaturation was measured by Bastiras and Wallace (1992) to be approximately 7.3 kcal/mol. The lower value of 3.8 kcal/mol obtained here for the free energy of urea denaturation of PGH shows that urea is not as potent a denaturant of PGH as GuHCl. The disulfide bonds contributed to the free energy of denaturation of PGH in urea as a reduced and carboxymethylated preparation of PGH lacks sufficient structure as measured by fluorescence spectroscopy. This indicates that the native-like conformation of PGH is dependent on the integrity of the large loop domain defined by the disulfide bond between cysteine residues 53 and 164 because the complete removal of the disulfide bonds in the reduced and carboxymethylated PGH (RCAM) sample is associated with a severe loss in native-like structure of the protein. The conformational stability of P-band was also assessed, and there appeared to be no significant difference to full-length PGH in the free energy of denaturation in urea. P-band has been shown in Puri *et al.* (1993) to lack the C-terminal loop domain, and the ΔG_{H_2O} of P-band in urea has been shown here to be similar to that of the full-length PGH while the reduced and carboxymethylated protein (RCAM-PGH) has no significant conformation as measured by intrinsic tryptophan fluorescence. These results suggest that the first disulfide, forming the large loop domain of PGH, provides significantly more energy to the conformational stability of PGH than the second disulfide, which forms the carboxy-terminal small loop domain. This has been also been observed for BGH by Holzman *et al.* (1986). However, the retention of a native-like conformation for PGH is dependent on disulfide bonds and, in particular, on the large loop domain because the far-UV CD spectrum of the RCAM-PGH is devoid of secondary structure. However, the contribution of the small loop domain is not as critical, this is because the far-UV spectrum of P-band is very similar to the far-UV CD spectrum of the native PGH (Puri *et al.*, 1993). This observation contrasts with those of Holzman *et al.* (1986), who observed that native-like conformation of BGH is only partially dependent on disulfide bonds, because the CD spectra at 222 nm of reduced BGH demonstrated considerable native-like structure. The observed lack in secondary structure in reduced and carboxymethylated PGH may reflect the modest value of 3.8 kcal/mol for the apparent free energy of denaturation in urea, compared to the reported value of 6.8 kcal/mol for BGH (Holzman *et al.*, 1986). The differences may also be explained by inherent differences in the contributing factors determining the structure and function of PGH. For instance, the conformational energy contributed by disulfide bonds in PGH is much less than the energy contributed by disulfides in BGH (Holzman *et al.*, 1986) and in thermostable enzymes such as bacteriophage T4 and lysozyme (Perry & Wetzel, 1984; Alber *et al.*, 1988). Other factors such as hydrophoic interactions (Lim *et al.*, 1989) and proline isomerization (Matthews *et al.*, 1987) may be more important in maintaining the conformational stability of PGH. Further work is required to verify the claim that the retention of the native-like conformation for PGH is dependent on disulfide bonds and, in particular, on the large loop domain. For instance, the use of expression mutants of recombinant PGH that lack disulfides would probably be better suited for answering the question of identifying the importance of disulfides to the conformational stability of PGH.

The change in fluorescence following the urea denaturation of PGH was used to follow the kinetics of folding. Kauffman *et al.* (1989) observed that both static and dynamic quenching of the tryptophan residue fluorescence occurred within the native states of PGH and BGH as both the quantum yields and lifetimes of intrinsic fluorescence increase on changing pH from 8 to 2. Kauffman *et al.* (1989) speculated that the chemical groups responsible for static and dynamic fluorescence quenching in the native states of PGH and BGH were in α-helical regions, near the center of the molecule, since the single Trp in PGH is centrally located within an α-helix of the hydrophobic core of PGH. The authors identified 10 residues as possible fluorescence quenchers: Gln(19), Lys-(30), Arg(108), Glu(111), Lys(112), Arg(125), Glu(126), Ser-(163), Tyr(175), and Lys(180) (Kauffman *et al.*, 1989). On the basis of these studies presented, it appears that urea denaturation decreases the quenching effect of these residues, and most probably shifts the Trp residue to a local environment of increased hydrophobicity, thus leading to the observed increase in PGH intrinsic fluorescence.

The time course of the "model" refolding pathway was investigated in detail with respect to the formation of a native-like state, as measured by intrinsic fluorescence spectroscopy and ANS spectroscopy and with respect to the formation of disulfides by SDS−PAGE and DTNB titrations. The results indicated that the formation of native structure, as assessed by fluorescence intensity, occurred much earlier than the formation of disulfide bonds. The rate of folding was observed to increase with protein concentration and with pH, whereas high concentrations of β-ME (≥50 mM) and urea (≥4 M) and 1 mM EDTA in the refolding buffer decreased the rate of folding (Figures 12 and 13). The

observation that EDTA slows down folding of PGH, as measured by intrinsic tryptophan fluorescence, suggests that disulfide formation is structurally important to the folding of the region in which the tryptophan residue is located (Figure 12). EDTA is believed to remove heavy metal cations which are reported in the literature to catalyze reoxidation and disulfide bond formation in proteins (Jaenicke & Rudolph, 1990).

The rates of disulfide formation in the model refolding pathway were monitored using the conventional Ellman assay, by monitoring changes in free sulfhydryl content using DTNB (Ellman, 1959). The rate of disulfide formation was much slower than the rate of folding. This observation is consistent with the "framework model" of folding, because it appears that the local environment surrounding the single tryptophan within the second α-helix of PGH obtains its native-like state much sooner than the formation of disulfides. The formation of disulfide bonds in PGH is a discrete process which occurs after a "framework" of protein structure is established. An example of another protein which appears to have a "framework" structure is α-lactalbumin (Kuwajima, 1977; Kuwajima *et al.*, 1987). A folding intermediate of this protein has been identified which has native-like secondary structure, yet lacks native-like tertiary structure while the protein disulfides are in the reduced state (Kuwajima *et al.*, 1987). The slower rate of disulfide formation in PGH suggests that a "framework" intermediate is a prerequisite for correct disulfide formation in PGH (Baldwin, 1989). Thus, internal or framework structure is required prior to the correct formation of disulfide bonds between Cys(53) and Cys(164) and between Cys(189) and Cys(181). The coincident slow reoxidation of PGH as assessed by DTNB titration suggests that the formation of the first (and possibly the second) is necessary for the formation of the final native state, as assessed by ANS hydrophobicity.

The results of ANS spectroscopy identified the presence of refolding intemediates with comparable hydrophobicity to the urea denatured state of the protein, within the first 10 min of folding. This suggests that early kinetic intermediates lack native-like tertiary structure. These results support the notion that the formation of a "nucleus" configuration is required for the formation of disulfide bonds, and these in turn, for the subsequent formation of the native state. The "framework model" for the folding of PGH is also consistent with an equilibrium denaturation study of BGH (Brems *et al.*, 1985) and, more recently, PGH (Bastiras & Wallace, 1992). These workers have also proposed a "framework" model for the folding of BGH and PGH.

An important aspect that must be considered is that self-association of growth hormone intermediates of folding have been very well documented (Brems, 1985; Bastiras & Wallace, 1992). Therefore, it may be possible that self-association of these intermediates could influence the observed rates of folding by the two methods (disulfide oxidation and fluorescence quenching) and that this effect could be more pronounced in one method more than the other. The observation in Figure 13B that the addition of the reducing agent, β-ME, in the intrinsic fluorescence studies, decreased the rate of folding even at a very low protein concentration of 0.05 mg/mL (2 μM PGH) where it can be hoped that self-association would be minimal would allow us to speculate that the rates, at least as measured by intrinsic fluorescence, are real and not influenced by self-association processes.

The rate of refolding of a protein is thought to occur according to first-order kinetics which is independent of protein concentration (Jaenicke & Rudolph, 1990). However, protein aggregation or self-association is claimed in the literature to follow predominantly second-order reaction kinetics (Cleland & Wang, 1990). Second-order reactions show a quadratic concentration dependence with respect to protein concentration. Sigmoidicity points to a sequential uni-bimolecular mechanism; however, coupled first-order reactions may also display sigmoidal profiles (Jaenicke & Rudolph, 1990). This means that it is essential that experiments be conducted at different concentrations because of what they can reveal about the nature of the refolding pathway; for instance, they can demonstrate the contribution of intermolecular disulfide pairing on the rate of disulfide oxidation.

We have attempted to determine the rates of refolding using intrinsic tryptophan and ANS fluorescence quenching and the rates of oxidation using the Ellman method. These were repeated at a number of different protein concentrations. The results in Figures 14 indicate that, first of all, the renaturation and reoxidation of recombinant PGH are coupled, which is consistent with the framework model of refolding (Baldwin, 1989). More importantly, the renaturaton of PGH as measured by intrinsic trptophan fluorescence occurs in the same time frame as the first phase of reoxidation of PGH as measured by the Ellman assay. Second, this first slow phase of reoxidation tends to be less dependent on protein concentration than the latter faster phase, indicative of sigmoidicity and, consequently, a sequential uni-bimolecular mechanism for its refolding and reoxidation (Figure 14). The ANS kinetic studies in Figure 17, where refolding was monitored at two different protein concentrations (at 4.5 and 18 μM), also showed that increasing protein concentration did not really affect the first and second phases of refolding at all. Furthermore, the observation in Figure 13B that the addition of the reducing agent, β-ME, in the intrinsic fluorescence studies also decreased the rate of folding is further evidence that the reoxidaton and renaturation as measured by intrinsic fluorescence are coupled. However, Figure 14 also demonstrates that, at high protein concentrations, the protein concentration dependence vanishes for the uni-bimolecular mechanism. This may be due to the fact that the first-order refolding reaction is rate limited by bimolecular or even oligomerization interactions. The results suggest that self-association is inherently coupled to the renaturation of PGH. This is also clearly seen in the refolding of the protein from the urea denatured state and also from the IB experiments. During renaturation, we attempted to decelerate the self-association as much as possible. For recombinant PGH, we have found that the addition of urea as a denaturant to a final concentration of 3.5 M and the addition of a redox pair β-ME/HED to a final concentration of 10 mM at a ratio of 10:1 were found to be optimal for the refolding of our model protein (Figure 13). We have also varied the pH and found the optimal pH to be pH 9.0. The reason why these conditions are optimal for the renaturation of PGH is probably because they appreciably quench the self-association processes that may lead to soluble aggregate or, at worst, insoluble aggregate formation, because the fast second phase of renaturation that may be intrinsically

coupled to the self-assocation of the protein is inhibited by the solution additives and pH conditions. The addition of EDTA and the reducing agent also assists to prevent side reactions that may lead to the eventual irreversible covalent inactivation of the protein that is currently claimed in the literature to be the mechanism behind the irreversible aggregation of proteins; however, the empirical optimum pH of 9 actually exacerbates protein inactivation (Volkin & Klibanov, 1989). This is particularly important, considering that long periods (order of hours for the reoxidation experiments) were required in the determination of the true final value for the rate of reoxidation. Under the optimized experimental refolding conditons evaluated for recombinant PGH, a balance between self-asssociation and renaturation was nevertheless obtained.

The framework model then clearly explains the experimental renaturation properties of recombinant PGH from the urea denatured state and also from *E. coli* inclusion bodies in the sense that it accounts for the observation that the refolding of recombinant PGH follows a uni-bimolecular mechanism which is coupled with its reoxidation. The evidence lies in the following obervations. First, the renaturaton of PGH as measured by intrinsic fluorescence occurs in the same time frame as the first phase of the reoxidation of PGH as measured in the thiol assay, and second, the rate of the first slow phase is less dependent of protein concentration than the second faster one. Also, self-association and renaturation appear to be coupled processes, which agrees with the recent observations of Bastiras *et al.* (1992), who characterized thermodynamically stable associated intermediates of refolding for recombinant porcine growth hormone.

The effect of using different solubilizing agents on the rate of folding of IB-derived PGH was also tested. GuHCl solubilized samples were shown to have the greatest rate constants of folding compared to urea and CTAC solubilized samples in the order: 6.4 M GuHCl > 9 M urea > 5% (w/v) CTAC. The different modes of solubilization may explain the observed differences in the observed rate constants. For instance, the changes may reflect degrees of denaturation. Thus, protein in the CTAC solubilized state is not as unfolded as in the urea and GuHCl solubilized state. Previously, CD analysis has shown that correctly refolded recombinant PGH comprises approximately 40−45% α-helix (Bastiras & Wallace, 1992). On the other hand, reduced, CTAC solubilized, that is, "partially unfolded" recombinant PGH still comprised approximately 10−15% α-helix (Puri & Cardamone, 1992; Cardamone *et al.*, 1994), whereas urea denatured and carboxymethylated, that is "unfolded" Met(1-190)PGH was devoid of secondary structure. It would appear that the notable differences in starting secondary structure between CTAC, urea, and GuHCl solubilized recombinant PGH affect the rate of *in vitro* refolding of recombinant PGH. The method of solubilization of IBs and the resultant difference in the starting secondary structure of recombinant PGH, particularly α-helical content, can be an *in vitro* factor that explains the difference in refolding efficiency between urea and CTAC solubilized recombinant PGH (Table 4). Again, these results are consistent with the "framework" model of protein folding (Baldwin, 1989), which postulates that the presence of localized secondary structures plays a central role in determining the rate of the folding pathway. These studies reinforce our earlier conclusions that the conditions used for the solubilization of insoluble recombinant proteins, particularly, the concentration of urea or GuHCl used, should be very carefully selected to give not only efficient solubilization but also the retention of some protein "framework" structure, prior to commencing *in vitro* refolding (Puri & Cardamone, 1992).

In summary, this study provides more information than simply an optimized recovery process for porcine growth hormone because we have detailed the important thermodynamic and kinetic parameters affecting the refolding of a recombinant protein under a number of solution conditions. More importantly, some insight on the relative importance of each of the disulfide bonds on its conformational stability has been gained. Recombinant porcine growth hormone characteristic of other proteins has perhaps some of the most arduous solution properties, and an understanding of what affects its *in vitro* refolding will be important for the utilization of many other types of recombinant growth factor proteins.

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