Reconstitution of the Holoenzyme Form of *Escherichia coli* Porphobilinogen Deaminase from Apoenzyme with Porphobilinogen and Preuroporphyrinogen: A Study Using Circular Dichroism Spectroscopy†

Sarah J. Awan,‡ Giuliano Siligardi,§ Peter M. Shoolingin-Jordan,|| and Martin J. Warren*,‡

Department of Molecular Genetics, Institute of Ophthalmology, University College London, Bath Street, London EC1V 9EL, England, Engineering and Physical Sciences Research Council National Chiroptical Spectroscopy and University of London Intercollegiate Research Service Optical Spectroscopy Services, Department of Pharmacy, King's College London, Manresa Road, London SW3 6LX, England, and Department of Biochemistry, School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, England

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ABSTRACT: Porphobilinogen deaminase (PBG-D), an early enzyme of the tetrapyrrole biosynthetic pathway, catalyzes the formation of a tetrapyrrole chain, preuroporphyrinogen, from four molecules of porphobilinogen (PBG). The PBG-D apoenzyme is responsible for the autocatalytic synthesis and covalent attachment of a dipyrromethane cofactor at its active site. In this paper an efficient method for the purification of *Escherichia coli* PBG-D apoenzyme using an affinity chromatography resin is reported. Circular dichroism (CD) spectra of apoenzyme and holoenzyme were recorded and significant differences in both the backbone and aromatic region of the spectra were observed. The differences in the spectra allowed the reconstitution of holoenzyme from purified apoenzyme with PBG and preuroporphyrinogen in solution to be monitored separately by CD. Apoenzyme incubated with preuroporhyrinogen gave a CD spectrum that was much more like the CD spectrum of holoenzyme than apoenzyme incubated with PBG. The results showed clearly that the cofactor was generated much more rapidly from preuroporphyrinogen than from PBG. Changes in the CD spectrum associated with the aromatic side-chain region, in particular the contribution assigned to phenylalanine-62, were found to correlate well with the activity of the reconstituted enzyme. Phenylalanine-62 is located in close proximity to the cofactor and acts as a sensitive probe to active-site changes. The stability of the holoenzyme and apoenzyme were compared with respect to both heat and susceptibility to proteolysis. The results were consistent with a model for the apoenzyme in which, in the absence of the cofactor, the three domains of the protein are held less rigidly together, thereby making the protein more susceptible to heat denaturation and proteolysis. The CD spectrum of the holoenzyme was found to be similar at both pH 5.1 and 7.4, suggesting that the crystal structure, determined at pH 5.1, is likely to be similar at physiological pH values.

A key early step in the biosynthesis of the tetrapyrrole-derived prosthetic groups such as heme, chlorophyll, and cobalamin (vitamin B_{12}) is the construction of the tetrapyrrole molecular framework from which all the prosthetic groups are derived (Warren & Scott, 1990; Jordan, 1991). This macrocyclic infrastructure is synthesized from four molecules of the monopyrrole porphobilinogen (PBG)[1] by the successive action of two enzymes, porphobilinogen deaminase (PBG-D) and uroporphyrinogen III synthase (Jordan 1994; Leeper, 1994). The former generates a linear tetrapyrrole, preuroporphyrinogen, by the deamination and consecutive polymerization of four molecules of PBG (Scheme 1) (Battersby *et al.,* 1979; Burton *et al.,* 1979). The latter enzyme is able to transform preuroporphyrinogen into uroporphyrinogen III in a process that requires the inversion of the terminal d ring by a mechanism that is still not fully understood (Jordan *et al.,* 1979; Battersby *et al.,* 1979; Jordan & Berry, 1980).

PBG-Ds have been studied from a range of sources and in all cases the protein has been found to be monomeric with a molecular mass of around 35 000 Da (Jordan, 1991). The enzyme performs a complex series of reactions involving deamination, carbon-carbon bond formation, and hydration (Scheme 1). The enzyme contains a unique dipyrromethane cofactor (Jordan & Warren, 1987; Hart *et al.,* 1987) which is linked covalently to the protein *via* a thioether bond of a cysteine residue, cysteine-242 in *Escherichia coli* PBG-D (Hart *et al.,* 1988; Miller *et al.,* 1988; Jordan *et al.,* 1988b). The cofactor is itself derived from two rings of the substrate PBG and is synthesized and inserted by the apo (cofactorless) enzyme (Hart *et al.,* 1988; Warren & Jordan, 1988; Scott *et al.,* 1989). Recent progress on the understanding of the action of the enzyme has come from the elucidation of the three-dimensional structure of the holoenzyme (Jordan *et al.,* 1992; Louie *et al.,* 1992, 1996; Hädener *et al.,* 1992). This has shown that the polypeptide chain is folded into three domains which enclose a large active-site cleft. The cofactor, which is attached to domain 3, inherently defines the active site of the enzyme and has aided the identification of important binding and catalytic groups. The active site is

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* To whom correspondence should be addressed.
‡ University College London.
§ King's College London.
|| University of Southampton.

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[1] Abbreviations: CD, circular dichroism; PBG, porphobilinogen; PBG-D, porphobilinogen deaminase; SDS−PAGE, denaturing sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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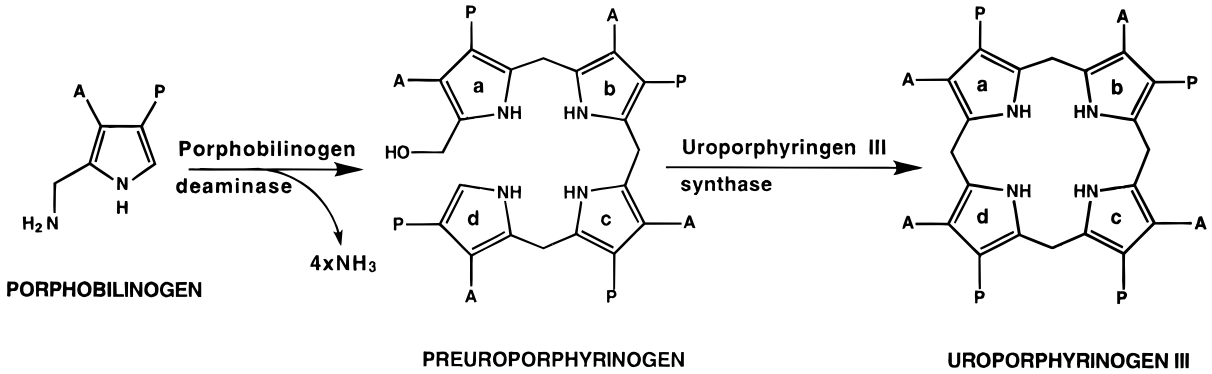
Scheme 1: Reaction Catalyzed by PBG-D[a]



[a] PBG-D catalyzes the polymerization and deamination of four molecules of PBG to give preuroporphyrinogen. The reaction is catalyzed in a discrete manner with the linear tetrapyrrole pieced together sequentially in the order of ring a followed by b, c, and d.

Table 1: Strain and Plasmid Description

strain/plasmid	genotype/properties	source/reference
TB1	JM83 *hsdR* (r_k^- m_k^+)	Baker *et al.* (1984)
RP523	*thr-1 leuB6 thi-1 lacY1 tonA21 supE44* λ^- F^- *hemB* heme permeable	Li *et al.* (1988)
pBM3	pUC18 with a *Bam*HI/*Sal*I fragment carrying *hemC*	Mbgeje (1989)
BM3	TB1 transformed with pBM3; overexpresses holo-PBG-D	Mbgeje (1989)
ApoBM3	RP523 transformed with pBM3; overexpresses apo-PBG-D	Scott *et al.* (1989)

lined with positively charged arginine side chains to counteract the negative charges of the cofactor and the oligopyrrole as it is synthesized. There would appear to be only one catalytically important amino acid, aspartate-84, in close proximity to the cofactor (Louie *et al.*, 1992; Woodcock & Jordan, 1994). Mutagenesis of this group to glutamate reduces the k_{cat} by 2 orders of magnitude but does not affect K_m. Mutagenesis of aspartate-84 to either alanine or asparagine totally abolishes enzymatic activity, although the mutants are still able to assemble the dipyrromethane cofactor (Woodcock & Jordan, 1994).

The role of aspartate-84 has not been fully elucidated, although it is likely that the group is involved as a general acid/base. In close proximity to aspartate-84 is phenylalanine-62, which is also stacked against the second ring (C2) of the cofactor. The role of the phenyl ring may be to maintain the aspartate carboxylic acid side chain in a protonated form and thus allow it to act initially as a general acid in the catalytic process (Louie *et al.*, 1992).

The conversion of apodeaminase into holodeaminase has been the focus of only limited investigation. Cofactorless enzyme (apoenzyme) has been generated by the expression of the *E. coli hemC* gene in *E. coli hemB* cells (Scott *et al.*, 1989). As these cells are unable to make PBG, the expressed PBG-D is denied the substrate for cofactor synthesis and thus apoenzyme accumulates. Alternatively, apodeaminase can be made from holodeaminase by acid hydrolysis of the thioether linkage between the cofactor and the holoenzyme (Hart *et al.*, 1988). After cleavage, apoenzyme can be resolubilized in urea and refolded by slow removal of the denaturant.

In this paper the technique of circular dichroism (CD) has been used to study structural aspects of the apo and holo forms of PBG-D. In the absence of a structure for the apoenzyme of PBG-D, the work presented was performed to investigate structural changes associated with the transformation of apoenzyme to holoenzyme. Furthermore, studies on the holoenzyme were used to confirm that the structure of the holoenzyme at pH 5 (Louie *et al.*, 1992), the pH at which the enzyme X-ray structure was solved, is likely to be very similar to that of enzyme at its pH optimum of pH 8.

MATERIALS AND METHODS

PD-10 columns, Sephadex G-75, and all fplc equipment and columns were purchased from Pharmacia. Mimetic Orange A6XL column resin was from Affinity Chromatography Limited. Tryptone and yeast extract were obtained from Difco Laboratories. All other chemicals were supplied by Sigma Chemical Co. unless stated otherwise.

Bacterial Strains and Vectors. The *E. coli* strains and vectors used in this investigation are described in Table 1. Strains were routinely grown in LB medium supplemented with the appropriate antibiotics (Sambrook *et al.*, 1989)

Preparation of Hemin Solution. Hemin was prepared as described by Sambrook *et al.* (1989). Briefly, hemin (30 mg) was dissolved in 0.2 N KOH (400 μL), H_2O (600 μL), and 1 M Tris/HCl, pH 7.8, (100 μL), to which was added ethylene glycol (4.8 mL). The solution was mixed by vortexing and centrifuged at 5000*g* for 5 min at 4 °C. Insoluble material was discarded and the hemin solution was used at 1 μL/mL of culture.

Synthesis of PBG. PBG was generated enzymatically by incubating purified 5-aminolevulinic acid dehydratase [500 units of purified *E. coli* enzyme obtained as described by Spencer and Jordan (1993)] with 5-aminolevulinic acid hydrochloride (1 g) in 10 mM phosphate buffer, pH 6.8 (2 L), containing 10 mM dithiothreitol at 37 °C for 17 h, in the dark and under an atmosphere of nitrogen. The pH of the solution was adjusted to 7.5, and the solution was applied to a column of Dowex 1-X8 (200−400 mesh) acetate. The column was washed with 1 L of water, after which the PBG was eluted with 0.2 M acetic acid and lyophilized. The PBG was further purified by recrystallization from dilute ammonia by adjusting the pH to 5 with 0.1 M acetic acid. The crystals were filtered, washed with ice-cold methanol, and freeze-dried. The crystals were stored at −20 °C in the presence of a desiccant.

Quantitation of PBG. Modified Ehrlich's reagent was freshly prepared by dissolving 1 g of *p*-dimethylaminobenzaldehyde in 42 mL of glacial acetic acid followed by the addition of 8 mL of perchloric acid (70%) as described previously (Mauzerall & Granick, 1956). A PBG solution was mixed with an equal volume of Ehrlich's reagent and the color was allowed to develop for 15 min. The absorption was measured at 555 nm and the concentration of PBG was calculated using the molar extinction coefficient $\epsilon_M = 6.02 \times 10^4$ M^{-1} cm^{-1} (Mauzerall & Granick, 1956).

Determination of PBG-D Activity. PBG-D activity was assayed by measuring the formation of uroporphyrin I. A reaction mixture consisting of 100 mM phosphate buffer, pH 8.0, containing 13 mM β-mercaptoethanol and enzyme (1−10 μg) was mixed in a total volume of 450 μL and warmed to 37 °C. The reaction was started by the addition of 100 nmol of PBG (50 μL); 80 μL aliquots of the reaction were removed at 5 min intervals and the reaction was terminated by addition of 5 N HCl (20 μL) to the aliquot. This was then diluted 10-fold with 1 N HCl and benzoquinone [10 μL, 0.1% (w/v) in methanol] was added to oxidize the uroporphyrinogen to uroporphyrin. The mixture was left on ice in the dark for 20 min. The absorption was measured at 406 nm and the activity of the enzyme was determined using the molar extinction coefficient $\epsilon_M = 5.48 \times 10^5$ M^{-1} cm^{-1}.

Protein Quantitation. Protein concentrations were determined by the method of Bradford (1976), using a commercial kit purchased from Bio-Rad. A standard curve was prepared with bovine serum albumin of known concentrations.

Purification of E. coli PBG-D. The *E. coli* strains (see Table 1 for all strains and plasmids) overexpressing the PBG-D holoenzyme (BM3) or apoenzyme (ApoBM3) were grown in LB medium containing ampicillin (100 μg/mL) overnight at 37 °C with shaking (200 rpm) in 2 L baffled flasks containing 1 L of medium. In the case of ApoBM3, hemin (1 μL/mL) was also added. The cells from 2 L of culture were harvested by centrifugation at 7000*g* for 20 min, to give about 5 g wet weight of cells, and were then resuspended in 20 mL of 0.1 M phosphate buffer, pH 8.0, containing 13 mM β-mercaptoethanol. The cells were broken by sonication, using an MSE Soniprep ultrasonic disintegrator, at an amplitude of 8−10 μm in five 1 min bursts with 2 min intervening periods for cooling.

Purification of Holoenzyme. The sonicated BM3 cells were heated to 60 °C and this temperature was maintained for 10 min with gentle stirring. The solution was then cooled on ice and the resulting extract was centrifuged (8000*g*) at 4 °C for 15 min. The supernatant was collected and ammonium sulfate was added slowly to the stirring supernatant at room temperature to give a 55% saturated solution (351 g/1 L of solution). The mixture was centrifuged (8000*g*), the supernatant was discarded, and the precipitate, containing the PBG-D, was collected.

The ammonium sulfate pellet was resuspended in 50 mM Tris/HCl buffer, pH 6.0, containing 13 mM β-mercaptoethanol and applied to the top of a column (150 cm × 2.5 cm) of Sephadex G-75. The column had been equilibrated in 50 mM Tris/HCl buffer, pH 6.0, containing 13 mM β-mercaptoethanol and 0.02% sodium azide and developed at a flow rate of 600 μL/min. The fractions containing the enzyme were pooled and dithiothreitol was added to a final concentration of 2 mM. The resulting solution was then concentrated in an Amicon ultrafiltration cell fitted with a PM-10 membrane. Holo-PBG-D was assessed for its purity by the measurement of its specific activity and by SDS−PAGE. If required, the protein was further purified by anion-exchange chromatography by fplc as described previously (Jordan *et al.*, 1988a).

Purification of Apoenzyme. The crude extract of the *E. coli* bacteria (apoBM3) containing the PBG-D apoenzyme, obtained after sonication and centrifugation, was applied directly to the top of a column (8 cm × 2.5 cm) of Mimetic Orange 1 A6XL. The column had been previously equilibrated in 200 mL of 20 mM Tris/HCl, pH 8.0, containing 7 mM β-mercaptoethanol.

The apoenzyme was eluted by developing the column sequentially with 40 mL of Tris/HCl buffer, pH 8.0, with buffer containing 100 mM NaCl, with buffer containing 250 mM NaCl, with buffer containing 500 mM NaCl, and with buffer containing 1 M NaCl (40 mL). Apo-PBG-D was detected by SDS−PAGE and its presence was confirmed using a reconstitution assay. The fractions containing the purified apoenzyme were subsequently pooled. The purified protein was desalted by gel filtration through a PD-10 column that had been previously equilibrated with 1 mM Tris/HCl buffer, pH 8.0, containing 2 mM dithiothreitol. The protein was freeze-dried and stored at −20 °C.

Reconstitution of Holo-PBG-D from the Apo Form with PBG. Holoenzyme was reconstituted from the apoenzyme by preincubation with a stoichiometric excess of PBG in 0.1 M potassium phosphate buffer, pH 8.0, containing 13 mM β-mercaptoethanol. Apoenzyme (50 μg) was incubated with between 4 and 10 molar equiv of PBG in a volume of 500 μL of 20 mM Tris/HCl buffer, pH 8.0, with 13 mM β-mercaptoethanol at 4 °C. At regular intervals (0, 5, 10, and 20 min for up to 2 h) 50 μL samples were removed from the incubation and were assayed for enzymatic activity as described above.

Generation of Preuroporphyrinogen and Reconstitution of Holo-PBG-D from the Apo Form with Preuroporphyrinogen. Preuroporphyrinogen (0.1 mmol) was generated by incubating holoenzyme (250 μg) with PBG (0.5 mmol) at 37 °C in 25 mM Tris/HCl, pH 9.3, containing 13 mM β-mercaptoethanol until all the PBG had been consumed (after about 10−15 min). The high pH and temperature (4 °C) were essential to prevent cyclization of the linear tetrapyrrole to uroporphyrinogen I. Under these conditions the preuroporphyrinogen was stable for at least 30 min. The preuroporphyrinogen was separated from the holoenzyme by filtration using an Amicon ultrafiltration cell fitted with a PM-10 membrane, followed by immediate fast-freezing of the preuroporphyrinogen in liquid nitrogen. For reconstitution of holo-PBG-D, preuroporphyrinogen was incubated in a 4−10-fold molar excess with apo-PBG-D in a volume of 500 μL and the incubation was followed as described above for reconstitution with PBG.

Circular Dichroism Spectroscopy Studies with PBG-D. The enzymes were prepared in 20 mM Tris/HCl, pH 8.0, containing 7 mM β-mercaptoethanol, typically to a final concentration of 1.2 mg/mL. CD spectra were recorded with nitrogen-flushed Jasco spectropolarimeters J-600 and J-720 using 4 s time constant, 10 nm/min scan speed, and a spectral bandwidth of 2 nm for J600 and 1 nm for J720, respectively. A 1 cm path length cell (Hellma) was used for the aromatic side-chain region (near UV) and a 0.02 cm cell for the backbone region (far UV). The spectra are reported as $\Delta\epsilon = \epsilon_L - \epsilon_R$ (M^{-1} cm^{-1}) based upon a mean molecular weight

per amino acid residue in accordance with pending IUPAC−IUB recommendations. The estimation of secondary structure content was performed using a partial least-square method (PLSplus of Grams/32 suite program) with a calibration data set of 16 proteins obtained from Hennessey and Johnson (1981).

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis under denaturing conditions (SDS−PAGE) was carried out using a 12% gel according to previously described methods (Laemmli, 1970; Sambrook *et al.*, 1989).

Proteolytic Cleavage. Purified PBG-D (100 μg) was subjected to digestion with trypsin (final concentration 1% w/w) in 20 mM Tris/HCl buffer, pH 8.0, for up to 16 h at 37 °C. The digestion was terminated by boiling the incubation mixture in SDS−PAGE sample disruption buffer (Sambrook *et al.*, 1989). The products of the proteolysis were analyzed by SDS−PAGE.

RESULTS AND DISCUSSION

Purification of Apodeaminase. Methods for the purification of *E. coli* PBG-D have provided large amounts of very pure protein facilitating the crystallization and eventual three-dimensional structure elucidation of the enzyme (Jordan *et al.*, 1988a, 1992; Louie *et al.*, 1992, 1996). However, the thermal instability and lability of the apoenzyme to proteolysis has made the purification of apoenzyme to complete homogeneity much more challenging (Scott *et al.*, 1989). The only published report on the purification of *de novo* apoenzyme is protein isolated from an *E. coli hemB* strain transformed with a *hemC* expression plasmid (Scott *et al.*, 1989), which affords inhomogenious protein.

To overcome the difficulties experienced with the purification of the apoenzyme, a one-step affinity procedure was devised. Crude cell extracts of ApoBM3 (*hemC* overexpressed in an *E. coli hemB* strain; Table 1) were applied to a column of Mimetic Orange, to which the apoenzyme was found to bind tightly. The column was washed in a stepwise fashion with increasing concentrations of salt and the bound apoenzyme was found eventually to elute in buffer containing 1 M NaCl in a near homogeneous form. Approximately 10 mg of purified apoenzyme could reproducibly be obtained from 1 L of ApoBM3 using the Mimetic Orange resin. An SDS−PAGE gel of the purified apo-PBG-D is shown in Figure 1. The nature of the interaction between Mimetic Orange and apodeaminase is unknown but the resin does not interact as tightly with holoenzyme. In fact, the resin can be used to separate apoenzyme from holoenzyme as the holo-PBG-D elutes from the Mimetic Orange resin largely in 0.5 M NaCl (data not shown).

Comparison of Apoenzyme and Holoenzyme by CD Spectroscopy. The CD data recorded in the far-UV CD region (185−250 nm) reflects the backbone secondary structure of the protein, representing the amide chromophore and the amide−amide interactions. Further information can be obtained from the protein by examining the near-UV CD region (250−320 nm), which reflects the local tertiary structure of the aromatic side chains of tryptophan (285−295 nm), tyrosine (270−280 nm), and phenylalanine (255−265 nm) (Strickland, 1974) and the disulfide bond of cystine (at about 250 and 300 nm) (Siligardi *et al.*, 1991).

E. coli PBG-D possesses two tryptophan, five tyrosine, and three phenylalanine residues but no disulfide bonds (Thomas & Jordan, 1986) (Figure 2). The crystal structure

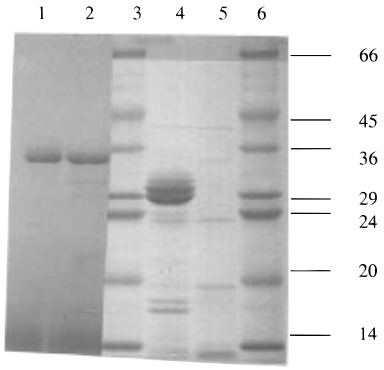


FIGURE 1: Purification of apodeaminase by Mimetic Orange Sepharose and the effect of trypsin on apoenzyme and holoenzyme. Lane 1 contains purified holo-PBG-D (10 μg), while lane 2 contains apo-PBG-D (10 μg) that has been eluted from a Mimetic Orange column in 1 M NaCl. The purified holo- and apoenzyme migrate on SDS−PAGE with a molecular mass of 35 kDa; the proteins migrate just ahead of the 35 kDa protein standard (lanes 3 and 6). Lanes 4 and 5 compare the effect of trypsin (1% w/w) on holo- and apoenzyme after incubation for 30 min at 37 °C. Holodeaminase (lane 4, 20 μg) is degraded slowly, producing a major proteolytic products with a molecular mass of about 29 kDa, whereas apoenzyme (lane 5, 20 μg) is degraded very rapidly. Molecular mass markers are shown in lanes 3 and 6.

FIGURE 2: Structure of PBG-D. The three-domain structure of the holoenzyme form of PBG-D is shown with the location of the aromatic residues within the structure highlighted. The enzyme contains only 10 aromatic amino acids; five tyrosines, three phenylalanines, and two tryptophans. The polypeptide chain of the protein is folded into three domains, marked 1, 2, and 3. The dipyrromethane cofactor is linked to domain 3 and is situated in the central catalytic cavity. Domain 1 contains one tyrosine, one tryptophan, and two phenylalanine residues; domain 2 contains two tryosine and one phenylalanine residues; and domain 3 contains two tyrosine and one tryptophan residues.

of the holoenzyme revealed that phenylalanine-62 is located in the enzyme active site and is stacked against the second ring of the cofactor (Louie *et al*., 1992, 1996) (Figure 2). It is conceivable that the aromatic side chain of phenylalanine-62 may act as an active-site reporter group as any change in the orientation of the aromatic ring (torsional angle χ_2) due to substrate interactions or rearrangement/denaturation of backbone structure will give rise to changes in sign and/or intensity to the CD contribution of the phenylalanine-62 side chain in the 255−265 nm region.

The spectra of apoenzyme and holoenzyme measured in the far-UV CD region are overlaid in Figure 3a. The secondary structure suggestions from these spectra are given in Table 2. The CD spectrum of holodeaminase suggest that the holoenzyme has an α-helical content of 26% and a

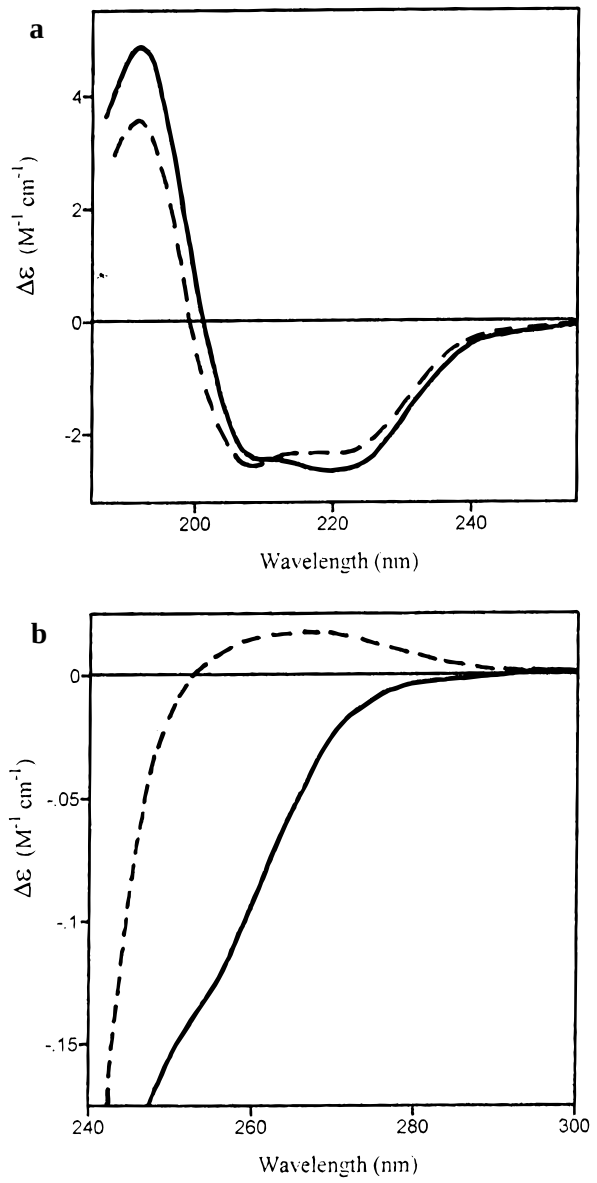


FIGURE 3: Far- and near-UV CD spectra of holoenzyme and apoenzyme. (a) Far-UV CD spectra of holoenzyme (solid line) and apoenzyme (dashed line) in 20 mM Tris/HCl buffer, pH 8.0, with 7 mM β-mercaptoethanol. The spectra are consistent with proteins of the α/β class and suggest that the apoenzyme contains 18% α-helix and 30% β-sheet, whereas the holoenzyme is estimated to contain 26% of both α-helix and β-sheet. (b) Near-UV CD spectra of holoenzyme (solid line) and apoenzyme (dashed line) in 20 mM Tris/HCl buffer, pH 8.0, with 7 mM β-mercaptoethanol. There are clear differences in the environment of the aromatic side chains of tryptophan (285−289 nm), tyrosine (270−280 nm), and phenylalanine (255−265 nm). The large differences mean that CD can be used to monitor the conversion of apo- to holoenzyme.

β-sheet content of 26%, whereas the X-ray-derived structure of the holoenzyme reveals that the enzyme has an actual α-helical content of 38% and a β-sheet content of 23%. Although the CD spectrum of holoenzyme is consistent with the protein belonging to the α/β class, it must be remembered that only the α-helical content from CD spectra can be taken with confidence. Other conformations including β-sheet, β-turns, extended polyproline II (P_{II}), and irregular loops give rise to less accurate estimations (Siligardi & Drake, 1995). Changing the buffer led to a relative change in the α-helical and β-sheet content; when the spectrum of holoenzyme was measured in sodium acetate at pH 7.4, the spectrum of

Table 2: Secondary Structure Estimations of Apoenzyme and Holoenzyme Structure Suggested from CD Spectroscopy in Comparison to the Values Obtained by X-ray Crystallography[a]

protein and conditions	α-helix (%)	β-sheet (%)	other (%)
apoenzyme (pH 8.0, Tris/HCl)	18	30	52
holoenzyme (pH 8.0, Tris/HCl)	26	26	48
holoenzyme (pH 7.4, NaAc)	32	30	38
holoenzyme (pH 5.0, NaAc)	32	30	38
actual holoenzyme (X-ray)	38	23	39

[a] The secondary structure content from CD data was carried out using a principal component regression (PCR) method of GRAMS/32 (Galactic Industries Corp.) with a calibration data set of 16 proteins obtained from Hennessey and Johnson (1981).

holoenzyme was consistent with a protein containing 32% α-helix and 30% β-sheet (Table 2).

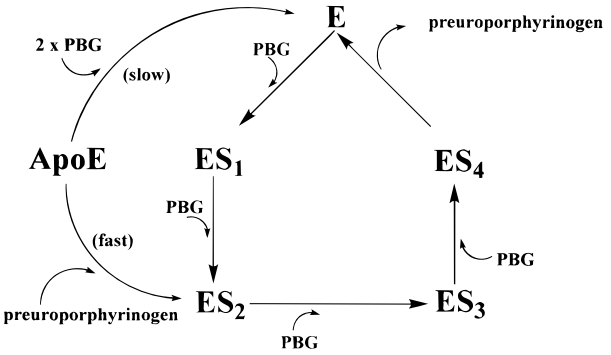
The CD spectra of apo-PBG-D was very different from that of the holoenzyme and was equivalent with a protein of 18% α-helix and 30% β-sheet content, implying that the cofactorless form of the protein is not unfolded. While the apoenzyme is suggested to contain 8% less helical structure than the holoenzyme, the CD data also implies that it contains 4% more β-sheet. A possible reason for the increase in the proportion of the α-helical content in the holoenzyme may be the stabilization of several loops, which may become more firmly held upon cofactor insertion, or alternatively it may represent the final folding of the long helix (residues 222−248, Figure 2) linking domains 1 and 3 on cofactor assembly.

The spectra of apoenzyme and holoenzyme measured in the near-UV CD region are overlaid in Figure 3b. The spectra show significant, measurable differences in the local environment of the five tyrosines and three phenylalanine residues. The spectra of apoenzyme and holoenzyme provide a reference to which subsequent spectra can be compared and allow the reconstitution of holoenzyme from apoenzyme to be monitored by CD spectroscopy (see later).

Reconstitution of Holoenzyme by the Addition of PBG to Apoenzyme. Previously it had been reported that apoenzyme can be converted to holoenzyme *in vitro* in yields of up to 40% by incubation of the cofactorless apoenzyme with PBG (Scott *et al*., 1989; Hart *et al*., 1988; Warren & Jordan, 1988). The conversion, however, displays a characteristic lag phase of around 30 min, after which time the conversion is observed at a faster rate. Although no reason was advanced for the lag phase, the recent discovery that preuroporphyrinogen is the preferred substrate for the cofactor (Shoolingin-Jordan *et al.*, 1996) offers a rational explanation. Thus apoenzyme will use PBG as a poor substrate for the cofactor synthesis but once holoenzyme has been generated, preuroporphyrinogen will be synthesized that will readily be utilized by the remaining apoenzyme, forming the ES_2 intermediate complex directly (Scheme 2). Hence the lag phase may represent the period of time for a few molecules of apoenzyme to incorporate PBG to yield holoenzyme. The reconstitution of holoenzyme from apoenzyme, with both PBG and preuroporphyrinogen, was monitored by CD spectroscopy to determine if the technique could yield any structural information concerning the reconstitution of apoenzyme from holoenzyme.

The reconstitution of holoenzyme from apoenzyme was anticipated as being most successfully examined in the near-UV CD region, as it was hoped that phenylalanine-62 would serve as a suitable marker for the active-site changes. The

Scheme 2: Transformation of Apoenzyme into Holoenzyme and the Catalytic Cycle of PBG-D[a]



[a] The enzyme proceeds through its catalytic cycle via discernible covalently linked enzyme intermediate forms. These forms represent the enzyme with one (ES_1), two (ES_2), three (ES_3), and four (ES_4) substrate molecules attached to the dipyrromethane cofactor. The addition of the final substrate molecule leads to hydration of the bound tetrapyrrole, resulting in the generation of free enzyme (E) and preuroporphyrinogen. Holoenzyme can be generated from the apoenzyme either by addition of two molecules of PBG or by the addition of preuroporphyrinogen. The latter gives rise to the direct formation of an ES_2 intermediate.

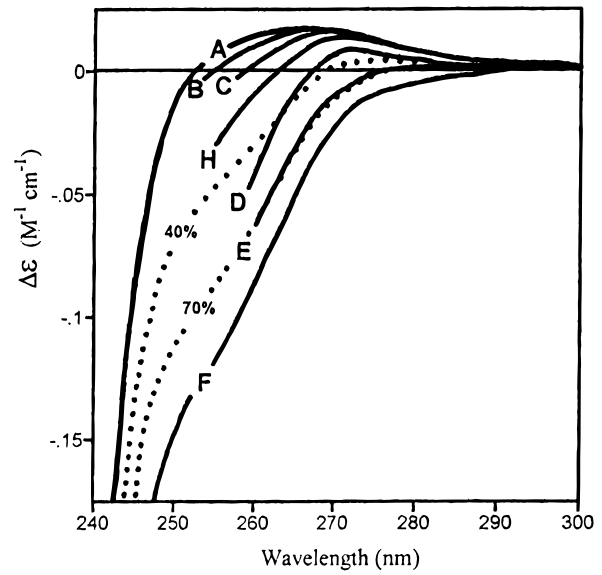


FIGURE 4: Effect of PBG and preuroporphyrinogen on the reconstitution of holoenzyme from apoenzyme. CD spectra were recorded in 20 mM Tris/HCl buffer, pH 8.0, with 7 mM β-mercaptoethanol in the aromatic side-chain region (240−320 nm). Spectra of apoenzyme at 4 °C (A), apoenzyme reconstituted with a 10-fold molar excess of PBG at $t = 0$ min at 4 °C (B) and at $t =$ 140 min at 4 °C (C), and apoenzyme incubated at 37 °C for 140 min with a 10-fold molar excess of PBG (H) are shown. Also shown are spectra obtained when apoenzyme was reconstituted with a 10-fold molar excess of preuroporphyrinogen at $t = 0$ min at 4 °C (D) and at $t = 140$ min (E) in comparison to the spectrum of holoenzyme (F). The dotted lines represent simulated spectra of 40% and 70% reconstituted holoenzyme generated from the spectra of apoenzyme and holoenzyme in the appropriate proportions. Data for spectra B, C, D, E, and H were not taken below 255 nm as the incubations were performed in the presence of a high concentration of β-mercaptoethanol, which interfered with the recording.

CD spectrum of apoenzyme that had been incubated with a 10-fold molar excess of PBG is shown at $t = 0$ min (spectrum B) and $t = 140$ min (spectrum C) in Figure 4. The spectrum of apo-PBG-D after reconstitution with PBG at 4 °C for $t =$ 140 min still maintained many of the characteristics of the apoenzyme CD spectrum (spectrum A) despite the 40% activity of the reconstituted holoenzyme recovered in this incubation (specific activity 12 μmol h^{-1} mg^{-1}). In particular, the CD spectrum showed both tryptophan residues and the majority of tyrosine residues still in an apoenzyme environment because of their retained positive CD sign. Only the local tertiary structure of phenylalanine residues with a change in CD sign from positive to negative could be seen as being affected by the conversion. A simulated CD spectrum representing 60% apoenzyme and 40% holoenzyme (Figure 4) was very different from the spectrum of the reconstituted holoenzyme at equilibrium, $t = 140$ min. A larger change in the CD spectrum was observed after reconstitution of the apo-PBG-D with PBG for 140 min at 37 °C, although this also exhibited only a 40% restoration of holo-PBG-D activity.

Why should spectra C and H (Figure 4), which are markedly different, produce similar reconstitution activity of holo-PBG-D? One possibility is that after reconstitution of apo-PBG-D with PBG for 140 min at 4 °C some cofactor will have been formed, generating approximately 5−10% holo-PBG-D (spectrum C). When this is incubated in the PBG-D assay to determine the activity of the reconstituted enzyme with a large excess of PBG, the small amount of reconstituted holo-PBG-D quickly generates preuroporphyrinogen, which is inserted into the remaining apo-PBG-D, thereby forming more active holo-PBG-D. When apo-PBG-D is reconstituted with PBG at 37 °C, more holo-PBG-D is generated, about 30% as observed in spectrum H (Figure 4), because it was reconstituted at a higher temperature. As apo-PBG-D does not reconstitute above 40%, when this sample is assayed the activity more closely correlates with the observed CD spectrum.

However, a comparison of spectra C and H to the 40% simulated spectrum (Figure 4) does not support the simple explanation that the partial enzymatic activity of the reconstituted holoenzyme from PBG is due to 40% of the apoenzyme converting to a holoenzyme conformation, as even spectrum H does not appear very similar to the simulated spectrum. A rather different mode of conversion has to be accounted for.

Reconstitution of Holoenzyme by the Addition of Preuroporphyrinogen to Apoenzyme As Monitored by Near-UV CD. The reconstitution of holoenzyme from apoenzyme by the addition of preuroporphyrinogen proceeds with much higher yields and at a much faster rate (Shoolingin-Jordan *et al.*, 1996). The structural changes induced by the addition of preuroporphyrinogen were investigated by CD. Apoenzyme was incubated with a 10-fold molar excess of preuroporphyrinogen at 4 °C and spectra were collected at various times. The spectra of preuroporphyrinogen incubated apoenzyme at $t = 0$ (spectrum D, Figure 4) and $t = 140$ min (spectrum E, Figure 4), together with the spectra of apoenzyme, holoenzyme, and PBG-incubated apoenzyme, are displayed in Figure 4.

Addition of preuroporphyrinogen to apoenzyme resulted in an immediate change in the environment of the phenylalanine and tyrosine residues (Figure 4, spectrum D). The reaction was so rapid that at $t = 0$ much of the change had already occurred and a CD profile that was closer to that of holoenzyme was observed (Figure 4, spectrum D). The CD spectrum in the tyrosine region (265−275 nm) was significantly positive in terms of CD sign, clearly indicative of several tyrosine residues still being in an apoenzyme environment, and similarly, the tryptophan residues were also reflecting an apoenzyme environment because of their

positive and relatively unchanged CD contributions. By the end of the incubation, $t = 140$ min, the CD spectrum (Figure 4, spectrum E) was very similar to that of holoenzyme. At this point the CD spectrum of the incubation of apoenzyme with preuroporphyrinogen was equivalent to a simulated spectrum of a mix containing apoenzyme and holoenzyme in the ratio of 30:70. These results confirm that preuroporphyrinogen is the preferred substrate of apoenzyme, leading to a rapid conversion of holoenzyme, in a conversion yield of around 70%. This yield is far greater than has been attained when PBG has been used for cofactor generation.

A question has to asked as to why the reconstituition does not improve beyond 70%. The most likely explanation is that some of the apoenzyme has been inactivated during the purification procedure. The cofactor is attached to cysteine-242 of the *E. coli* PBG-D and in this respect the apo-PBG-D is particularly sensitive to sulfhydryl reagents such as *N*-ethylmaleimide (Awan, 1996).

CD Changes on the Addition of PBG to Holoenzyme. Evidence for the occurrence of conformational changes during the catalytic cycle of holoenzyme had been obtained from demonstrating the increased susceptibility of the holoenzyme to thiol-directed modifying reagents during the catalytic cycle (Warren & Jordan, 1988; Warren *et al*., 1995). This had provided strong evidence for the relative movement of domains 2 and 3 with respect to one another. The binding of PBG to wild-type holoenzyme was therefore examined by CD spectroscopy to investigate whether conformational changes could be observed by changes to either the near- or far-UV CD spectrum. No overall change in the secondary structure of the enzyme−substrate intermediate as compared to the free holoenzyme was observed (data not shown).

Spectra were also recorded in the near-UV CD region for the addition of PBG to holoenzyme (Figure 5a). In this region, it was anticipated that significant changes may be recorded, especially as the positioning of phenylalanine-62 against the substrate-binding site would suggest that the local environment of this residue may change during the catalytic cycle of the enzyme. Indeed, the addition of PBG to the holoenzyme at 4 °C (Figure 5a) induced an immediate change in the CD spectrum of the tryptophan side-chain region (285−295 nm) that was followed by subsequent changes in the phenylalanine side-chain region (255−265 nm) until the reaction had reached completion at $t = 30$ min, when no further CD changes were observed. The small changes in the CD spectrum of the tryptophan region are consistent with the relative movements of domains 1 or 2, where each of the tryptophan residues is located (Figure 2), while the changes in the phenylalanine region are more likely to reflect the reactions at the catalytic site. At about 295 nm (Figure 5b), the change in CD sign at equilibrium was clearly indicative of a change in environment of both tryphtophan residues. It is conceivable that the three tyrosine residues, close in space to the two tryptophan residues (Figure 2), would experience similar tertiary structure perturbations. This result provides the first physical evidence for the role of phenylalanine-62 in the catalytic process of the enzyme. However, this conclusion does not preclude any contribution that may be made to the spectrum from the two other phenylalanines (at positions 89 and 108) in the protein.

Effect of Heat on Apoenzyme and Holoenzyme Structure As Monitored by CD. The apoenzyme and holoenzyme have been observed to display very different characteristics upon heat treatment at 60 °C (Scott *et al*., 1989). In general, the

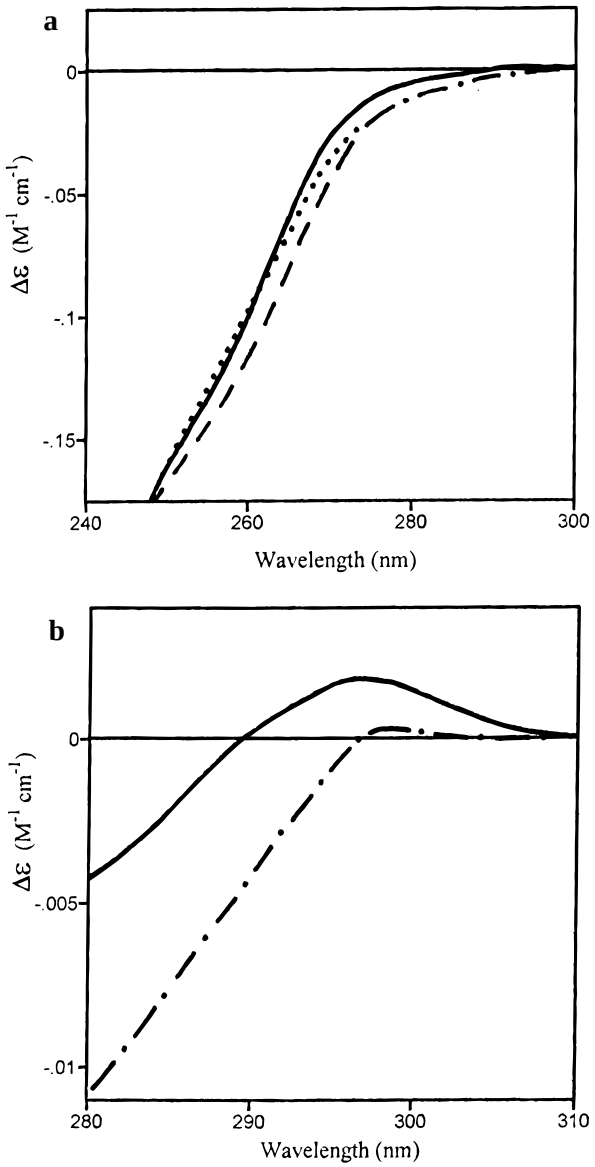


FIGURE 5: Effect of substrate on the UV-CD spectrum of holoenzyme. (a) CD spectrum of holoenzyme in the aromatic side-chain region (240−320 nm) in 20 mM Tris/HCl buffer, pH 8.0, with 7 mM β-mercaptoethanol without substrate (solid line) and with 4 molar equiv of PBG at $t = 0$ (dashed line) and at $t = 30$ min (dotted line). (b) Magnification of part of panel a highlighting the change in CD sign.

holoenzyme is found to be relatively stable to heat (Jordan *et al*., 1988a) and does not lose significant activity or structure after heat treatment at 60 °C for 10 min, as analyzed by both gel electrophoresis and enzyme activity assay after cooling. The apoenzyme analyzed under the same conditions, however, shows a dramatic loss of activity and structure. When apo- and holoenzyme were studied by CD under similar thermal conditions, different temperature dependencies in terms both of thermal denaturation and structural recovery were observed. The overlaid far-UV CD spectra for holoenzyme at 60 and 25 °C can be seen in Figure 6a. The spectra showed that, at 60 °C, the holoenzyme secondary structure was disrupted but that, on cooling, the native structure was recovered. In contrast, the backbone CD spectrum of apoenzyme showed a structural denaturation on heating from 25 to 60 °C that was largely irreversible on cooling back to 25 °C. In the aromatic side-chain region, the thermal denaturation at 60 °C of apoenzyme was experienced by many aromatic residues, in particular the

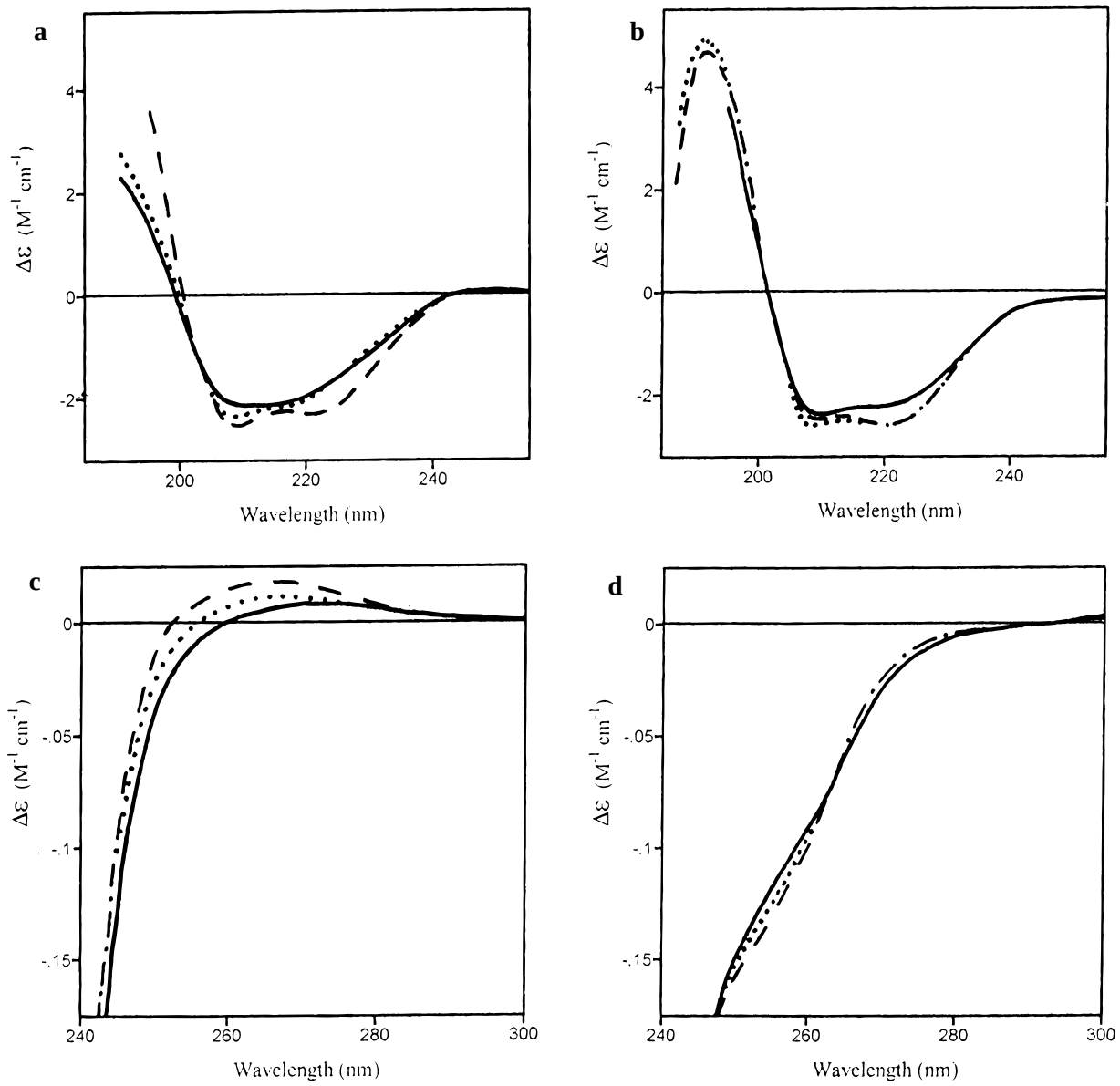


FIGURE 6: Effect of temperature on the far- and near-UV CD spectrum of apoenzyme and holoenzyme. (a) CD spectra of apoenzyme in 20 mM Tris/HCl buffer, pH 8.0, with 7 mM β-mercaptoethanol in the backbone region (185−260 nm) at 25 °C (dashed line) and 60 °C (solid line) and cooled to 25 °C (dotted line). The perturbations in the backbone with temperature appear not to be reversible with apoenzyme. (b) CD spectra of holoenzyme in 20 mM Tris/HCl buffer, pH 8.0, with 7 mM β-mercaptoethanol in the backbone region (185−260 nm) at 25 °C (dashed line) and 60 °C (solid line) and cooled to 25 °C (dotted line). The perturbations in the backbone with temperature appear to be largely reversible with the holoenzyme. (c) CD spectra of apoenzyme in 20 mM Tris/HCl buffer, pH 8.0, with 7 mM β-mercaptoethanol in the aromatic side-chain region (240−320 nm) at 25 °C (dashed line) and 60 °C (solid line) and cooled to 25 °C (dotted line). The changes in the aromatic side-chain environment that occur on heating to 60 °C appear not to be reversible on cooling. (d) CD spectra of holoenzyme in 20 mM Tris/HCl buffer, pH 8.0, with 7 mM β-mercaptoethanol in the aromatic side-chain region (240−320 nm) at 25 °C (dashed line) and 60 °C (solid line) and cooled to 25 °C (dotted line). The changes in the aromatic side-chain environment that occur on heating to 60 °C appear to be largely reversible on cooling

phenylalanine and tyrosine residues (Figure 6c), as identified by a decreased overall $\Delta\epsilon$ intensity. A partial recovery of the $\Delta\epsilon$ intensity related to the local tertiary structure of the aromatic residues was observed on cooling the apoenzyme to 25 °C (Figure 6c). Holoenzyme showed significant CD changes only in the 255−260 nm region on heating to 60 °C that were attributed to phenylalanine residues (Figure 6d). These changes were largely recovered on cooling to 25 °C (Figure 6d).

Thus the thermal lability of the apoenzyme appears to be due to an irreversible structural denaturation of the secondary and tertiary structure. In the holoenzyme the thermal denaturation of the protein is reversible, presumably as the presence of the cofactor favors the correct folding of the protein into its active conformation.

Stability of Apoenzyme Compared to Holoenzyme with Regard to Proteolysis by Trypsin. The instability of the apoenzyme can also be visualised by examination of the effect of trypsin on apodeaminase compared to holodeaminase (Figure 1). Holoenzyme is cleaved at several defined points; initially at lysine-64, generating a major 29 kDa fragment, and subsequently at lysine-158 and arginine-278 (Warren, 1988). Degradation of the holoenzyme is slow, and even after 30 min at 37 °C with a high concentration of trypsin (1% w/w) the 29 kDa fragment can be visualized by SDS−PAGE. Conversely, apo-PBG-D is rapidly degraded by the protease such that no major fragments can be detected (Figure 1). The apo-PBG-D is not cleaved in the absence of trypsin when left at 37 °C for the same period of time, indicating that no endogenous proteases are present. The

enhanced susceptibility of apodeaminase to proteolysis by trypsin suggests that, in comparison to holoenzyme, the apoenzyme has a less tightly folded structure, allowing greater accessibility to the protease. The crystal structure of the holoenzyme has shown that there are few contacts between the three domains of the holoenzyme and that the majority of contacts which stabilize the association between the three domains of the protein are formed between the enzyme and the cofactor (Louie *et al*., 1992, 1996). Thus the absence of the cofactor may allow for greater flexibility between the domains and make the protein more susceptible to thermal denaturation and proteolytic degradation. However, a structure for the apoenzyme in which the protein is totally unfolded has also been proposed and supported by NMR studies (Scott *et al*., 1989). In this case it has been suggested that the addition of the cofactor to apoenzyme leads to folding of the protein. It is possible that the observed NMR signals of the apoenzyme may be due to denaturation of the protein in the NMR tube during data collection. The CD spectrum of apodeaminase presented in Figure 3 is not consistent with an unfolded protein.

Comparison between the CD Spectrum of Holoenzyme at pH 8.0 and 5.0. Native *E. coli* PBG-D has an optimal activity around pH 8.5 (Jordan *et al*., 1988a). The enzyme is catalytically inactive below pH 6.0 and the enzyme is irreversibly inactivated when the pH drops below pH 4.0 (Jordan *et al*., 1988a). Crystal growth of the enzyme is most effective in conditions around pH 5.0 (Jordan *et al.*, 1992), and the structure currently available for PBG-D is one that has been solved at pH 5.0 (Louie *et al.*, 1992, 1996). However, the crystals of the holoenzyme grown at pH 5.0 shatter upon addition of substrate, indicating that although catalytically inactive at this pH, the enzyme is able to interact with PBG (S. Wood, personal communication). The validity of a structure obtained from PBG-D at a pH where the enzyme is inactive was therefore investigated by CD spectroscopy.

The holoenzyme was resuspended in 20 mM sodium acetate buffer, pH 7.4, and the spectrum was recorded. Sodium acetate buffer was used for the experiment as it was the buffer in which crystallization of the enzyme was achieved. The overlaid spectra are given in Figure 7a. In 20 mM sodium acetate buffer, pH 7.4, the holoenzyme (Figure 7a) maintained its α/β type of CD spectrum but an overall increased $\Delta\epsilon$ intensity was indicative of an higher content of α-helix conformation than that observed in Tris/HCl buffer, pH 7.8 (Figure 3a). The secondary structure estimation from CD data alone was consistent with a 32% α-helix content in sodium acetate, pH 7.4, and 26% in Tris/HCl buffer, pH 7.8 (Table 2). The X-ray-derived structure shows a protein with 38% α-helix content (Louie *et al*., 1996). The far-UV CD spectrum of holoenzyme appeared unchanged at the lower pH, indicating that the backbone of the protein was not in an altered conformation at pH 5.0. Thus PBG-D crystallized at pH 5.0 is likely to have a similar structure to enzyme crystallized at pH 7.4. The holoenzyme was also monitored for the drop from pH 7.4 to 5.0 in the near-UV CD region under the same conditions. The two spectra can be seen in Figure 7b. In sodium acetate buffer, pH 5, no significant changes were observed in the backbone region, suggesting an overall unchanged backbone conformation (Figure 7a). A small but significant change was observed, however, in the aromatic side-chain region that was assigned to the phenylalanine contribution (Figure 7b),

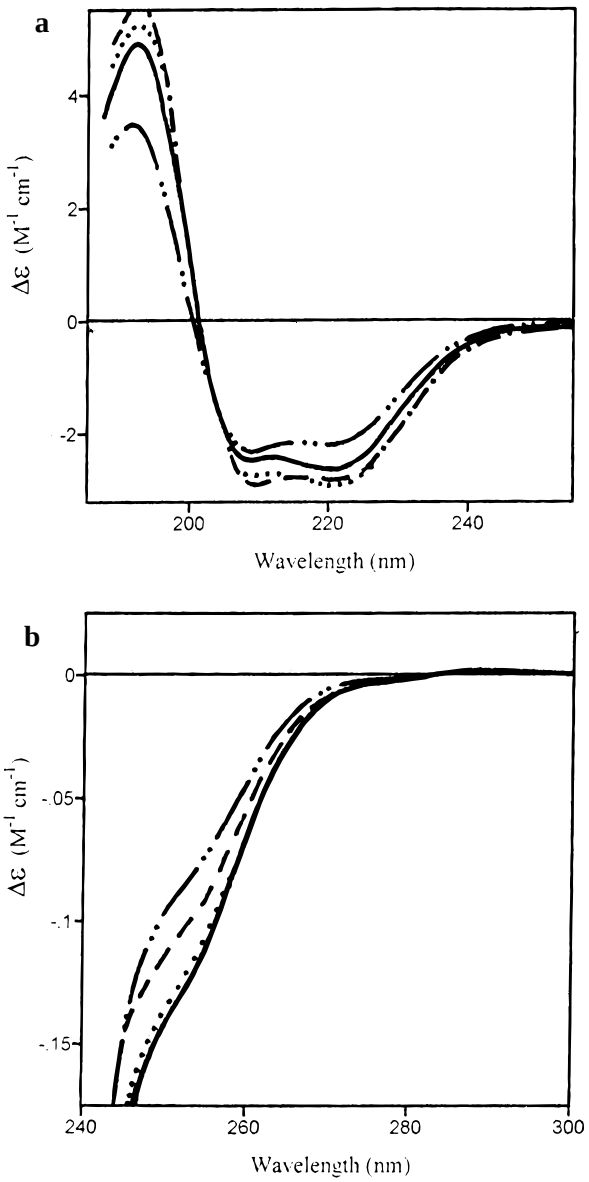


FIGURE 7: Effect of different buffers and pH on far- and near-UV CD spectrum of apoenzyme and holoenzyme. (a) CD spectra of holoenzyme in the backbone region (185−260 nm) in 20 mM Tris/HCl buffer, pH 8.0, with 7 mM β-mercaptoethanol (solid line) and 100 mM sodium acetate buffer, pH 7.4 (dotted line), pH 5.0 (dashed line), and pH 3.7 (dotted−dashed line). In sodium acetate buffer at pH 7.4 and 5.0 the holoenzyme is observed to have a higher proportion of α-helix, which is more consistent with the amount of α-helix observed in the crystal structure of the enzyme. At pH 3.7 some secondary structure is lost as the protein becomes irreversibly denatured. (b) CD spectra of holoenzyme in the aromatic side-chain region (240−320 nm) in 20 mM Tris/HCl buffer, pH 8.0, with 7 mM β-mercaptoethanol (solid line) and 100 mM sodium acetate buffer, pH 7.4, (dotted line), pH 5.0 (dashed line), and pH 3.7 (dotted-dashed line).

perhaps reflecting a more protonated environment. This further confirmed the validity of a PBG-D structure obtained at pH 5.

Effect of Lowering the pH of the Holoenzyme to pH 3.7. Previous work has shown that the holoenzyme becomes irreversibly inactivated below pH 4.0 (Jordan *et al*., 1988). Such a loss of activity suggests that the holoenzyme may be irreversibly denatured below this point. It would be anticipated, therefore, that major changes would be observed in either or both the far- and near-UV CD regions at the lower pH values. The spectra of holoenzyme at both pH 7.4 and 3.7 are shown in Figure 7, panels a and b. The far-UV CD

spectra showed that some secondary structure had been lost at pH 3.7 (Figure 7a). Lowering the pH of the holoenzyme from 7.4 to 3.7 brought about changes in the local environment of the tyrosine and particularly the phenylalanine residues as shown in Figure 7b. A greater change was observed than for the effect of heating holoenzyme to 60 °C. This may reflect the irreversibility of the low pH on the activity of the enzyme.

In summary, a rapid one-step procedure for the purification of *E. coli* apo-PBG-D was achieved by affinity chromatography using Mimetic Orange resin. The availability of large quantities of very pure apoenzyme facilitated a detailed study of the conversion of apo- to holoenzyme. The reconstitution of apo- to holoenzyme was found to occur at a much faster rate and with greater efficiency when preuroporphyrinogen was used as a substrate as compared to PBG, consistent with recent kinetic findings (Shoolingin-Jordan *et al.*, 1996). The cofactorless form (apoenzyme) of PBG-D is both thermounstable and susceptible to proteolysis in comparison to the holoenzyme. However, analysis by CD spectroscopy has shown that the apoenzyme is not unfolded, as suggested previously from NMR data, but rather exhibits a spectrum consistent with the presence of well-defined regions of α-helix and β-sheet. The instability of the apoenzyme is therefore attributed to the important interactions made between the cofactor and the three domains of the protein, which increase the rigidity of the structure as the cofactor makes over 26 interactions with the protein structure (Louie *et al.*, 1996). CD is an excellent tool to study the transformation of the apoenzyme into holoenzyme as the near-UV CD spectrum of apoenzyme is markedly different from that of holoenzyme. In particular, the different environments of the phenylalanine and tyrosine residues are most striking.

The CD spectrum of preuroporphyrinogen-regenerated holoenzyme was found to be very similar to the CD spectrum of holoenzyme, whereas the CD spectrum of PBG-regenerated apoenzyme maintained many of the characteristics of the apoenzyme CD spectrum. CD was also used to probe the structure of apoenzyme and holoenzyme at different temperatures. The results showed that although the holoenzyme was able to regain its secondary structure after heating to 60 °C, the apoenzyme was not. Apoenzyme was also shown to be more sensitive to proteolysis by trypsin. The lability of the protein to both heat and proteolysis is consistent with a holoenzyme tertiary structure that is more organized and compact, reflecting the large number of interactions between the three domains and the cofactor.

Finally, the CD spectrum of the holoenzyme was found to be very similar at both pH 5.0 and 7.4 in acetate buffer. This is an important observation since the crystal structure of the holoenzyme was determined at pH 5.0, a pH at which the enzyme is catalytically inactive. This would suggest that the tertiary structure is broadly similar at both pH values.

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