Evidence for Participation of Aspartate-84 as a Catalytic Group at the Active Site of Porphobilinogen Deaminase Obtained by Site-Directed Mutagenesis of the *hemC* Gene from *Escherichia coli*

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ABSTRACT: The role of aspartate-84, an invariant residue in the active site cleft of *Escherichia coli* porphobilinogen deaminase, has been investigated by site-directed mutagenesis. Substitution of aspartate-84 by glutamate results in an enzyme that retains less than 1% of its activity and which can form highly stable enzyme–intermediate complexes. Substitution of aspartate-84 by either alanine or asparagine, however, results in proteins unable to catalyze the formation of preuroporphyrinogen but which, nevertheless, appear able to assemble the dipyrromethane cofactor. The mechanisms of the tetramerization reaction and cofactor assembly are discussed.

Porphobilinogen deaminase (EC 4.3.1.8) catalyzes the tetramerization of porphobilinogen to give preuroporphyrinogen, a highly unstable 1-(hydroxymethyl)bilane (Burton *et al.*, 1979; Battersby *et al.*, 1979a), which is then transformed by uroporphyrinogen III synthase into uroporphyrinogen III (Jordan *et al.*, 1979; Jordan & Berry, 1980). Uroporphyrinogen III is the first cyclic intermediate of the tetrapyrrole biosynthesis pathway and the ubiquitous precursor of all other tetrapyrroles [reviewed in Jordan (1991)]. The deaminase links the four pyrrole rings in an ordered, stepwise mechanism in which ring A of the tetrapyrrole macrocycle is first to bind to the enzyme, followed by rings B, C, and finally D (Jordan & Seehra, 1979; Battersby *et al.*, 1979b). Enzyme–intermediate complexes representing each of these steps, ES, ES_2, ES_3, and ES_4, respectively, have been isolated, and their individual properties have been studied (Anderson & Desnick, 1980; Berry *et al.*, 1981; Warren & Jordan, 1988; Aplin *et al.*, 1991).

Porphobilinogen deaminase is unique among enzymes in that it possesses a dipyrromethane cofactor derived from two molecules of porphobilinogen (Jordan & Warren, 1987; Hart *et al.*, 1987) which acts as a primer for the enzymic reaction. The cofactor is linked covalently to cysteine-242 in the *Escherichia coli* enzyme through the C1 ring while the C2 ring forms the covalent attachment site for the substrate during the tetramerization reaction (Scheme 1). This results in a chain of six pyrrole units, all attached covalently to the protein (Jordan & Warren, 1987; Hart *et al.*, 1987), that are cleaved by hydrolysis to yield the tetrapyrrole product, preuroporphyrinogen, thus regenerating the holoenzyme with the dipyrromethane cofactor intact (Scheme 1). The cofactor is self-assembled by the deaminase and, once formed, remains a permanent component of the active site (Jordan & Warren, 1987). There is substantial evidence to suggest that the dipyrromethane system contributes significantly to protein folding and stability (Warren & Jordan, 1988; Scott *et al.*, 1989). Detailed investigations with the *E. coli* porphobilinogen deaminase pointed to the existence of a single catalytic site composed of a substrate binding site (S) and a cofactor binding site (C) (Warren & Jordan, 1988). The results from site-directed mutagenesis (Jordan & Woodcock, 1991; Lander *et al.*, 1991) highlighted the role of several conserved arginines for binding the negatively charged acetate and propionate side chains of the dipyrromethane cofactor and substrate at the two sites. These proposals have been borne out by the crystal structure of the *E. coli* enzyme (Louie *et al.*, 1992) that identifies the role of arginines 131, 132, and 155 at site C and arginines 11, 149, and 155 at site S (see Figure 1).

The catalytic mechanism of the condensation reaction is thought to involve the deamination of porphobilinogen at the substrate binding site (S) to give an azafulvene (or equivalent species) which acts as an electrophile for attack by the unsubstituted nucleophilic α-position of an enzyme-bound pyrrole ring (Scheme 2). This can be either the C2 ring of the dipyrromethane cofactor or the terminal unit of an enzyme–intermediate complex, ES, ES_2, or ES_3, located at the C binding site. The mechanism would require a suitably located negatively charged group to facilitate the development of positive charge on the pyrrole nitrogen atoms of the reacting pyrrole units.

A comparison of the deduced primary structures from a number of porphobilinogen deaminases shows that seven acidic residues, five aspartates (D46, D76, D84, D106, and D209) and two glutamates (E65 and E231), are invariant in all species (Thomas & Jordan, 1986; Raich *et al.*, 1986; Stubnicer *et al.*, 1988; Beaumont *et al.*, 1989; Sharif *et al.*, 1989; Petricek *et al.*, 1990; Keng *et al.*, 1992; Dr. A. G. Smith, personal communication). One of these invariant acidic residues, aspartate-84, is located in a highly conserved region of the *E. coli* sequence, V-H-S-M-K-D-V-P, extending from residue 79 through 86 (Thomas & Jordan, 1986). The crystal structure of the *E. coli* enzyme (Louie *et al.*, 1992) has revealed that aspartate-84 is positioned near the proposed S and C pyrrole binding sites, within hydrogen-bonding distance (3 Å) of the pyrrole NH groups of the dipyrromethane cofactor (Figure 1), making it a strong contender as a catalytic group.

This paper reports the results of site-directed mutagenesis in which aspartate-84 was substituted by alanine, glutamate, and asparagine. The results of experiments performed on these mutants provide powerful evidence that aspartate-84 does indeed play a key role in the tetramerization reaction

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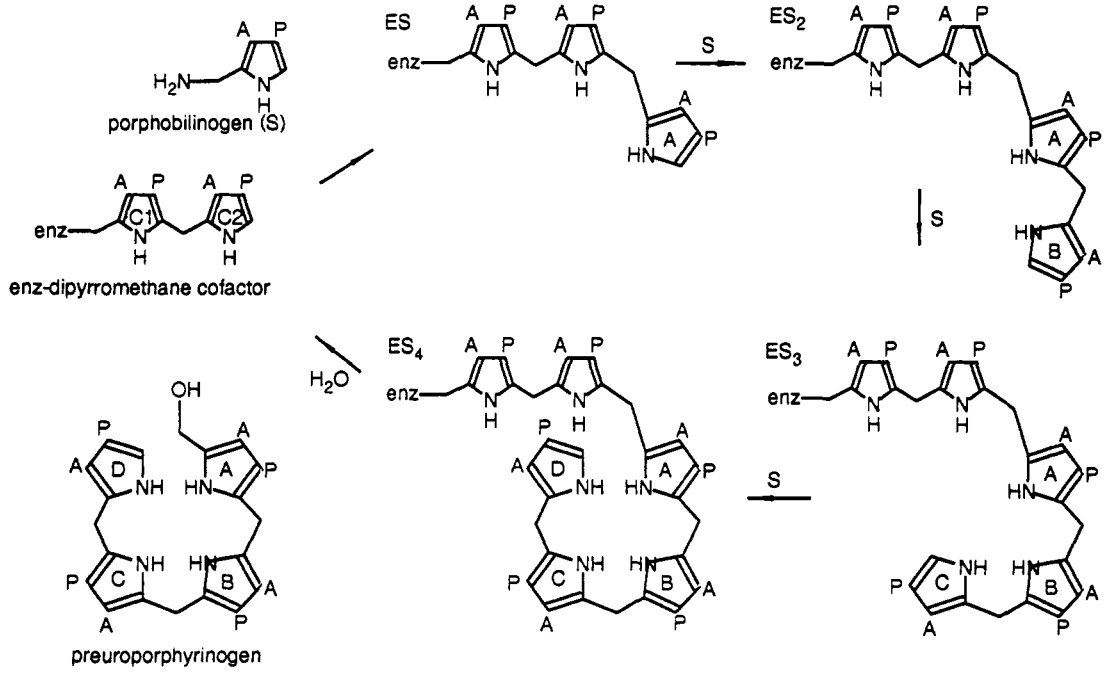
Scheme 1: Assembly of the Tetrapyrrole Product Preuroporphyrinogen Attached to the Dipyrromethane Cofactor of Porphobilinogen Deaminase[a]



[a] A = acetate; P = propionate; C1 and C2 represent the two pyrrole rings of the dipyrromethane cofactor; A, B, C, and D indicate the four substrate molecules in order of attachment to the cofactor; enz represents the deaminase covalently attached by cysteine-242 to the dipyrromethane cofactor through a thioether linkage.

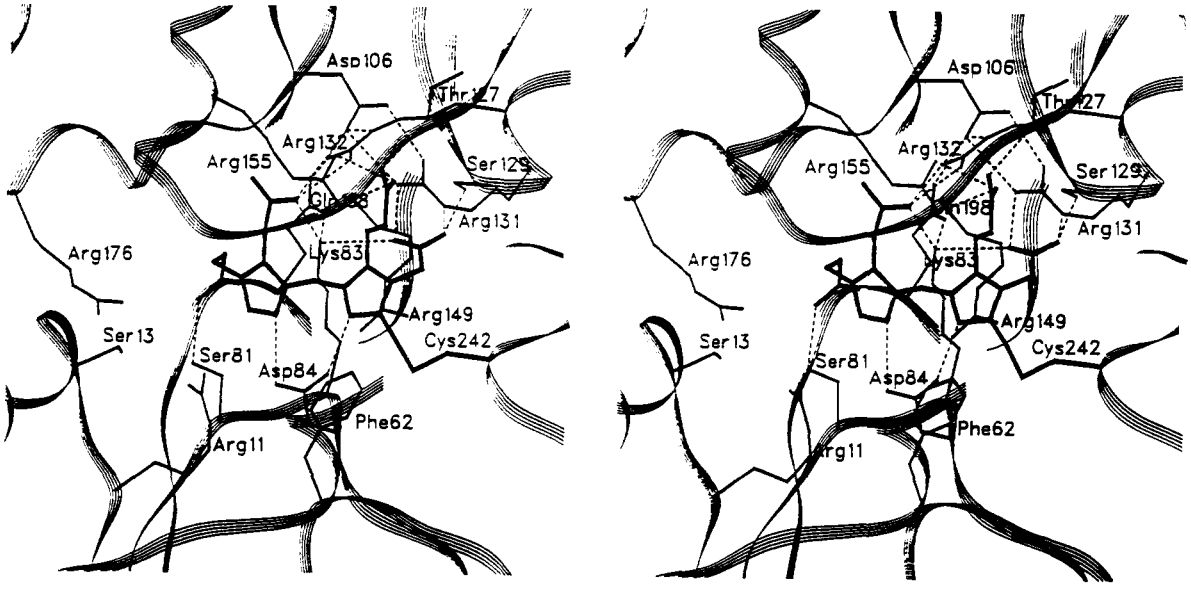


FIGURE 1: Catalytic cleft of *E. coli* porphobilinogen deaminase. The dipyrromethane cofactor is bound through the acetate and propionate side chains to arginine-131, -132, and -155 and lysine-83. Aspartate-84 is situated within hydrogen-bonding distance of both pyrrole nitrogen hydrogen atoms of the dipyrromethane cofactor. The vacant substrate binding site (S) comprises arginine-11, -149, and -155 that interact with the acetate and propionate side chains of the substrate. Asparate-84 can hydrogen bond to the hydrogen atoms attached to the pyrrole nitrogen and the amino group of the substrate. Arginine-176 is situated at the back of the catalytic cleft and is too far distant to bind directly to the substrate but is well positioned to interact with the negatively charged side chains of the enzyme-intermediate complexes.

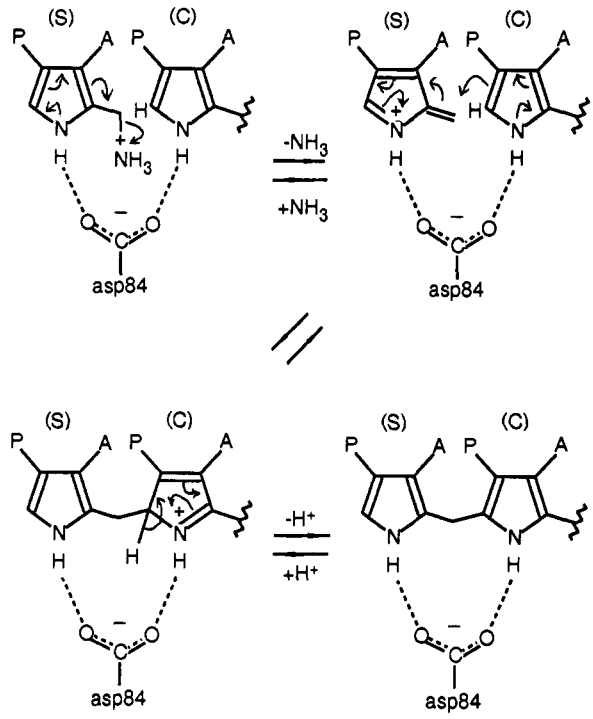
catalyzed by the porphobilinogen deaminase. The results from the studies on these mutants also throw light on the possible mechanism of the cofactor assembly process.

MATERIALS AND METHODS

Materials. Porphobilinogen was synthesised from 5-aminolevulinic acid using homogeneous porphobilinogen synthase (Jordan & Seehra, 1986). All laboratory reagents were purchased from BDH Chemicals or Sigma Chemical Co., both at Poole, Dorset, U.K. FPLC requisites and PD10 columns were from Pharmacia, Milton Keynes, Bucks, U.K. Mutagenesis kits, restriction endonucleases, and [α-[^{35}S]thio]-dATP were purchased from Amersham International, Bucks, U.K. Mutagenic primers (Table 1) were prepared using an Applied Biosystems Model 381A DNA synthesizer with Applied Biosystems reagents.

Bacterial Strains Used for Mutagenesis and Expression. Mutagenesis was performed in M13mp19 harboring a *Bam*HI–*Hin*dIII fragment obtained from plasmid pST46 containing the *hemC* gene (Thomas & Jordan, 1986). The *hemC*

Scheme 2: Mechanism of the Reaction Catalyzed by Porphobilinogen Deaminase[a]



[a] The mechanism proceeds in the following stages: binding of porphobilinogen to the S (substrate) site and deamination to the azafulvene; tautomerism of the ring bound at the C (cofactor) site (ring C2, A, B, or C); formation of the C–C bond; loss of the hydrogen atom at the newly substituted α-position of the ring at site C.

Table 1: Oligonucleotides Used To Generate the Aspartate-84 Mutants

wild-type sequence	CTCAATGAAAGATGTGCC
D84A mutant sequence	CTCAATGAAAGCTGTGCC
D84E mutant sequence	CTCAATGAAAGAAGTGC
D84N mutant sequence	CTCAATGAAAAATGTGC

mutants D84A, D84E, and D84N were generated using the single-mismatched oligonucleotides shown in Table 1 (mismatches are underlined) by the method of Nakamaye and Eckstein (1986). The mutated DNAs were characterized by restriction enzyme analysis and by sequencing each of the DNA inserts. The *Bam*HI–*Sal*I fragments containing the mutated *hemC* genes were then subcloned into the *Bam*HI–*Sal*I site of pUC18 to give plasmids pSWD84A, pSWD84E, and pSWD84N. These plasmids were used to transform strain TB1 to generate the recombinant bacterial strains, SWD84A, SWD84E, and SWD84N. The recombinant strains were selected by growth on LB medium containing ampicillin (50 μg/mL) and were used to express the mutant proteins.

Purification of Wild-Type and Mutant Porphobilinogen Deaminase Proteins. Wild-type and mutant strains were grown in 5-L amounts of LB medium containing ampicillin (50 μg/mL). Cells were harvested and disrupted by sonication, and the proteins in the cell-free extract were analyzed by PAGE in the presence of SDS (Laemmli, 1970). The strain TB1 transformed with pUC18, without the *hemC* insert, produces insignificant levels of porphobilinogen deaminase activity although it harbors the intact *hemC* gene of the bacterial chromosome. Wild-type porphobilinogen deaminase was purified to homogeneity from a recombinant strain of *E. coli* as previously described (Jordan *et al.*, 1988) except that 5 mM 2-mercaptoethanol was included in the buffers to prevent undue oxidation of the dipyrromethane cofactor. Mutant deaminases were purified to homogeneity using a similar method. Purifications of inactive mutants were monitored by PAGE in the presence of SDS. Porphobilinogen deaminase activity was assayed as previously described (Jordan *et al.*, 1988) by determining the rate of formation of the product preuroporphyrinogen from porphobilinogen (see also Scheme 1). This was accomplished by chemical cyclization of preuroporphyrinogen to uroporphyrinogen I in acid followed by oxidation to uroporphyrin I (ϵ = 540 000 L/mol). Enzyme activity is expressed as micromoles of uroporphyrin I formed per hour per milligram of protein (Jordan *et al.*, 1988). The K_m and V_{max} values were determined for the D84E mutant and compared to those of the wild-type enzyme.

Reaction of Wild-Type and Mutant Deaminases with Ehrlich's Reagent. The presence of the dipyrromethane cofactor in the wild-type and mutant deaminases was followed by reacting an equal volume of enzyme with modified Ehrlich's reagent (Mauzerall & Granick, 1956), and the mixture was scanned immediately over the wavelength range 350–650 nm using a Hitachi U-2000 spectrophotometer (Figure 3). The reaction was monitored at intervals for 15 min (Jordan & Warren, 1987; Warren & Jordan, 1988).

Formation of Enzyme Intermediate Complexes by Wild-Type and Mutant Porphobilinogen Deaminases. The ability of purified mutant proteins to form the intermediate complexes ES, ES_2, and ES_3 was investigated by FPLC essentially according to Warren and Jordan (1988). Porphobilinogen deaminase (30 nmol) in 50 mM Tris·HCl buffer, pH 7.5, was mixed with various molar ratios of porphobilinogen (see Figure 4), and any enzyme–intermediate complexes were characterized by chromatography using a MonoQ HR/5 FPLC column. The enzyme–intermediate complexes were eluted using a linear gradient of NaCl (0–300 mM) in a total volume of 20 mL. Protein was monitored continuously at 280 nm.

RESULTS AND DISCUSSION

General Properties of Aspartate-84 Mutants. The purified porphobilinogen deaminase mutant proteins D84A, D84E, and D84N each gave a single protein band of M_r = 35 000 ± 3000 on SDS–PAGE, similar to that of the wild-type protein, establishing that there had been no significant proteolytic digestion of the enzymes during the growth of the recombinant strains SWD84A, SWD84E, and SWD84N.

Non-denaturing PAGE revealed the double protein band profile characteristic of the *E. coli* porphobilinogen deaminase holoenzyme (Jordan *et al.*, 1988). The D84E mutant migrated similarly to the wild-type deaminase, as predicted for a species with a similar overall charge. However, D84A and D84N mutants each migrated with a slightly lower mobility consistent with the replacement of aspartate-84 with an uncharged amino acid (Figure 2). Since the mobility of apodeaminase is substantially lower than that of the wild-type enzyme on PAGE (Warren & Jordan 1988; Scott *et al.*, 1989; Jordan & Woodcock, 1991), due to the absence of the dipyrromethane cofactor (which contributes four negatively charged groups), these observations suggest that all three mutant deaminases exist in their holoenzyme form.

K_{cat} and K_m of Aspartate-84 Mutants. The k_{cat} values for the purified mutant enzymes were compared to that of the wild-type enzyme. The D84E mutant showed a specific activity of 0.3 μmol/h/mg, less than 1% of that shown by the wild-type porphobilinogen deaminase (43 μmol/h/mg). The D84A and D84N mutant enzymes were found to be catalytically inactive, and therefore no kinetic analysis was possible. The K_m of the D84E mutant for porphobilinogen was 16 ±

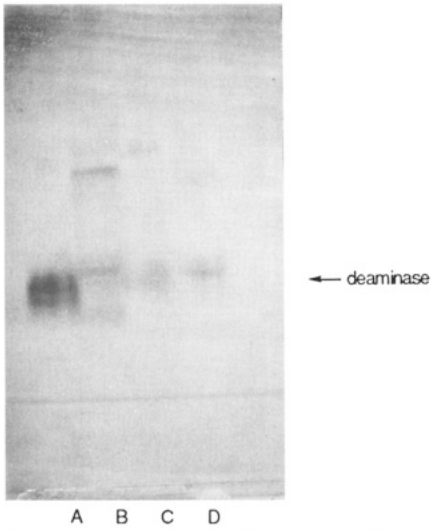


FIGURE 2: Non-denaturing PAGE of wild-type and mutant purified porphobilinogen deaminase enzymes. Purified proteins were separated on non-denaturing polyacrylamide gels and stained with Coomassie brilliant blue: track A, wild-type enzyme; track B, D84A mutant; track C, D84E mutant; track D, D84N mutant.

4 μM, similar to that of the wild–type (19 ± 7 μM). The k_{cat}/K_m values for the wild type and the D84E mutant were calculated as 26.30 and 0.06 s^{-1} M^{-1}, respectively.

Dipyrromethane Cofactor Status of Mutant Deaminases. The dipyrromethane cofactor of the wild-type porphobilinogen deaminase is slowly oxidized to a yellow chromophore in the absence of a reducing agent (Jordan *et al.* 1992), and the appearance of a colored protein is therefore diagnostic for the presence of the holoenzyme with its attached dipyrromethane cofactor. The observation that all three aspartate-84 mutant enzymes became reddish-pink in color when freeze-dried at pH 7.0 in the absence of a reducing agent provided further evidence that they all contained the dipyrromethane cofactor. The increased oxygen sensitivity of the cofactor in the mutant enzymes compared to that in the wild type is predictable since aspartate-84 forms hydrogen bonds with the two NH groups from the C1 and C2 rings of the cofactor (Louie *et al.*, 1992) and accounts, in part, for the exceptional stability of the dipyrrole system when linked to the protein. The colored, oxidized forms of the mutant enzymes, D84A and D84N, migrated on non-denaturing PAGE with a slightly faster mobility (data not shown) compared to the reduced forms, probably as a result of conformational changes induced in the protein by the transition from the dipyrromethane to the dipyrromethene structure, or a related chromophore (Jordan *et al.*, 1992).

The presence of the dipyrromethane cofactor in each mutant protein was further confirmed by reaction of the proteins with modified Ehrlich's reagent, which gives a color reaction with α-unsubstituted pyrroles. Reaction of the wild-type enzyme with this reagent (Figure 3a) gives initially a characteristic purple color (λ_{max} = 565 nm) that changes to an orange chromophore (λ_{max} = 495 nm) over a period of 10 min (Jordan & Warren, 1987). When the purified D84E mutant was reacted with Ehrlich's reagent, spectra similar to that of the wild-type holoenzyme were obtained (Figure 3b) although the reaction was slightly faster. The reaction of the D84A and D84N mutants with Ehrlich's reagent was more rapid, however, giving spectra in which the absorption maximum at 565 nm had largely disappeared after 1 min and had been replaced by the maximum at 495 nm (panels c and d, respectively, of Figure 3). The more rapid reaction of these

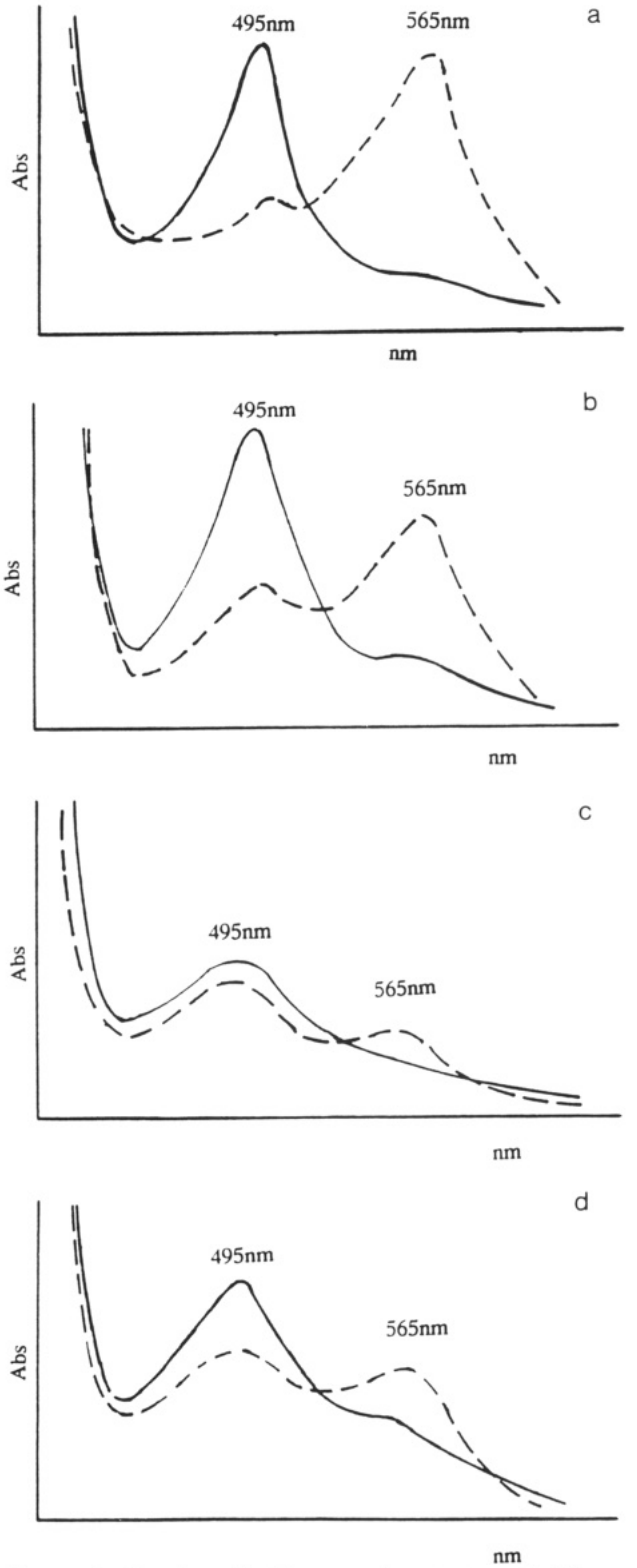


FIGURE 3: Reaction of wild-type and mutant porphobilinogen deaminases with Ehrlich's reagent. Spectra were determined after 1 min (– –) and 15 min (—): (a) wild type; (b) D84E mutant; (c) D84A mutant; (d) D84N mutant.

mutants with Ehrlich's reagent may be attributed to the altered protein environment of the cofactor. The above observations thus taken together confirm that all three mutant proteins are able to assemble the dipyrromethane cofactor.

Characterization of the Reaction of Aspartate-84 Mutants with Porphobilinogen Using FPLC Analysis. Porphobilinogen deaminase and its enzyme–intermediate complexes show characteristic elution patterns during FPLC on MonoQ

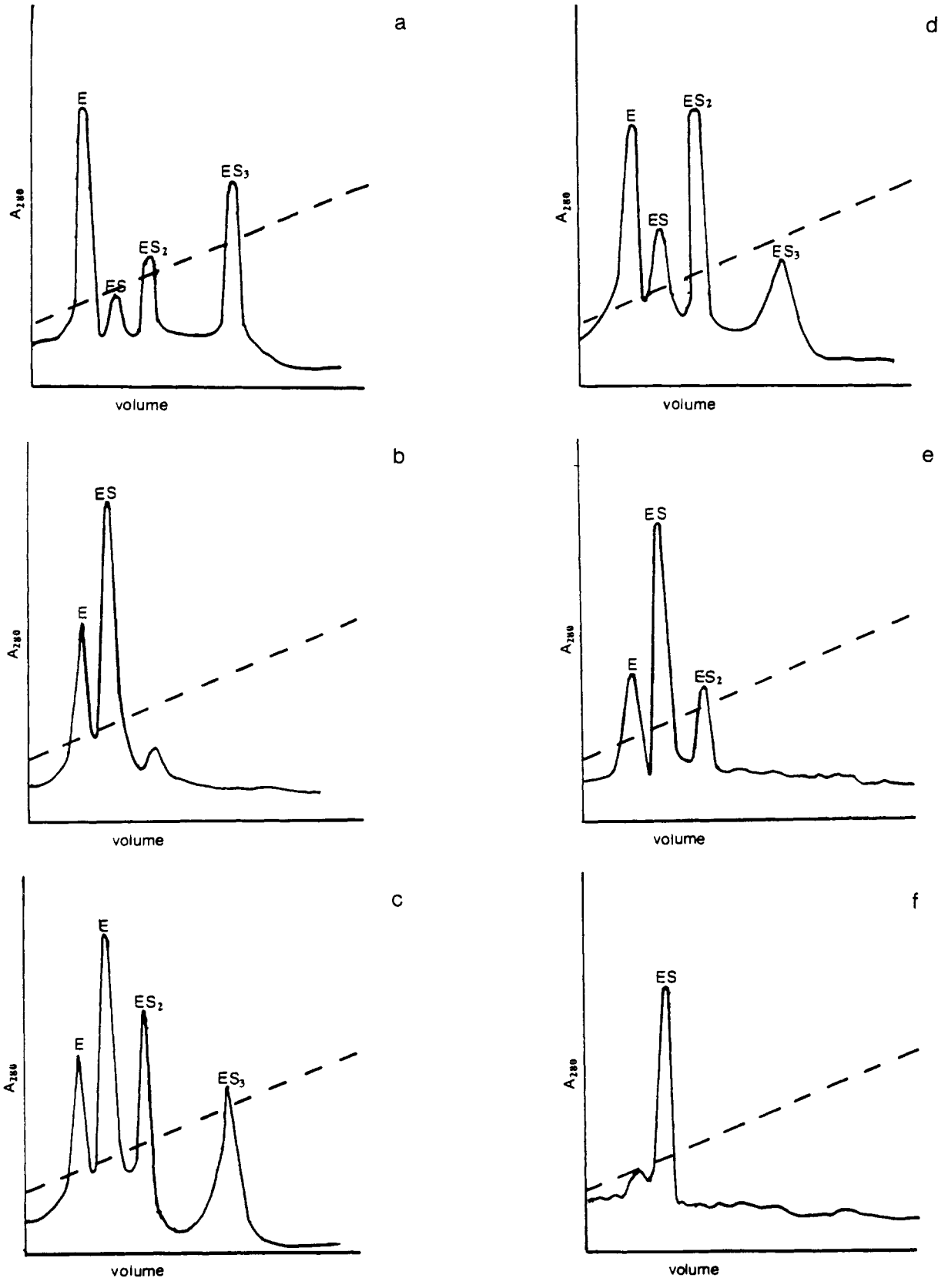


FIGURE 4: Reaction of wild-type and mutant porphobilinogen deaminases with porphobilinogen and analysis by FPLC: (a) enzyme–intermediate complexes of wild-type porphobilinogen deaminase generated by adding 5 equiv of porphobilinogen; (b) ES from sample a after heating for 15 min at 37 °C; (c) enzyme–intermediate complexes generated by adding 1 equiv of substrate to D84E mutant; (d) as in c but with 5 equiv of porphobilinogen; (e) as in c but with 20 equiv of porphobilinogen; (f) sample d after purification of ES and incubation for 15 min at 37 °C.

HR5/5 columns (Warren & Jordan, 1988). The wild-type holoenzyme elutes from the column at a salt concentration of 240 mM, whereas the enzyme–intermediate complexes, being more negatively charged due to the presence of additional substrate acetate and propionate groups, elute at higher salt concentrations (Figure 4a). FPLC of each of the three purified

mutant holodeaminases gave single peaks (data not shown) that eluted at the same salt concentration (240 mM) as the wild-type enzyme (see Figure 4a).

The D84E mutant holoenzyme was analyzed extensively by FPLC to determine the nature of its interaction with the substrate porphobilinogen. Wild-type porphobilinogen deaminase reacts with porphobilinogen in a characteristic manner to give a family of enzyme–intermediate complexes (Figure 4a) which rapidly interconvert (Figure 4b) when incubated in the absence of further substrate at 37 °C.

When purified D84E mutant holoenzyme was incubated with porphobilinogen, the formation of enzyme–intermediate complexes was markedly different. With 2 molar equiv of porphobilinogen (Figure 4c), the major peak from D84E was ES with very little ES_2 being formed (compare with Figure 4a). When the substrate:enzyme concentration ratio was increased to 5:1, ES_2 was produced, but ES remained as the major component (Figure 4d). Further raising of the porphobilinogen concentration to 10- and 20-fold molar excess (Figure 4e) was necessary to produce ES_3, although the proportion of the lower order complexes, ES and ES_2, still predominated. These observations indicate that the catalytic cycle of the enzyme, while operative, is impaired at all stages of substrate coupling, suggesting that aspartate-84 is intimately involved in the tetramerization reaction. The inactive D84A and D84N mutants revealed no change in chromatographic behavior on incubation with porphobilinogen consistent with their inability to catalyze the tetramerization reaction.

Stability of Enzyme–Intermediate Complexes of D84E Mutant Deaminase. Wild-type porphobilinogen deaminase catalyzes the reverse reaction to release the terminal pyrrole unit from the intermediate complexes as porphobilinogen in the presence of high concentrations of the product ammonia (Pluscec & Bogorad, 1970). Hydroxylamine and methoxyamine can also act as ammonia analogs in this reaction (Pluscec & Bogorad, 1970; Davies & Neuberger, 1973). The release of the terminal pyrrole unit from any of the enzyme–intermediate complexes can also occur by hydrolysis to yield the analogous hydroxy derivative of porphobilinogen (Warren & Jordan, 1988). Since hydroxyporphobilinogen is a reasonable substrate for the enzyme (Battersby *et al.*, 1979c), this can react with free enzyme, or any of the enzyme–intermediate complexes, to give finally the product preuroporphyrinogen. The active site machinery is thus able to catalyze deamination *and* amination as well as the analogous dehydration and hydration reactions with water (Warren & Jordan, 1988).

It would be expected, therefore, that if aspartate-84 participates as a catalytic residue, the reverse reaction to release substrate (or hydroxyporphobilinogen) from enzyme–intermediate complexes, as well as the forward reaction, would also be affected. Consequently, the enzyme–intermediate complexes of the D84E mutant should be far more stable than the equivalent intermediates of the wild-type enzyme. This is borne out by the fact that the mutant D84E in crude cell extracts exists as a mixture of enzyme–intermediate complexes even after extensive heat treatment, a procedure which results in the formation of the free enzyme in the case of the wild-type enzyme.

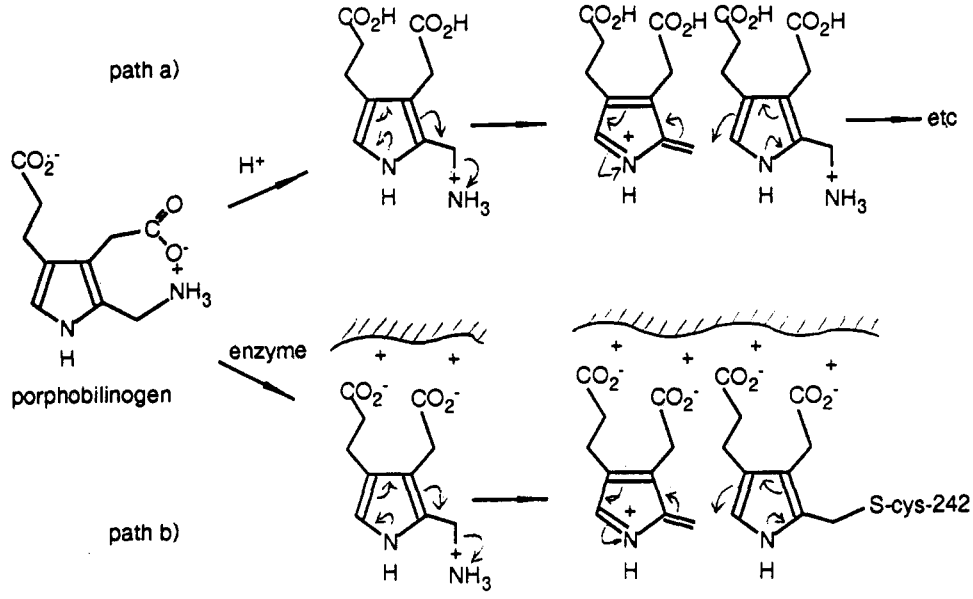
To investigate fully the properties of the D84E mutant, the ES intermediate complex of the mutant was isolated by FPLC (from the experiment in Figure 4d), as described in the Materials and Methods section, and incubated in buffer at 37 °C for 15 min. Panels b and f of Figure 4 compare the result of incubation on the wild-type and D84E mutant ES complexes, respectively. The data indicate that the ES complex of the D84E mutant was extremely stable with little indication of decomposition to E (Figure 4f). In contrast the ES complex (as with all wild-type enzyme–intermediate complexes) of the wild-type porphobilinogen deaminase was extremely labile and was freely transformed to other enzyme–intermediate complexes and free enzyme under similar conditions (Figure 4b).

Mechanistic Role of Aspartate-84 in the Polymerization Reaction. The reaction catalyzed by porphobilinogen deaminase involves the deamination of porphobilinogen at the S site to give an electrophilic azafulvene (or equivalent species), the nucleophilic attack from the unsubstituted α-position of the C2 ring of the dipyrromethane cofactor (or the terminal ring of an enzyme–intermediate complex) at the C site to form the C–C bond, and finally, loss of the α-hydrogen atom. These stages are shown in Scheme 2. In the holoenzyme, aspartate-84 hydrogen bonds with the pyrrole NH groups of the cofactor (Figure 1); however, on substrate binding it is also ideally placed to interact with the substrate NH group. Thus aspartate-84 can stabilize the positively charged nitrogen atoms both in the electrophilic azafulvene substrate and in the nucleophilic pyrrole ring tautomer by providing a strategically placed negatively charged group (Scheme 2). The bidentate nature of aspartate is particularly well suited to this role (Gandour, 1981). It is also possible that aspartate-84 could function as an acid to protonate the product ammonia and then as a base to accept the acidic proton from the newly substituted pyrrole α-position. Although the pH optimum of the enzyme (pH 8.4) is rather high for aspartate-84 to exist in a protonated form, the close proximity of the hydrophobic phenylalanine-62 may alter the p*K*a of the group sufficiently to permit this role.

The greatly reduced k_{cat} but essentially unaltered K_m exhibited by the D84E mutant is consistent with a catalytic role for aspartate-84 in the tetramerization reaction. Glutamate has the same charge as aspartate but is one C–C bond longer, which would place the side chain in an unfavorable position for optimal interaction with the two reacting pyrrole rings at the S and C sites. In triose phosphate isomerase, in which an unprotonated glutamate interacts by hydrogen bonding with the -OH groups of the *cis*-enediol, the converse site-directed mutation of glutamate-165 to aspartate leads to a dramatic reduction in the k_{cat}/K_m ratio (Knowles, 1991; Blacklow *et al.*, 1991).

Cofactor Assembly and Mechanistic Conclusions. The finding that the D84E mutant contains the dipyrromethane cofactor is not unexpected since this protein shows a low, but nevertheless significant, enzymic activity. However, the presence of a cofactor in the inactive D84A and D84N mutants can only satisfactorily be explained if the mechanism involved in the tetramerization reaction is different from that of cofactor assembly. Evidence from site-directed mutagenesis studies (Jordan & Woodcock, 1991; Lander *et al.*, 1991) and from the X-ray structure (Louie *et al.*, 1992) is in accord with this possibility since arginine-11 and -155, both of which are crucial for substrate binding at the S site, are not essential for cofactor assembly. However, site-directed mutants of either arginine-131 or -132, residues that make up the cofactor binding C site, cannot assemble the cofactor, and the proteins exist as inactive apoenzymes. Different mechanisms for cofactor assembly and the tetramerization reaction could also explain why the dipyrromethane cofactor, once assembled, remains attached permanently to the active site during turnover or

Scheme 3: Disruption of the Porphobilinogen Intermolecular Ion Pair (Evans *et al.*, 1985) by (a) Protonation and (b) Binding to Positively Charged Enzyme Groups



heat treatment, whereas covalently bound substrate can be released enzymically by reaction with nitrogenous bases or hydrolysis.

To understand how the D84A and D84N mutant apoproteins, despite the absence of the key aspartate-84 group, are able to react with two molecules of porphobilinogen, it is essential to consider the chemical properties of porphobilinogen itself. At physiological pH this pyrrole is quite stable; however, if heated in acid, it polymerizes rapidly to yield a mixture of the uroporphyrinogens in high yield (Mauzerall, 1960; Frydman *et al.*, 1971). A major contribution to the stability of porphobilinogen is thought to be the intermolecular ion pair between the negatively charged acetate and the positively charged aminomethyl side chains (Evans *et al.*, 1985). Under physiological conditions this ion pair may prevent the aminomethyl side chain from adopting an optimal stereoelectronic conformation to permit deamination where the ammonium leaving group is perpendicular to the plane of the pyrrole ring. However, protonation of the acetate and propionate groups in acid would be expected to disrupt the ion pair, freeing the aminomethyl group to facilitate deamination (Scheme 3a). Acid would also diminish the reverse reaction by reducing the concentration of unprotonated ammonia.

The possibility arises therefore that cofactor assembly occurs by the interaction of porphobilinogen, through the acetate and propionate side chains, with the positively charged enzyme groups, arginine-131 and -132 and lysine 83, at the C site (Scheme 3b). Since these key groups are intact in the D84A and D84N mutants, it is envisaged that binding of porphobilinogen in this way would free the charged aminomethyl side chain and permit deamination to the azafulvene. This unstable species would lead to C-alkylation of cysteine-242 to install the C1 ring of the cofactor. The porphobilinogen molecule destined for the C2 ring of the cofactor could interact in a similar fashion to react with the free α-position of the C1 ring to complete the cofactor assembly. Such an enzyme-facilitated reaction could explain why cofactor assembly occurs far more slowly than the tetrapolymerization reaction in the wild-type deaminase (Warren & Jordan, 1988; Scott *et al.*, 1989). It is quite likely that the normal cofactor assembly process in the wild-type deaminase is similar to that observed in the mutants and is independent of the tetramerization reaction to form preuroporphyrinogen. However, a detailed study of the cofacor assembly process is needed to resolve this matter fully.

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REFERENCES

Anderson, P. M., & Desnick, R. J. (1980) *J. Biol. Chem. 225*, 1993–1999.

Aplin, R. T., Baldwin, J. E., Pichon, C., Roessner, C. A., Scott, A. I., Schofield, C. J., Stolowich, N. J., & Warren, M. J. (1991) *Bioorg. Med. Chem. Lett. 1*, 503–507.

Battersby, A. R., Fookes, C. J. R., Gustafson-Potter, K. E., Matcham, G. W. J., & McDonald, E. (1979a) *J. Chem. Soc., Chem. Commun.*, 1155–1158.

Battersby, A. R., Fookes, C. J. R., Matcham, G. W. J., & McDonald, E. (1979b) *J. Chem. Soc., Chem. Commun.*, 539–541.

Battersby, A. R., Fookes, C. J. R., Matcham, G. W. J., McDonald, E., & Gustafson-Potter, K. E. (1979c) *J. Chem. Soc., Chem. Commun.*, 316–319.

Beaumont, C., Porcher, C., Picat, C., Nordmann, Y., & Grandchamp, B. (1989) *J. Biol. Chem. 264*, 14829–14834.

Berry, A., Jordan, P. M., & Seehra, J. S. (1981) *FEBS Lett. 129*, 220–224.

Blacklow, S. C., Lui, K. D., & Knowles, J. R. (1991) *Biochemistry 30*, 8470–8476.

Burton, G., Fagerness, P. E., Hosozawa, S., Jordan, P. M., & Scott, A. I. (1979) *J. Chem. Soc., Chem. Commun.*, 202–204.

Davies, R. C., & Neuberger, A. (1973) *Biochem. J. 133*, 471–492.

Evans, J. N. S., Fagerness, P. E., Mackenzie, N. E., & Scott, A. I. (1985) *Magn. Reson. Chem. 23*, 939–944.

Frydman, R. B., Reil, S., & Frydman, B. (1971) *Biochemistry 10*, 1154–1160.

Gandour, R. D. (1981) *Bioorg. Chem. 10*, 169–176.

Hart, G. J., Miller, A. D., Leeper, F. J., & Battersby, A. R. (1987) *J. Chem. Soc., Chem. Commun.*, 1762–1765.

Hart, G. J., Miller, A. D., Beifuss, U., Leeper, F. J., & Battersby, A. R. (1989) *J. Chem. Soc., Perkin Trans I*, 1979–1993.
Jordan, P. M. (1991) *in New Comprehensive Biochemistry 19* (Jordan, P. M., Ed.; Neuberger and van Deenen, Series Eds.) pp 1–66, Elsevier, Amsterdam.
Jordan, P. M., & Seehra, J. S. (1979) *FEBS Lett. 104*, 364–366.
Jordan, P. M., & Berry, A. (1980) *FEBS Lett. 112*, 86–88.
Jordan, P. M., & Seehra, J. S. (1986) *Methods Enzymol. 123*, 427–434.
Jordan, P. M., & Warren, M. J. (1987) *FEBS Lett. 225*, 87–92.
Jordan, P. M., & Woodcock, S. C. (1991) *Biochem. J. 280*, 445–449.
Jordan, P. M., Burton, G., Nordlov, H., Schneider, M. M., Pryde, L. M., & Scott, A. L. (1979) *J. Chem. Soc., Chem. Commun.*, 204–205.
Jordan, P. M., Thomas, S. D., & Warren, M. J. (1988) *Biochem. J. 254*, 427–435.
Jordan, P. M., Warren, M. J., Mgbeje, B. I. A., Wood, S. P., Cooper, J. B., Louie, G. V., Brownlie, P., Lambert, R., & Blundell, T. L. (1992) *J. Mol. Biol. 224*, 269–271.
Keng, T., Richard, C., & Larocque, R. (1992) *MGG, Mol. Gen. Genet. 234*, 233–243.
Knowles, J. R. (1991) *Philos. Trans. R. Soc. London, B 332*, 115–121.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lander, M., Pitt, A. R., Alefounder, P. R., Bardy, D., Abell, C., & Battersby, A. R. (1991) *Biochem. J. 275*, 447–452.
Louie, G. V., Brownlie, P. D., Lambert, R., Cooper, J. B., Blundell, T. L., Wood, S. P., Warren, M. J., Woodcock, S. C., & Jordan, P. M. (1992) *Nature (London) 359*, 33–39.
Mauzerall, D. (1960) *J. Am. Chem. Soc. 82*, 2601–2605, 2605–2609.
Mauzerall, D., & Granick, S. (1956) *J. Biol. Chem. 205*, 435–442.
Nakamaye, K. L., & Eckstein, F. (1986) *Nucleic Acids Res. 14*, 9679–9698.
Petricek, M., Rutberg, L., Schroder, I., & Hederstedt, L. (1990) *J. Bacteriol. 172*, 2250–2258.
Pluscec, J., & Bogorad, L. (1970) *Biochemistry 9*, 4736–4742.
Raich, N., Romeo, P.-H., Dubart, A., Beaupain, D., Cohen-Solal, M., & Goossens, M. (1986) *Nucleic Acids Res. 14*, 5955–5968.
Scott, A. I., Clemens, K. R., Stolowich, N. J., Santander, P. J., Gonzalez, M. D., & Roessner, C. A. (1989) *FEBS Lett. 242*, 319–324.
Sharif, A. L., Smith, A. G., & Abell, C. (1989) *Eur. J. Biochem. 184*, 353–359.
Stubnicer, A. C., Picat, C., & Grandchamp, B. (1988) *Nucleic Acids Res. 16*, 3102.
Thomas, S. D., & Jordan, P. M. (1986) *Nucleic Acids Res. 14*, 6215–6226.
Warren, M. J., & Jordan, P. M. (1988) *Biochemistry 27*, 9020–9030.