Contribution of the DDDD motif of *H. influenzae e* (P4) to phosphomonoesterase activity and heme transport

Thomas J. Reilly[a], Bruce A. Green[b], Gary W. Zlotnick[b], Arnold L. Smith[a,*]

[a] *Department of Molecular Microbiology and Immunology, University of Missouri Medical School, DC044.00, M616 Medical Sciences Bldg., Columbia, MO 65212, USA*
[b] *Wyeth-Lederle Vaccines, 211 Bailey Road West, Henrietta, NY 14586-9728, USA*

Received 3 January 2001; accepted 27 February 2001

First published online 19 March 2001

Edited by Gianni Cesareni

Abstract ***Haemophilus influenzae*** **lipoprotein *e* (P4) is a member of the DDDD phosphohydrolase superfamily and mediates heme transport. Each of the aspartate residues of the signature motif is required for phosphomonoesterase activity, as none of the *e* (P4) single D mutants (D64A, D66A, D181N, and D185A) possessed detectable phosphomonoesterase activity. These results suggest that the signature motif is essential to the phosphomonoesterase activity of lipoprotein *e* (P4). When assessed for phosphomonoesterase-dependent heme transport activity in *Escherichia coli hemA* strains, plasmids containing D181N and D185A retained heme transport as indicated by aerobic growth while D64A and D66A did not. We conclude that phosphomonoesterase activity is not required for heme transport. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Lipoprotein *e* (P4); DDDD phosphomonoesterase; Heme transport; *Haemophilus influenzae*

1. Introduction

Haemophilus influenzae is a Gram-negative coccobacillus, a common commensal and occasional pathogen of the human respiratory tract; it requires both heme and nicotinamide adenine disnucleotide (NAD) for aerobic growth [1]. Macromolecules in the outer membrane of this organism not only directly mediate interactions at the host–pathogen interface, but are also essential for acquisition and transport of requisite nutrients and cofactors including heme and NAD. The outer membrane of *H. influenzae* possesses up to 36 proteins [2] of which six, designated P1–P6, represent major protein constituents [3]. While interest in these major proteins has been for potential vaccine components, their biological role is only beginning to be determined.

H. influenzae lipoprotein *e* (P4) is one outer membrane protein to which important physiological roles have been assigned. This surface-localized 28-kDa lipoprotein is found in all strains of *H. influenzae*, is encoded by the *hel* gene and is antigenically conserved within and between strains [4]. In separate studies, *e* (P4) was found to mediate the transport of both heme [5] and nicotinamide nucleotides [6] into this heme and NAD-requiring organism. Lipoprotein *e* (P4) was initially purified as a potential component of a non-typeable *H. influenzae* vaccine [4]. Our laboratory purified, characterized the protein as an acid phosphatase [7] and produced a soluble recombinant form of the enzyme [8].

Bacterial non-specific acid phosphatases (NSAPs) are found as soluble enzymes associated with the periplasmic space or as constituents of the bacterial membrane. They generally have broad substrate specificity and have optimum activity at acidic to neutral pH. Results from biochemical characterization of the enzymes and amino acid relatedness suggest that NSAPs constitute three distinct classes of enzymes designated A, B, or C [9]. Results from amino acid sequence alignment [10], its identification as a bacterial lipoprotein [4], and enzymatic characterization [7] suggest that *H. influenzae* lipoprotein *e* (P4) belongs to NSAP class C [9]. These enzymes, along with those of class B and related plant phosphatases belong to an enzyme superfamily designated DDDD phosphohydrolases, so named for the presence of two pairs of requisite aspartic acid residues present in separate domains of the proteins and separated by a variable spacer region [9].

While sequence alignments suggest these aspartic acid residues are essential for enzymatic activity of the bacterial nonspecific acid phosphatases in this superfamily, direct demonstration of their importance has not been reported. The purpose of the present study was two-fold: (1) to determine whether each of the aspartic acid residues of the DDDD signature motif is required for enzymatic activity of *e* (P4) and (2) to determine if phosphomonoesterase activity of *H. influenzae* lipoprotein *e* (P4) was necessary for one of the protein's physiological roles, heme transport. Site-directed mutants were constructed at each of the four aspartic acid residues, sequenced and assessed for enzymatic activity as well as presence of the *e* (P4) antigen. An aspartate presumed to be irrelevant, D49 was also changed to an alanine to control for the loss of the carboxylate on the overall p*I* of the enzyme. Plasmids containing wild-type or mutant *hel* genes were transformed into *hemA* strains of *Escherichia coli* and assessed for their ability to mediate heme transport indicated by aerobic growth.

2. Materials and methods

2.1. Bacteria and growth conditions

E. coli strains RK1065 (*F, leu, pro, hemA*) and SASX77 (*lacY1,*

*Corresponding author. Fax: (1)-573-882-4287.
E-mail: smithal@health.missouri.edu

Abbreviations: ALA, aminolevulinic acid; *e* (P4), *H. influenzae* lipoprotein *e* (P4); LB, Luria Bertani; NSAP, non-specific acid phosphatase; pNPP, *p*-nitrophenylphosphate

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PII: S0014-5793(01)02294-3

glnV44, hem48, trpA43, rpsL134(strR), *malT1*(λ^R), *metB1*) were obtained from Dr. Robert Kadner (University of Virginia) and the *E. coli* Genetic Stock Center respectively and grown in the presence of aminolevulinic acid unless otherwise indicated. *E. coli* strain DH5α used for plasmid manipulation and amplification was obtained from BRL. *E. coli* transformants were grown at 37°C on LB agar or in broth containing the appropriate antibiotic (chloramphenicol 34 μg/ml or ampicillin 100 μg/ml) prior to assessment of phosphomonoesterase activity, or identification of wild-type or mutant lipoprotein *e* (P4) antigen by Western blot analysis. *E. coli* transformants containing wild-type or mutant forms of the *hel* gene in pBluescript were also screened on tryptose phosphate (Difco Laboratories) methyl green indicator media containing phenolphthalein diphosphate for assessment of phosphatase activity according to the method of Riccio et al. [11].

2.2. Determination of specific phosphomonoesterase activity

Specific phosphomonoesterase activity of transformed *E. coli* strains was measured using a discontinuous colorimetric assay in microtiter wells as described previously [7]. The 0.2-ml standard assay mixture contained 0.2 M sodium acetate, pH 5.5, 0.1 mM $CuSO_4$, 1.0 mM *p*-nitrophenylphosphate (pNPP), and varying amounts of sample. The mixtures were incubated at 37°C for 15 min with constant agitation. The reaction was stopped by addition of 100 μl 0.5 M glycine, pH 10.0. Concentration of *p*-nitrophenol produced was measured with a Dynatech MR5000 microtiter plate reader at 410 nm using an extinction coefficient of 18.3 mM^{-1} cm^{-1}. Enzyme preparations were diluted in 50 mM sodium acetate pH 6.0 containing 200 mM NaCl to a concentration that resulted in a linear response with increasing amounts of sample. One unit of enzyme activity was defined as the amount of activity required to convert 1 nmol substrate to product per hour at 37°C. Protein concentrations were determined using bicinchoninic acid (BCA protein assay reagent, Pierce) as described previously [12]. Bovine serum albumin was used as the standard.

2.3. Construction of lipoprotein e (P4) phosphomonoesterase mutants

The wild-type *hel* gene was cloned into the *Bam*HI site of pBluescript and designated phel3 [7], and was used as the template for PCR-mediated mutagenesis. Site-directed mutations were generated using the QuickChange site-directed mutagenesis kit according to the manufacturer's (Stratagene) instructions. The following oligonucleotide PCR primers and their reverse complement (not shown) were synthesized at the University of Missouri DNA Core. Degenerate nucleotides are indicated in bold.

F48C-GCAAAAGTTGCAT**GC**GATCACGCAAAAG
D64A-GCGGTTGTGGCTG**C**TTTAGATGAAACTATGTTAG
D66A-GCGGTTGTGGCTGATTTAG**C**TGAAACTATGTTAG
D181N-CGTACTTTATGTAGGT**A**ATAACTTAGATGACTTCG
D185A-GGTGATAACTTAGATG**C**CTTCGGTAATACCG

The mutagenesis primers were coincubated with plasmid phel3 through 16 cycles of PCR amplification. One cycle consisted of the following three steps: (i) 30 s at 95°C, (ii) 1 min at 55°C, and (iii) 9 min at 68°C. PCR-generated replicons were selected for transformation by 60 min incubation with the restriction enzyme *Dpn*I. Plasmids containing mutated *hel* genes were transformed into *E. coli* DH5α by the heat-shock method. Transformants obtained after overnight incubation on LB agar containing ampicillin (100 μg/ml) were screened for the presence of phosphomonoesterase activity by the phenolphthalein diphosphate methyl green assay. Plasmids were isolated from those clones devoid of activity using Promega Wizard Plus miniprep kit. The presence of the desired mutation was confirmed by DNA sequencing. Wild-type and mutant *hel* genes were then cloned into the *Eco*RV/*Xba*I site of pACYC184, transformed into *E. coli* DH5α, and grown on LB agar containing chloramphenicol (34 μg/ml). Plasmid DNA was isolated from individual clones as previously described, transformed into *E. coli* strains RK1065 and SASX77B and grown on LB agar containing chloramphenicol (34 μg/ml) and aminolevulinic acid (25 μg/ml). Heme transport activity by wild-type and mutant lipoprotein *e* (P4) in *E. coli hemA* strains was assessed by aerobic incubation at 37°C on LB agar containing chloramphenicol 34 μg/ml and heme (equine hemin HCl, Sigma) 15 μg/ml.

2.4. Polyacrylamide gel electrophoresis and detection of lipoprotein e (P4) by Western blot

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Laemmli [13]. Polyacrylamide gels were 3% T stacking and 11% T resolving. Western blot detection of *e* (P4) was performed as described previously [14]. Purified mouse anti-*H. influenzae e* (P4) monoclonal antibody EPR5-2.1 was used as the primary antibody (1:10 000 dilution) and rabbit anti-mouse IgG (heavy and light chain; Sigma) conjugated to alkaline phosphatase was used as the secondary antibody (1:10 000 dilution).

3. Results

To determine if the conserved aspartic acid residues of the DDDD phosphomonoesterase motif, D64, D66, D181, and D185 in lipoprotein *e* (P4) are necessary for phosphatase activity and for heme transport activity, mutations in the *hel* gene were generated at each of these positions. Site-directed mutagenesis of the *hel* gene resulted in the following substitutions: D64A, D66A, D181N, and D185A. Overnight cultures of *E. coli* strain DH5α transformed with plasmids bearing mutated *hel* genes possessed specific phosphatase activity equal to that seen with the control transformant containing pBluescript. When incubated overnight at 37°C on tryptose phosphate agar containing phenolphthalein diphosphate (1 mg/ml), methyl green (50 μg/ml), and ampicillin (100 μg/ml) only transformants harboring the wt *hel* gene produced green-stained colonies indicative of an active phosphomonoesterase (Fig. 1). When analyzed for specific phosphomonoesterase activity using the discontinuous colorimetric assay, *E. coli* transformed with *hel* gene mutants possessed enzyme activity at approximately 10% of the level of activity as an *E. coli* strain transformed with a plasmid bearing wt *hel* (Fig. 2). Reduction in enzymatic activity was not attributed to seques-



Fig. 1. Detection of phosphomonoesterase in *E. coli* DH5α transformed with pBluescript vector control, phel3 wt, or phel3 mutants plated on tryptose phosphate methyl green phenolphthalein diphosphate indicator media. Phosphatase-producing clones grow as green-stained colonies whereas phosphatase-negative clones grow as unstained colonies. The mutants are F48C, D48A, D66A, D181N and D185A; wt = wild-type and vector is *E. coli* DH5α with pBluescript.

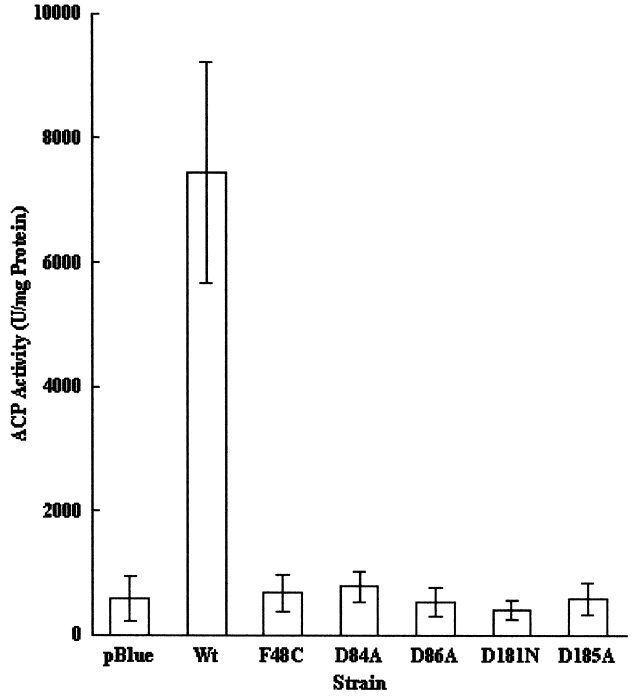


Fig. 2. Phosphomonoesterase specific activity of wild-type and mutant *e* (P4) constructs as measured by the discontinuous enzyme assay described in Section 2. Average activity (open bars) of five independent samples for each clone is shown with standard deviation (error bars) indicated for each.

tration of the enzyme as assay of French press lysates of these strains resulted in no significant increase in phosphatase activity (data not shown). Western blot analysis (Fig. 3) indicated that *E. coli* containing the wt (lane 3) and each of the mutants (lanes 4–8) produced similar levels of an ~28-kDa antigen identified as lipoprotein *e* (P4) with anti-*H. influenzae* *e* (P4) monoclonal antibody EPR5-2.1. Sequence analysis of the mutated *hel* genes confirmed the presence of the predicted mutations. Purified mutant proteins were devoid of detectable levels of phosphomonoesterase activity in both the discontinuous colorimetric assay using pNPP as substrate and when assessed by zymogram assay conducted on purified mutant

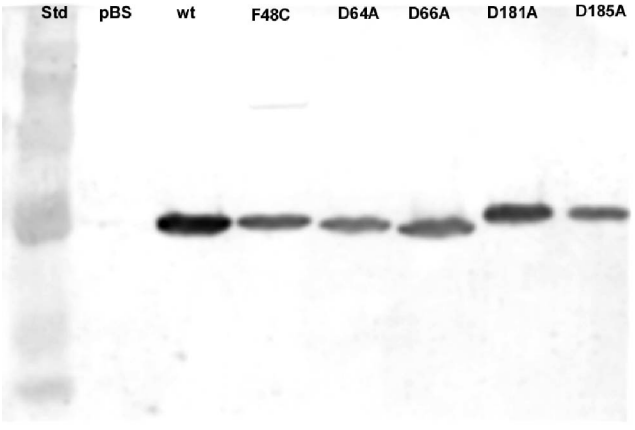


Fig. 3. Western blot detection of lipoprotein *e* (P4) antigen with mouse monoclonal antibody EPR5-2.1. Amersham prestained standards (lane 1), 25 μg of *E. coli* transformants (as indicated) was loaded in lanes 2–8.

enzymes as described by Rossolini et al. [15] (and unpublished results). These data indicate that each of the conserved aspartic acid residues of the DDDD signature motif of lipoprotein *e* (P4) is required for phosphomonoesterase activity. Not all aspartic acid residues present in *e* (P4) were required for phosphomonoesterase activity as *E. coli* transformants containing a D49A *hel* gene have enzyme activity on the indicator plates (Fig. 1).

To determine if phosphomonoesterase activity was required for lipoprotein *e* (P4)-mediated transport of exogenous heme, wild-type and mutant *hel* genes were cloned into pACYC184 and transformed into *E. coli hemA* strains RK1065 and SASX77B. Both strains are defective in aminolevulinic acid synthetase, auxotrophic for ALA, and used as reporter strains for gene products involved in heme transport which is essential for aerobic growth.

After overnight growth in LB containing chloramphenicol and aminolevulinic acid, all transformed strains were found to produce *e* (P4) by Western blot (data not shown). None of the transformed strains of RK1065 nor SASX77B containing wt or a mutant *hel* gene grew aerobically on LB agar without ALA; all transformants grew aerobically in its presence (Table 1). When tested for their ability to transport exogenous heme, two of the four plasmids carrying *e* (P4) D mutants in *hemA* strains grew aerobically, indicating that they mediated transport of heme (Table 1): specifically *e* (P4) with substitutions D181N and D185A, were able to transport heme. Thus, phosphomonoesterase activity is not required for heme transport as these mutants were found to lack detectable phosphatase activity in both crude and purified preparations. In contrast, neither the D64A nor D66A mutants mediated heme transport as indicated by lack of aerobic growth of the *hemA* strains harboring plasmids with these mutations. The lack of aerobic growth of the D64 and D66 mutants may reflect proximity effects on the putative heme-binding site (K45–H50) of lipoprotein *e* (P4).

In conjunction with the above studies, a lipoprotein *e* (P4) predicted to be defective in transport of exogenous heme was

Table 1
Assessment of lipoprotein *e* (P4) heme transport activity in *E. coli* *hemA* strains

Plasmid	LB chlor	LB chlor/ALA	LB chlor/heme
Strain RK1065			
pACYC184	−	+	−
phel22 wt	−	+	+
phel22 F48C	−	+	−
phel22 D64A	−	+	−
phel22 D66A	−	+	−
phel22 D181N	−	+	+
phel22 D185A	−	+	+
Strain SASX77			
pACYC184	−	+	−
phel22 wt	−	+	+
phel22 F48C	−	+	−
phel22 D64A	−	+	−
phel22 D66A	−	+	−
phel22 D181N	−	+	+
phel22 D185A	−	+	+

Vector control, wt, or mutant hel genes present on pACYC184 were transformed into *hemA* strains of *E. coli* and plated on LB agar containing the above constituents and placed at 37°C for 24 h. Presence of at least one single well-defined colony is indicated by (+); no growth is indicated by (−).

made by creating a mutation in the conserved phenylalanine residue (F48) of the putative heme-binding site KVAFDH [5]. Incorporated nucleotide changes were predicted to result in the concomitant creation of a unique *Sph*I site in phel3 and substitution of F48 with a cysteine residue in *e* (P4) producing KVACDH. All transformants analyzed after mutagenesis contained the *Sph*I site and were found to possess the predicted mutation by DNA sequencing. The F48C transformant was positive for the 28-kDa antigen as assessed by Western blot (Fig. 3, lane 4) using monoclonal antibody EPR5-2.1. Interestingly this mutation not only resulted in a mutant incapable of heme transport in *E. coli hemA* strains (Table 1) but also resulted in production of lipoprotein *e* (P4) devoid of phosphomonoesterase activity (Figs. 1 and 2). Mature lipoprotein *e* (P4) is devoid of free sulfhydryl groups as its only cysteine residue is covalently linked to a diacylglyceryl moiety attached to the N-terminal cysteine of the lipoprotein [4,16]. Replacement of phenylalanine 48 with a cysteine may therefore have resulted in formation of *e* (P4) dimers through the formation of disulfide bonds between mutant proteins preventing substrate access to the active site. However, the lack of enzymatic activity could not be attributed to formation of such dimers as no significant increase in activity was observed in the presence of 1 mM mercaptoethanol, a concentration which had no deleterious effect on the phosphomonoesterase of crude or purified *e* (P4) [7]. These data suggest that while F48 is an essential component of the heme-binding motif as previously suggested [5], it is also important for phosphomonoesterase activity of *H. influenzae* lipoprotein *e* (P4).

4. Discussion

The goals of this study were to determine the following: (1) are the four aspartic acid residues of the DDDD phosphohydrolase signature motif essential for phosphomonoesterase activity of lipoprotein *e* (P4), and (2) is enzymatic activity necessary for *e* (P4)-mediated heme transport into *E. coli* strains defective in heme biosynthesis.

Identification as a bacterial lipoprotein [4], presence of the DDDD signature motif [10], and biochemical characterization of lipoprotein *e* (P4) are all consistent with this enzyme's inclusion in this phosphohydrolase superfamily. Results from this study indicate that the DDDD motif is essential to the phosphomonoesterase activity of lipoprotein *e* (P4) and suggest that these residues may be indispensable to the enzymatic activity of all members of the superfamily. These results are consistent with those obtained with two other enzymes of the superfamily [17,18]. Collet et al. have demonstrated that the first aspartic acid residue of the signature motif present in other enzymes in the family, a phosphomannomutase [17] and a phosphoserine phosphatase [18] is essential to catalysis through formation of a covalent acyl-phosphate intermediate. The fourth aspartic acid residue of the motif binds magnesium, a requisite divalent cation [18]. Since lipoprotein *e* (P4) does not catalyze the hydrolysis of phosphoserine and requires divalent copper, assessment of the contribution of the signature motif of the human enzyme to phosphomonoesterase activity of *e* (P4) is unlikely to yield a lipoprotein defective in enzymatic activity. While the first and fourth aspartic acid residues were found to be essential to catalytic activity in *e* (P4) and are now thought to participate in catalysis as characterized by Collet et al., the functions of the requisite second and third aspartic acid residues of the DDDD motif in lipoprotein *e* (P4) have yet to be elucidated.

While lipoprotein *e* (P4)'s role in heme transport has been reported [5], the mechanism by which this substrate is translocated across the outer membrane has yet to be defined. A mechanism of phosphomonoesterase-dependent heme transport is not supported by these data as two of the four *e* (P4) D mutants (D181N and D185A) mediated the transport of heme as indicated by aerobic growth of *E. coli hemA* strains. While presence of residual phosphomonoesterase activity in either crude or purified preparations of the enzyme is below the level our detection methods cannot be discounted, the fact that all members of the DDDD superfamily possess this signature motif suggests that each of the residues is essential for enzymatic activity. As expected, mutation of the conserved phenylalanine residue, F48, of the heme-binding site resulted in a mutant protein incapable of heme transport. Unexpectedly this mutation also abrogated phosphomonoesterase activity. Gapped sequence alignment of *e* (P4) with *S. equisimilis* LppC, *Streptococcus pyogenes* LppA, *Flavobacterium meningoseptium* OlpA, *Helicobacter pylori* HP 1285, and *E. coli* AphA suggests that the conserved residue at position 48 of these phosphatases is leucine [19]. Preliminary results suggest that mutation of the F48C mutant to C48L with concomitant loss of the *Sph*I site does not restore *e* (P4) phosphomonoesterase activity. Since *e* (P4) is the only member of the group shown to possess a heme-binding site and to transport heme it may be that presence of this phenylalanine residue represents an evolutionary compromise for phosphomonoesterase activity and heme transport.

Initial observations of *e* (P4)-mediated heme transport in *H. influenzae* have been recently discounted [6]. New experimental evidence suggests that observed lack of *H. influenzae e* (P4) mutants growth under aerobic conditions was a deficiency in NAD uptake and not in heme transport. While relevant with respect to *H. influenzae* growth in vitro, these mutants were not point mutations, but produced through random insertion of transposons leading to disruption of the *hel* gene [5]. In addition the NAD concentration required for aerobic growth of the mutant is 100-fold greater than the minimum required for aerobic growth of the parent. Irregardless, these observations fail to explain specific effect of lipoprotein *e* (P4) on *hemA* strains of *E. coli* when grown in the presence of exogenous heme and *E. coli hemA* strains have no requirement for NAD. The ability of lipoprotein *e* (P4) to transport exogenous heme into *E. coli hemA* strains appears specific as mutation in the heme-binding motif resulted in lack of growth under aerobic conditions. Incorporation of these D mutants into *H. influenzae* and analysis of their effects on in vitro and in vivo growth are currently in progress.

Acknowledgements: We thank Dr. Robert Kadner for his kind gift of the *E. coli hemA* strain RK1065, the University of Missouri DNA core for synthesis of all mutagenesis oligonucleotide primers, and Dr. Richard Finkelstein for use of the French press. This study was supported by T32 AI07276 (T.J.R.) and AI44002 (A.L.S.).

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