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Biochemical characterization of protoporphyrinogen dehydrogenase and protoporphyrin ferrochelatase of *Vibrio vulnificus* and the critical complex formation between these enzymes

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ABSTRACT

Background: Protoporphyrin IX (PPn), an intermediate in the heme biosynthesis reaction, generates singlet oxygen upon exposure to UV light. It has been proposed that PPn is channeled directly to ferrochelatase within a protoporphyrinogen dehydrogenase (PgdH1)-protoporphyrin ferrochelatase (PpfC) complex as a way to avoid this damaging side reaction. However, the PgdH1-PpfC complex has not been characterized, and the question of how heme affects the activities of PgdH1 has not been addressed.

Methods: Protein interactions were explored through pull-down assays and western blotting, and the importance of this complex *in vivo* was examined using inter-species combinations of the two proteins. The purified PgdH1-PpfC complex was characterized kinetically and used for heme binding studies.

Results: In *Vibrio vulnificus*, PgdH1 and PpfC formed an 8:8 heterohexadecameric complex that was important for maintaining PPn at low levels. PpfC catalyzed PPn efficiently whether or not it was part of the complex. Notably, heme was a noncompetitive inhibitor of *V. vulnificus* PgdH1, but a competitive inhibitor of the human protoporphyrinogen oxidase PgoX.

Conclusion: The PdgH1-PpfC complex is important for protective channeling of PPn and for efficient catalysis of free PPn. The production of PPn by PgdH1 is regulated by feedback inhibition by heme.

General significance: Both proteobacteria and eukaryotes have evolved mechanisms to prevent the harmful accumulation of the heme biosynthesis intermediate PPn. The data presented here suggest two previously unknown mechanisms: the channeling of PPn through the PgdH1-PpfC complex, and the direct inhibition of PgdH1 activity (PgoX activity as well) by heme.

1. Introduction

Heme is a cyclic tetrapyrrole containing ferrous iron. As a ubiquitous prosthetic group, heme is found in organisms throughout the biosphere [1]. It mediates a variety of biochemical and regulatory processes [2]. Eukaryotes and gram-negative bacteria typically synthesize heme through a protoporphyrin-dependent pathway ("the canonical pathway") [1], whereas gram-positive bacteria and sulfate-reducing bacteria (including archaea) employ two different coproheme-dependent alternative pathways. Coproheme of gram-positive bacteria is derived from coproporphyrinogen III (CPgen) [3,4], whereas that of sulfate-reducing bacteria is synthesized from uroporphyrinogen [5]. Although the canonical and non-canonical pathways differ, they have the enzymatic steps during which 5-aminolevulinic acid (ALA) is converted to uroporphyrinogen in common.

Protoporphyrinogen dehydrogenases (PgdH1 and PgdH2, formerly known as HemG and HemJ, respectively) and protoporphyrinogen oxidase (PgoX, formerly known as HemY) catalyze the penultimate step of the protoporphyrin-dependent heme biosynthesis pathway, oxidizing protoporphyrinogen IX (PPgen) to protoporphyrin IX (PPn) [1]. Three nonhomologous isofunctional PPgen-oxidizing enzymes have been identified through phylogenic analysis, and these include Y-type, G-type, and J-type [6]. Y-type PgoX, which is oxygen-dependent and contains FAD, is widespread in both eukaryotes and aerobic bacteria

Abbreviations: ALA, 5-Aminolevulnic acid; CPgen, Coproporphyrinogen III; PPgen, Protoporphyrinogen IX; PPn, Protoporphyrin IX; CgdC, Coproporphyrinogen decarboxylase; CgdH, Coproporphyrinogen dehydrogenase; PgoX, Protoporphyrinogen oxidase; PgdH1, Protoporphyrinogen dehydrogenase (G-type); PgdH2, Protoporphyrinogen dehydrogenase (J-type); PpfC, Protoporphyrin ferrochelatase; CgoX, Coproporphyrinogen oxidase; CpfC, Coproporphyrin ferrochelatase; SD, Standard deviation.

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Table 1
Bacterial strains and plasmids.

Strains and plasmids	Relevant characteristics	Source or Reference
V. vulnificus		
MO6–24/O	Clinical isolate, Type strain, Rf^r	[63]
ATCC 29307	Clinical isolate, Type strain	[64]
AR	ATCC29307, Rf^r	[65]
HN1	AR, deletion in *cgdH* (formerly known as *hemN*)	[25]
HF1	AR, deletion in *cgdC* (formerly known as *hemF*)	[25]
PD1	MO6–24/O, deletion in *pgdH1*	This study
PDF1	MO6–24/O, deletion in *pgdH1* and *ppfC*	This study
W-415	MO6–24/O + pRK415	This study
W-$D_{(V)}$	MO6–24/O + pRK-$D_{(V)}$	This study
W-$F_{(V)}$	MO6–24/O + pRK-$F_{(V)}$	This study
D-$D_{(V)}$	PD1 + pRK-$D_{(V)}$	This study
D-415	PD1 + pRK415	This study
W-$D_{(V)}F_{(V)}$	MO6–24/O + pRK-$D_{(V)}F_{(V)}$	This study
DF-$D_{(V)}F_{(V)}$	PDF1 + pRK-$D_{(V)}F_{(V)}$	This study
DF-$D_{(V)}F_{(E)}$	PDF1 + pRK-$D_{(V)}F_{(E)}$	This study
DF-$D_{(E)}F_{(V)}$	PDF1 + pRK-$D_{(E)}F_{(V)}$	This study
DF-$D_{(E)}F_{(E)}$	PDF1 + pRK-$D_{(E)}F_{(E)}$	This study
DF-415	PDF1 + pRK415	This study
N-$D_{(V)}F_{(V)}$	HN1 + pRK-$D_{(V)}F_{(V)}$	This study
F-$D_{(V)}F_{(V)}$	HF1 + pRK-$D_{(V)}F_{(V)}$	This study
E. coli		
DH5α *phe*	*supE*44 Δ*lacU*169 (Φ80 *lacZ* ΔM15) *hsdR*17 *recA1 endA1 gyrA96 thi*-1 *relA*1 *phe*::Tn*10d*Cm	[66]
S17–1	C600::RP-4 2-(Tc::Mu)(Km::Tn7) *thi pro hsdR hsdM*$^+$*recA*	[30]
S17–1 λ*pir*	S17–1, λ*pir* lysogen	[30]
BL21 (DE3)	*E. coli* B F^- *dcm ompT hsdS*(r_B^- m_B^-) *gal* λ(DE3)	Stratagene
BL21 (DE3) C43	*E. coli* B F^- *dcm ompT hsdS*(r_B^- m_B^-) *gal* λ(DE3)	[32]
Plasmids		
pDM4	Cm^r; *ori* R6K Mob RP4	[27]
pRK415	Tc^r; *ori* IncP Mob RP4 *lacZ*α	[28]
pRSET-A	Ap^r; Expression vector for N-terminally his_6-tagged protein	Invitrogen
pET29a	Km^r; Expression vector for C-terminally his_6-tagged protein	Novagen
pDM-PD	Cm^r; pDM4 + 885-bp *ApaI*/*Sac*I fragment containing the internally deleted *V. vulnificus pgdH1*	This study
pDM-PF	Cm^r; pDM4 + 1078-bp *ApaI*/*Sac*I fragment containing the internally deleted *V. vulnificus ppfC*	This study
pRK-$D_{(V)}$	Tc^r; pRK415 + *Hin*dIII/*Xba*I 793-bp fragment containing *V. vulnificus pgdH1*	This study
pRK-$F_{(V)}$	Tc^r; pRK415 + *Xba*I/*Bam*HI 1045-bp fragment containing *V. vulnificus ppfC*	This study
pRK-$D_{(V)}F_{(V)}$	Tc^r; pRK415 + *Hin*dIII/*Bam*HI 1771-bp fragment containing *V. vulnificus pgdH1* and *V. vulnificus ppfC*; For expression of strep-tag-fused $PgdH1_{(V)}$ and his_6-tag-fused $PpfC_{(V)}$ in *V. vulnificus*	This study
pRK-$D_{(V)}F_{(E)}$	Tc^r; pRK415 + *Hin*dIII/*Bam*HI 1714-bp fragment containing *V. vulnificus pgdH1* and *E. coli ppfC*; For expression of strep-tag-fused $PgdH1_{(V)}$ and his_6-tag-fused $PpfC_{(E)}$ in *V. vulnificus*	This study
pRK-$D_{(E)}F_{(V)}$	Tc^r; pRK415 + *Hin*dIII/*Bam*HI 1615-bp fragment containing *E. coli pgdH1* and *V. vulnificus ppfC*; For expression of strep-tag-fused $PgdH1_{(E)}$ and his_6-tag-fused $PpfC_{(V)}$ in *V. vulnificus*	This study
pRK-$D_{(E)}F_{(E)}$	Tc^r; pRK415 + *Hin*dIII/*Bam*HI 1558-bp fragment containing *E. coli pgdH1* and *E. coli ppfC*; For expression of strep-tag-fused $PgdH1_{(E)}$ and his_6-tag-fused $PpfC_{(E)}$ in *V. vulnificus*	This study
pRS-$D_{(V)}$	Ap^r; pRSET-A + 525-bp *Pst*I/*Eco*RI fragment containing *V. vulnificus pgdH1*; For expression of $PgdH1_{(V)}$ fused with N-terminal his_6-tag in *E. coli*	This study
pET-$F_{(V)}$	Km^r; pET29a + 960-bp *Nde*I/*Sal*I fragment containing *V. vulnificus ppfC*; For expression of $PpfC_{(V)}$ fused with C-terminal his_6-tag in *E. coli*	This study
pRS-$X_{(H)}$	Ap^r; pRSET-A + 1434-bp *Bam*HI/*Hin*dIII fragment containing *H. sapiens pgoX* synthesized in an *E. coli* codon-optimized sequence; For expression of $PgoX_{(H)}$ fused with N-terminal his_6-tag in *E. coli*	This study

[6,7]. Y-type coproporphyrinogen oxidase of *Bacillus subtilis* ($CgoX_{(B)}$, formerly known as $HemY_{(B)}$), which oxidizes CPgen to coproporphyrin III [8], bears some structural similarity to the mitochondrial PgoX used in canonical heme biosynthesis in *Nicotiana tabacum* [9]. There are differences between these Y-type enzymes, however, in that hemin is a competitive inhibitor of $CgoX_{(B)}$ [10], whereas cobalt-PPn is a non-competitive inhibitor of the mitochondrial PgoX of *Homo sapiens* ($PgoX_{(H)}$) [11]. G-type PgdH1 is mostly restricted to the γ-proteobacteria group (*e.g. Escherichia coli*, *Salmonella* sp., *Vibrio* sp.), though it is also found in the eukaryote *Leishmania* [12]. It is an FMN-containing enzyme and uses quinone as an electron acceptor rather than oxygen [13]. Thus, it is linked to the electron transport chain, allowing the bacteria to synthesize heme irrespective of oxygen tension [14]. J-type PgdH2 is a recently identified third type of PPgen-oxidizing enzyme that is found in both α-proteobacteria and cyanobacteria [6,15,16] and is thought to be oxygen independent [1] but has not been extensively studied.

Protoporphyrin ferrochelatase (PpfC, formerly known as HemH) and corpoporphyrin ferrochelatase (CpfC, formerly also known as HemH) catalyze the iron chelation of PPn and coproporphyrin in protoporphyrin-dependent and coproheme-dependent pathways, respectively [1,3]. Most PpfCs that have been studied have a highly conserved catalytic domain [17] and are associated with either the mitochondrial inner membrane or the bacterial cytoplasmic membrane [18]. Since iron is usually a limiting metal for many organisms, PpfCs play an important role in interacting with various proteins to mediate iron metabolism and iron transport [19–21].

PPn levels must be kept low because in the presence of light, PPn generates reactive singlet oxygen which can damage the cell. It has been proposed that in order to keep cellular PPn levels low, this molecule is channeled from one enzyme to the next through a molecular interaction between PgoX and PpfC [7,21,22]. The complex that forms between these two enzymes was visualized using immunoprecipitation and immuno-electron microscopy in the cyanobacterium *Thermosynechococcus elongatus* [23]. However, how this complex affects the kinetic properties of the individual constituent enzymes has not been