The YbgC protein encoded by the *ybgC* gene of the *tol-pal* gene cluster of *Haemophilus influenzae* catalyzes acyl-coenzyme A thioester hydrolysis

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Abstract This paper examines the catalytic function of the protein YbgC, encoded by the *ybgC* gene of the *tol-pal* gene cluster in *Haemophilus influenzae*. The YbgC protein, a homologue of the *Pseudomonas* sp. strain CBS3 4-hydroxybenzoyl-coenzyme A thioesterase, conserves the active site Asp residue associated with thioesterase activity. The *H. influenzae ybgC* gene was cloned and overexpressed in *Escherichia coli*. The recombinant protein was purified and tested for thioesterase activity towards acyl-CoA and acyl-*N*-acetylcysteamine thioesters. The YbgC protein catalyzes the hydrolysis of short chain aliphatic acyl-CoA thioesters, while the D18N YbgC mutant protein (prepared to serve as a control) does not. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

1. Introduction

The Tol-Pal system, which is present in a wide variety of Gram-negative bacteria, is important for maintenance of cell envelope integrity and may function in the transport of materials through the periplasm (for a recent review see [1]). In *Escherichia coli*, the *tol-pal* gene cluster encodes seven proteins: YbgC is a cytoplasmic protein, TolA, TolQ, TolR are inner membrane proteins, TolB and YbgF are periplasmic proteins and Pal is a peptidoglycan-associated lipoprotein. Homologues to this same gene cluster are found in the α- and γ-subdivisions of proteobacteria [2]. The function of the YbgC protein, and its relation to that of the Tol-Pal system, is not known [2]. The amino acid identity between paired YbgC proteins of the Tol-Pal systems found in *E. coli*, *Haemophilus influenzae*, *Vibrio cholerae*, *Pseudomonas aeruginosa* and *Rhodobacter capsulatus* ranges from 41 to 62%.

The amino acid sequence of the YbgC protein is homologous to the *Pseudomonas* sp. strain CBS3 4-hydroxybenzoyl-CoA (4-HBA-CoA) thioesterase [3]. Based on recent, unpublished structure determinations of 4-HBA-CoA thioesterase–inhibitor complexes, the primary elements of catalysis in the thioesterase are known to consist of the active site loop residue Asp17 and the backbone amide NH of the N-terminal residue (Tyr24) of the active site α-helix (see Fig. 1). Asp17

functions in base catalysis while the positive pole of the α-helix, and the H-bond formed with its N-terminal NH, polarize the substrate thioester $C=O$. These elements are conserved in the YbgC protein (Fig. 1A), suggesting that it too may function as a thioesterase. In this paper we report the cloning, mutation and expression of the *H. influenzae ybgC* gene and the functional characterization of the wild-type and D18N mutant YbgC proteins.

2. Materials and methods

All restriction enzymes and the T4 DNA ligase were purchased from Gibco BRL. Oligonucleotide primers were synthesized by Gibco BLC. DNA sequencing was performed by the DNA Sequencing Facility of the University of New Mexico. All biochemicals, including the acyl-CoAs, were purchased from Sigma. Western transfer reagents and the XCell II Blot module were purchased from Novex. Protein N-terminal sequencing was performed at the Protein Microsequencing Facility of the Clinical Neuroscience Branch, NIMH (Bethesda, MD, USA). The propionyl-s-*N*-acetylcysteamine was prepared by reacting 0.36 g of *N*-acetylcysteamine with 290 µl of propionyl chloride in 5 ml of anhydrous tetrahydrofuran for 1 h at 25°C. The reaction mixture was filtered and the filtrate was chromatographed on a silica gel column using hexane:acetone 1.2:0.8.

2.1. Cloning, expression and purification

The *ybgC* gene from *H. influenzae* was amplified by PCR using the clone GHICB25 obtained from ATCC (Manassas, VA, USA) and *PfuTurbo* DNA polymerase (Stratagene). Primers containing restriction endonuclease cleavage sites *Nde*I and *Bpu*1102I were used. The amplification protocol employed 30 cycles of denaturation at 95°C, annealing at 45°C, and elongation at 72°C. The pET-3a vector (Novagen), which was cut with the restriction enzymes 5′-*Nde*I and 3′-*Bpu*1102I, was ligated to the isolated gene. The ligation product named HI0386-pET-3a was used to transform *E. coli* XL1-Blue competent cells (Stratagene). Plasmid was prepared using a QIAprep Spin Miniprep Kit (Qiagen). The gene sequence was confirmed by DNA sequencing. The recombinant plasmid was used to transform BL21(DE3) competent cells (Novagen). The transformed cells were grown at 30°C in 1.5 l Luria broth containing 50 µg/ml carbenicillin to an $OD_{600nm} \approx 0.8$ and then induced using 0.4 mM IPTG until an $OD_{600nm} \approx 2.0$ was reached. The cells were harvested by centrifugation and the cell pellet was resuspended in ice-cold 50 mM K^+HEPES buffer (pH 7.5) containing 10% $(NH_4)_2SO_4$, 2 mM dithiothreitol (DTT) and 0.2 mM phenylmethylsulfonyl fluoride. The cells were lysed in a French press cell then centrifuged. The supernatant was loaded onto a butyl Sepharose 4 Fast Flow column (Amersham Pharmacia Biotech) equilibrated with 10% ammonium sulfate in 50 mM K^+HEPES buffer. A 10–0% linear gradient of ammonium sulfate in 50 mM K^+HEPES buffer was used to elute the protein. The desired fractions (eluted at 5% ammonium sulfate) were pooled, concentrated, and dialyzed against 50 mM K^+HEPES (pH 7.5) containing 0.1 M KCl and 1 mM DTT. The sample was loaded onto a Bio-Gel HTP Gel Hydroxyapatite (Bio-Rad) column and eluted with 400 ml of a linear gradient of 0.01–0.5 M K_2HPO_4 in 50 mM K^+HEPES buffer (pH 7.5). The desired fractions (eluted at 0.4 M K_2HPO_4) were

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pooled, concentrated, and dialyzed against 10 mM K+HEPES (pH 7.5) containing 0.05 M KCl and 1 mM DTT. The sample solution was then chromatographed on a FPLC MonoQ HR 16/10 column using a linear gradient of 0–0.5 M KCl in 10 mM K+HEPES (pH 7.5) as eluant. The desired fractions (eluted at 0.15 M KCl) were concentrated and dialyzed against 10 mM K+HEPES (pH 7.5) containing 0.2 M KCl. The yield of homogeneous YbgC protein was 5 mg protein/g wet cell.

2.2. YbgC protein N-terminal sequencing

The YbgC protein sample was chromatographed on a 16% SDS–PAGE gel using Tris (25 mM)–glycine (192 mM) (pH 8.3) as the running buffer. The protein was transferred to a polyvinylidene difluoride (PVDF) membrane by using a Novex XCell II blot module and Tris (12 mM)–glycine (96 mM) transfer buffer (pH 8.3). The resulting PVDF membrane was stained using Bio-Safe Coomassie blue dye (Bio-Rad). The target protein band was excised and subjected to automated N-terminal sequencing.

2.3. D18N YbgC mutant protein

Mutagenesis was done using a PCR strategy based on HI0386-pET-3a as template, commercial primers, the PCR kit supplied by Stratagene, and the Techgene thermal cycler manufactured by TECHNE. PCR-amplified DNA was cloned into pET-3a (Stratagene) for expression in E. coli BL21(DE3). The mutated gene was verified by DNA sequencing. The D18N mutant protein was purified as described above for the wild-type YbgC protein and shown to be homogeneous on the basis of SDS–PAGE gel analysis. The yield of the pure protein was 4 mg/g wet cell.

2.4. HPLC fixed-time assay of YbgC-catalyzed hydrolysis of propionyl-CoA

The reaction solution initially contained 7.4 µM YbgC protein and 10 mM propionyl-CoA in 50 mM K+HEPES/0.2 M KCl/1 mM DTT (pH 7.5, 25°C). At various times, 2.5 µl aliquots were removed and mixed with 100 µl of 1 mM HCl (pH 3.0). The YbgC protein was removed from the quenched sample by using a 5 kDa Ultrafree micro filter (Millipore). A Rainin Dynamax HPLC system equipped with a reversed-phase C-18 column (Beckman Ultrasphere; 4.6×250 mm), pre-equilibrated in 30% solvent B and 70% solvent A, was used to separate coenzyme A (CoASH) and propionyl-CoA (260 nm detection) from the protein-free sample. A linear methanol gradient (30–80% solvent B in 10 min) and hold 80% solvent B for 5 min was employed to elute the column at a flow rate of 0.8 ml/min. Solvent A is 0% methanol and 10 mM K+Pi (adjusted to pH 6.5 with H₃PO₄), and solvent B is 40% methanol and 10 mM K+Pi (adjusted to pH 6.5 with H₃PO₄). The retention times of CoASH and propionyl-CoA are 5.3 and 10.9 min, respectively.

2.5. Continuous spectrophotometric assay of YbgC thioesterase activity

Hydrolysis reactions of the acyl-thioester substrates were monitored at 25°C by measuring the absorbance of 5-thio-2-nitrobenzoate at 412 nm, which was formed by reacting DTNB (5,5′-dithio-bis(2-nitrobenzoic acid)) with the CoASH or N-acetylcysteamine liberated from the acyl-CoA or acyl-N-acetylcysteamine substrate, respectively. To allow high substrate concentrations to be used in the assays, reactions were carried out in a quartz cuvette having a 1 mm light path. Each assay reaction (200 µl) contained YbgC protein (7.7 µM), acyl-CoA substrate (0.5–30 mM), DTNB (2 mM), KCl (0.2 M) and 50 mM K+HEPES, pH 7.5. The kinetic parameters of V_{max} and K_m were determined from initial velocity data, measured as a function of substrate concentration, using Eq. 1 and the computer program of Cleland [5].

$$V = V_{max}[A]/([A] + K_m) \qquad (1)$$

[A] is the substrate concentration, V is the initial velocity, V_{max} is the maximum velocity and K_m is Michaelis constant. The k_{cat} was calculated from the ratio of V_{max} and the total enzyme concentration. The enzyme concentration was determined using the Bradford method [6].

3. Results and discussion

The H. influenzae ybgC gene was cloned and overexpressed in E. coli. The YbgC protein was purified to homogeneity by column chromatography (see SDS–PAGE gel of purified protein in Fig. 2) in a yield of 5 mg/g wet cell. The N-terminal sequence, MLDNGFSFPVRVYYEDTDAG, determined for the recombinant protein is the same as that predicted on the basis of the ybgC gene sequence. The purified YbgC protein is soluble in K+HEPES buffer (pH 7.5) containing 0.2 M KCl and 1 mM DTT, and is stable to prolonged storage in this solution at 4°C.

The substrate activities of various acyl-CoA thioesters were tested at 25°C and pH 7.5. Within the detection limit (∼0.005 s⁻¹ turnover rate) of the spectroscopic assay employed, 4-hydroxybenzoyl-CoA, lauroyl-CoA (C12:0), arachidoyl-CoA (C20:0) and arachidonoyl-CoA (C20:4) were found to be inactive as substrates for YbgC protein-catalyzed hydrolysis. Several short chain aliphatic acyl-CoA thioesters were, however, found to be active substrates. A fixed-time HPLC-based assay was used to demonstrate the formation of the carboxylic acid and CoASH products and then the spectrophotometric assay was used to measure the steady-state kinetic constants (Table 1). In a separate experiment, the thioesterase activity of the YbgC protein towards propionyl-CoA was measured at pH 7.5 and 8.6. The two activities were equivalent.

The k_{cat} values observed for the two most active substrates tested, propionyl-CoA and isobutyryl-CoA, range from 0.5 to 0.6 s⁻¹ while the K_m values range from 11 to 16 mM (Table 1). The modest k_{cat}/K_m values suggest that these acyl-CoA thioesters are similar, but not identical, to the actual physiological substrate.

The structure of the physiological substrate may differ from propionyl-CoA and isobutyryl-CoA at its acyl group, or its thiol group, or both. To test for the preference of a CoA thiol group, we prepared propionyl-s-N-acetylcysteamine for reaction with the YbgC protein. Whereas propionyl-CoA was hydrolyzed with a $k_{cat} = 0.44$ s⁻¹ and $K_m = 11$ mM, no reaction was observed with propionyl-s-N-acetylcysteamine (30 mM) incubated with YbgC protein (7.7 µM) under the same conditions used with propionyl-CoA (25°C, pH 7.5). This result
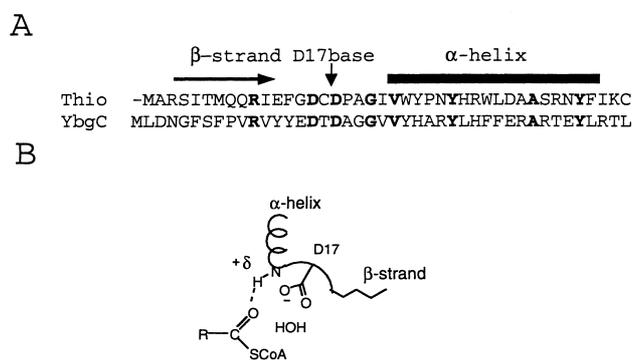
Fig. 1. A: N-terminal sequence regions of the 4-HBA-CoA thioesterase from Pseudomonas sp. strain CBS-3 (Thio) and the YbgC protein in H. influenzae. The sequence alignment was generated using the 3-D PSSM homology search program [4]. The secondary structural elements observed in the X-ray crystal structure of the 4-HBA-CoA thioesterase [3] are labeled along with those predicted for the YbgC protein by the 3-D PSSM program. Asp17, which is positioned in the 4-HBA-CoA thioesterase active site to function in base or nucleophilic catalysis, is labeled. The backbone amide NH of Tyr24, located at the N-terminus of the active site α-helix, is found just downstream of Asp17. These active site groups are illustrated in B.

A

B
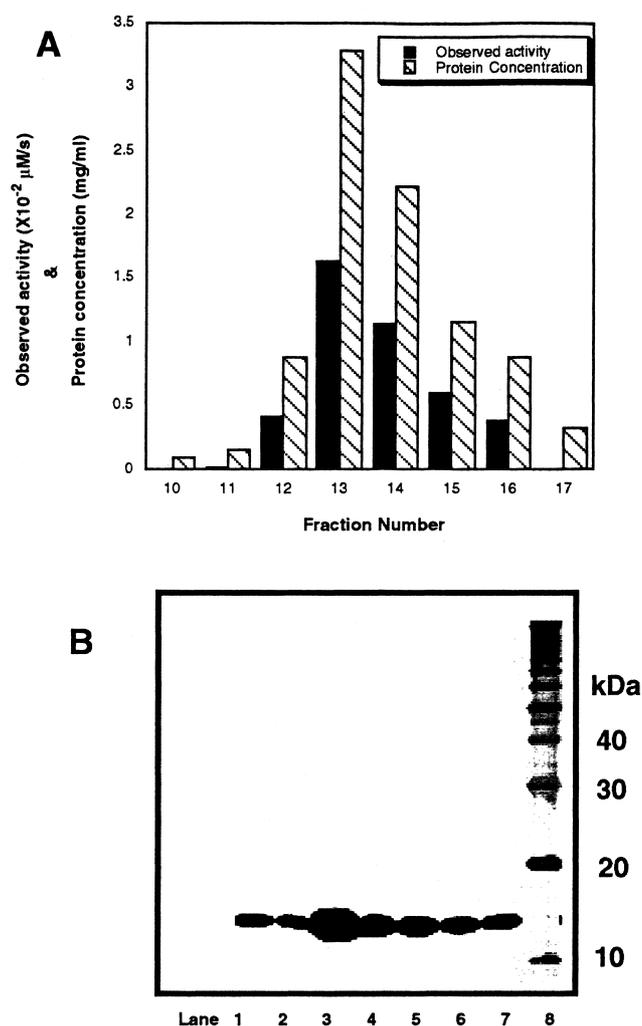
Fig. 2. A: Observed rates of propionyl-CoA hydrolysis ($\times 10^{-2}$ μM/s) catalyzed by the YbgC protein contained in 25 μl aliquots taken from each FPLC MonoQ column fraction. The protein concentration (mg/ml) of each fraction is also shown (see Section 2 for details). B: SDS–PAGE analysis of fractions 11–17 (labeled as lanes 1–7) pictured in A. The gel was stained with Coomassie blue dye. Lane 8 shows the Gibco 10 kDa protein ladder.

shows that the YbgC protein prefers the acyl-CoA thioester over the acyl-s-N-acetylcysteamine thioester as substrate.

The YbgC thioesterase activity towards propionyl-CoA was measured for each column fraction obtained during the final purification step of YbgC on the FPLC MonoQ column. As shown in Fig. 2, the thioesterase activity precisely correlates with the amount of YbgC present. This result, along with the size of the k_{cat} value, is strong evidence that the thioesterase activity observed with the YbgC protein is intrinsic and not the result of contamination by a highly active thioesterase. To test this conclusion the D18N YbgC mutant protein was prepared. This mutant is lacking the active site Asp residue, which in the 4-HBA-CoA thioesterase homologue functions in catalysis. The D18N YbgC mutant protein was purified in the same manner as the wild-type YbgC protein, but unlike the wild-type enzyme, it does not catalyze the hydrolysis of

Table 1
Kinetic constants measured for YbgC-catalyzed hydrolyses of acyl-CoA thioesters at pH 7.5 and 25°C

	Acyl-CoA Substrate	K_m (mM)	k_{cat} (s^{-1})
	Propionyl-CoA	11 ± 1	0.44 ± 0.04
	iso-Butyryl-CoA	16 ± 2	0.54 ± 0.05
	n-Butyryl-CoA	24 ± 7	0.17 ± 0.03
	D,L-β-hydroxybutyryl-CoA	20 ± 7	0.05 ± 0.01
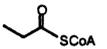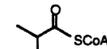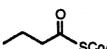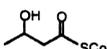
propionyl-CoA. Thus, we can attribute the thioesterase activity observed to the wild-type YbgC protein with reasonable certainty.

We conclude that the *ybgC* gene associated with the *tol-pal* gene cluster in *H. influenzae* encodes a protein that displays a significant level of thioesterase activity towards short chain acyl-CoA thioesters. Acyl-CoA thioesterases are known to perform a wide range of cellular functions [7]. It is not yet clear how the thioesterase activity of the YbgC protein may be related to the function of the Tol-Pal system. It is noteworthy that YbgC protein is representative of a large group of structurally related 'unknown proteins' in bacteria whose encoding genes are nestled among gene clusters functioning in the synthesis and/or export of extracellular substances. It is thus tempting to speculate that the 4-HBA-CoA thioesterase scaffold has been recruited to perform specific functions in bacteria associated with the structure and transport operations of the cell envelope. The determination of the three-dimensional structure of YbgC, which is under way, should provide insight into the structure of its physiological substrate through definition of the enzyme active site.

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