Mutagenesis identifies a conserved tyrosine residue important for the activity of uroporphyrinogen III synthase from *Anacystis nidulans*

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Abstract Uroporphyrinogen III synthase from the cyanobacterium *Anacystis nidulans* was overproduced in *Escherichia coli* and analyzed by site specific mutagenesis. Of the nine conserved amino acids altered, only a single tyrosine mutant (Y166F) showed any significant decrease in activity suggesting this residue is critical for proper substrate binding and/or catalysis.

Key words: Uroporphyrinogen III synthase; Mutagenesis; Tyrosine

1. Introduction

Uroporphyrinogen III (urogen III), the common precursor to the 'pigments of life' heme, siroheme, chlorophyll, cobalamin and factor F430, is derived by the enzymatic cyclization and rearrangement of the D ring of hydroxymethylbilane (HMB) by urogen III synthase (U3S) (reviewed in [1]) whilst non-enzymatic cyclization of HMB without rearrangement results in the non-functional isomer, urogen I (Fig. 1). The structure of human U3S has recently been reported [2] accompanied by a mutagenic study which suggested that, since no catalytic amino acid residue could be identified among those conserved in homologous enzymes, U3S functions simply by holding the substrate in a conformation favorable to urogen III formation.

The favored mechanism for the synthesis of urogen III features a spiro intermediate (Fig. 1) in which the enzyme functions to hold HMB in a configuration favorable to urogen III formation and to provide a proton source to facilitate azafulvene formation by removal of the hydroxyl group at C-20. Indeed, azafulvene formation and cyclization to afford urogen I occur at physiological and slightly alkaline pH but the enzymatic reaction leading to urogen III is much faster [1]. In addition, azafulvene formation at C-20 is absolutely dependent on U3S at pH 10.8 using purified *Escherichia coli* enzyme which lends support to a role more than just structural for the catalytic activity [3].

We report here the overproduction of U3S from *Anacystis nidulans* and the requirement of a conserved tyrosine residue for optimal activity of the enzyme.

2. Materials and methods

2.1. Materials

Porphobilinogen (PBG) was purchased from Sigma. PBG deaminase (specific activity = 25 U/mg) was purified free from U3S activity as previously described [4]. I_2/trichloroacetic acid (TCA) solution was made by dissolving 25 mg of I_2 and 50 mg of KI in 5.0 ml of 10% TCA. PCR primers and sequencing services were provided by the Gene Technologies Laboratory, Biology Department, TAMU. Pfu-Turbo polymerase was purchased from Stratagene. Restriction enzymes and T4 DNA ligase were from New England Biolabs. The expression vector pET28a was purchased from Novagen.

2.2. Overproduction and purification of *A. nidulans* U3S

A. nidulans U3S was overproduced with a 6×His-tag to facilitate purification by ligating a PCR product containing the *A. nidulans hemD* gene [5] into pET28a using the *Nhe*I and *Hin*dIII restriction sites and transformation of the resultant plasmid into *E. coli* strain BL21DE3 to afford strain CR586. The sequence of the forward PCR primer was CGC GCG GCT AGC ATG GCT GAG CAG CCG CTG ATC GGT AAA ACC which provided the *Nhe*I site (underlined) and the first 10 codons of the *A. nidulans hemD* gene. The sequence of the reverse primer was CTG CAG AAG CTT **TTA** CTG GGT TTG GCG CGC CCA TTG TTC GAT CGC which provided the *Hin*dIII site (underlined), a stop anticodon (bold), and the anticodons of the last 10 amino acids. The PCR product was generated and ligated into pET28a using standard procedures [6].

For expression of U3S and mutant enzymes, the strains were grown with good aeration at 37°C in LB medium containing 50 μg/ml kanamycin to an A_{600} of 0.8, induced by addition of 0.4 mM IPTG and incubated at 25°C for 16 h. Production of the protein at 37°C resulted in accumulation of the protein in insoluble inclusion bodies. The cells were collected by centrifugation, resuspended in lysis buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl) and lysed by sonication. The lysate was centrifuged at 10 000×*g* for 10 min and U3S was purified from the supernatant (soluble fraction) via the N-terminal His-tag provided by pET28a using Talon resin (Clontech) and following instructions given by the supplier. Protein purity and solubility were monitored by SDS–PAGE using the system of Laemmli [7].

2.3. Mutagenesis of the *A. nidulans hemD* gene

U3S mutants were constructed using the QuikChange™ site-directed mutagenesis procedure (Stratagene). Presence of the mutations and the integrity of the rest of the gene were confirmed by sequencing.

2.4. U3S assay

U3S activity was monitored by the production of urogen III as determined by a modified lag assay [8]. For each reaction, 50 μl PBG deaminase and 50 μl of U3S-containing solution (lysate or purified enzyme) were mixed and preincubated for 1 min at 37°C. 50 μl of PBG (0.2 mg/ml at 37°C) was added to start the reaction. After

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Abbreviations: urogen, uroporphyrinogen; U3S, uroporphyrinogen III synthase; uro, uroporphyrin; HMB, hydroxymethylbilane; PBG, porphobilinogen

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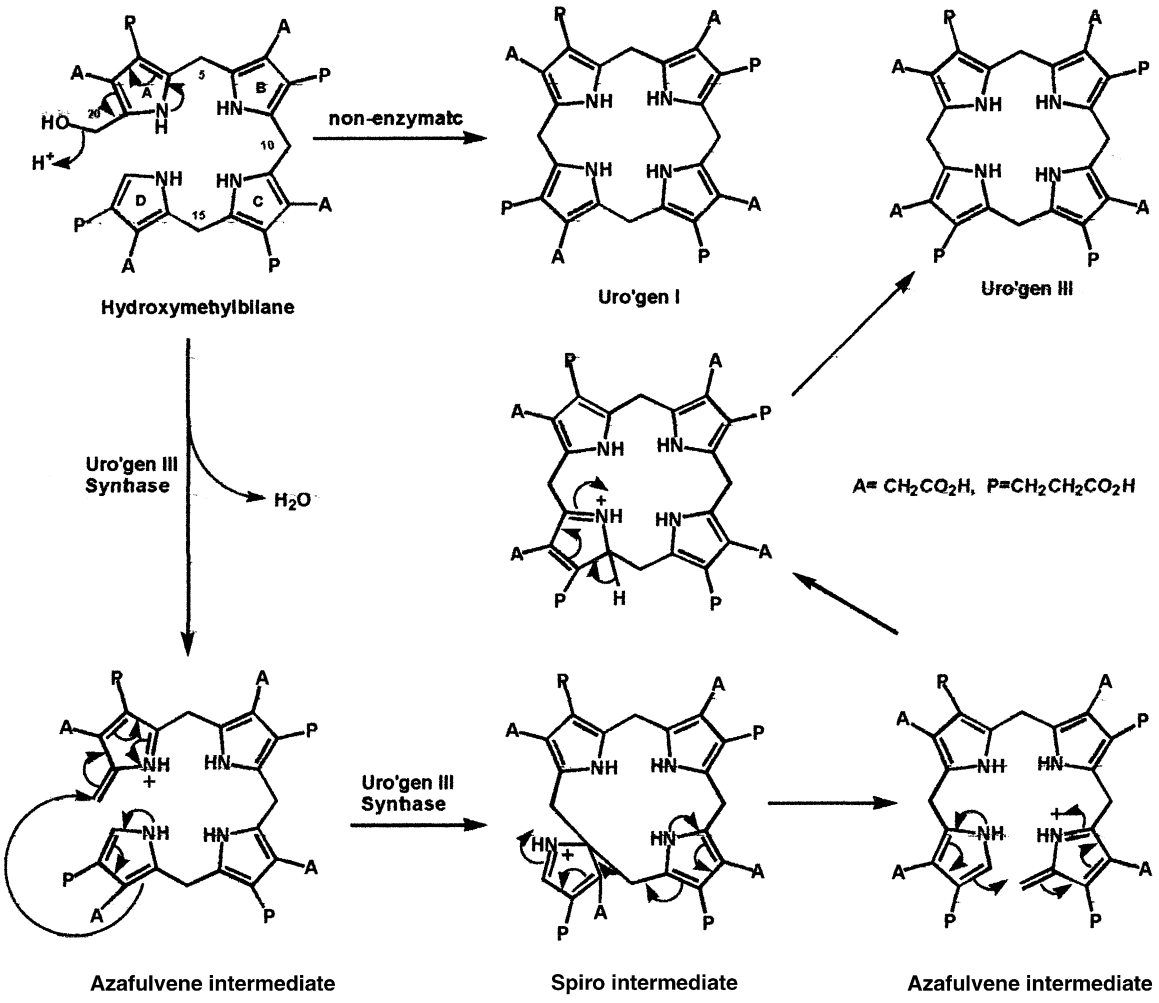

Fig. 1. The proposed mechanism for the biosynthesis of urogen III from HMB by U3S featuring a spiro intermediate.

1 min, 150 μl of I_2/TCA was added to stop the reaction and oxidize urogens to uroporphyrins (uros). After 4.0 min at room temperature, 5.0 μl of saturated sodium metabisulfite was added to quench excess I_2, and the solution was centrifuged for 1 min in a microfuge. The supernatant was diluted 20-fold with 1.0 N HCl and the absorbance measured at 405 nm. A background value, determined from a control sample of 50 μl of buffer without U3S, was subtracted from the sample A_{405} and the amount of urogen III synthesized calculated from the difference using $E = 518\,000\ M^{-1}\ cm^{-1}$ for the extinction coefficient of uros in HCl. Alternatively, the products were analyzed by high-performance liquid chromatography (HPLC) separation of the uro isomers in the free acid form [9]. One unit is defined as the amount of U3S required to synthesize 1.0 nmol of urogen III in 1 min.

3. Results and discussion

Based on the nucleotide sequence of the *A. nidulans hemD* gene and the corresponding amino acid sequence for U3S (Fig. 2) [5], a vector for the overexpression of the gene in *E. coli* was constructed which afforded high levels of active wild-type U3S (Fig. 3, Table 1). Site-directed mutagenesis provided mutants of the amino acid residues most highly conserved among homologous U3S enzymes (R15Q, S68A, T100A, D112G, Y166F, and S194A, Fig. 2). The relative activities of soluble cell fractions containing the wild-type or mutant enzymes were determined with the lag assay (Table 1). In this coupled assay, the substrate, HMB, is generated from PBG and PBG deaminase and is partially consumed by non-enzymatic cyclization during the reaction (Fig. 1). Therefore, care must be taken that substrate does not become limiting. Thus, rather than using a single enzyme concentration, a series of dilutions of the soluble cell fractions were tested and the highest dilution still showing significant activity over background was chosen to estimate relative activity. This assay indicated that only the alteration Y166F had any significant effect on the activity of the enzyme (Table 1). To confirm this finding, the wild-type and Y166F mutants were purified and the specific activity of each determined with the lag assay (Fig. 3, Table 1). In a separate experiment, HPLC analysis of the reaction products [9] gave a higher specific activity for both

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  1  MAEQPLIGKT ILTTRAAGQS SPFAAQLRAA GAAVIEMPTL EIGPPSSWLP
 51  LDEAIAAIAD FDWLILASAN AVEAVQQRLA AQQKSWSDVP CAIAVVGQKT
101  AQVLAAQGGK ADYIPPEFIA ESLVEHFPQP VAGQRLLFPR VETGGREQIT
151  QALQSQGAIV VEVPAYESRC PSQIPDDALI ALRQAHLNLI SFTSSKTVRN
201  FCQLMASNLG VDWSARISGV AIASIGPQTS ITCQELLGRV EVEAQEYTLD
251  GLLLAIEQWA RQTT*
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Fig. 2. Amino acid sequence of U3S from *A. nidulans*. The residues that were altered for this work are shown in bold.

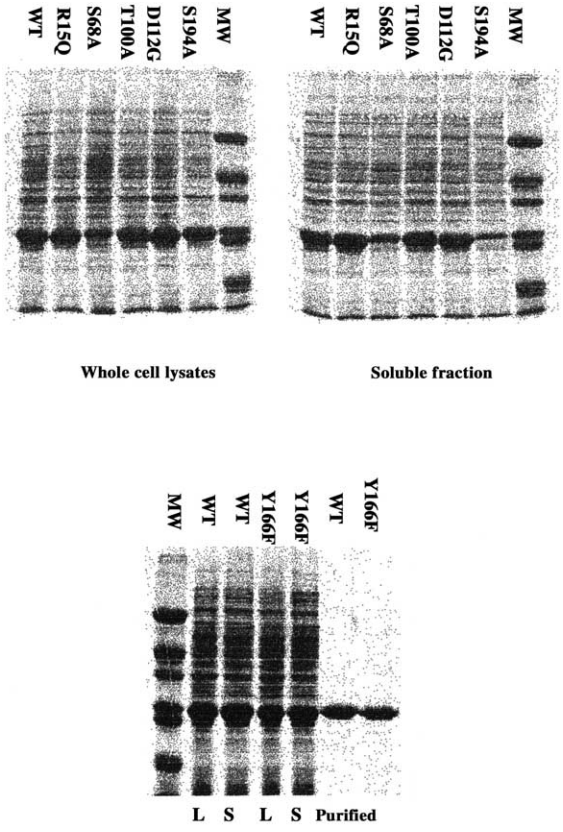

Fig. 3. SDS–PAGE determination of the relative levels of production and solubility of wild-type and mutant U3S enzymes. Also shown (bottom gel) are wild-type and Y166F enzymes purified by a single step batch metal affinity procedure. WT, wild-type; L, whole cell lysate; S, soluble fraction; MW, molecular weight markers from the top: 66K, 45K, 36K, 29K, 24K, 14K.

the wild-type enzyme (160 000 U/mg) and the Y166F mutant enzyme (2660 U/mg). While the Y166F mutant enzyme is not completely inactive, the specific activity of the purified enzyme was only 0.6–1.6% of that of the purified wild-type enzyme indicating that this tyrosine residue is essential for activity, either by directing the binding of the substrate in the correct conformation or by providing a functional group for catalysis, or both. If the latter is the case, this result is somewhat surprising because tyrosine is not generally considered a good proton donor as are lysine and arginine. However, mutating the only conserved arginine (R15, which is functionally conserved as a lysine in some organisms) to glutamine had no effect on enzyme activity. T229 and at least one of three closely clustered arginines of *A. nidulans* U3S (R135, R140 and R146) are also found in U3S of most other organisms, but R135Q, R140Q, R146Q and T229A mutants retained 50–100% of the wild-type activity (not shown).

Some of the mutations apparently caused misfolding of the protein (e.g. S68A and, especially, S194A) since they were less soluble, but Y166F remains as soluble as wild-type U3S (Fig. 3). The soluble S68A and S194A, however, maintain activity (Table 1).

The above results are consistent with the previously reported mutagenic study of human U3S with the exception of the greatly reduced activity of the Y166F mutant. The analogous mutant of human U3S (Y168F) apparently retained about 50% of the activity of the wild-type enzyme [2]. This result may be misleading, however, since substrate may have been limiting in the assay described to monitor the activity of this enzyme.

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Table 1
Lag assay results for determination of the activity of *A. nidulans* U3S mutants relative to wild-type enzyme

	Fold dilution of cell extract[a]							Relative activity (%)[b]
	10	100	200	400	800	1600		
w.t.	–	5.3	5.3	5.9	4.7	3.4		100
R15Q	–	5.6	5.8	6.0	5.5	5.3		≥100
S68A	–	5.5	5.5	4.6	3.0	–		≥100
T100A	–	5.5	4.8	4.0	2.7	–		25
D112G	–	5.3	5.5	5.3	3.7	–		50
S194A	5.9	2.7	–	–	–	–		
blank	1.7	1.4	1.4	1.8	1.8	2.1		
	Fold dilution of cell extract[a]							Relative activity (%)[b]
	10	20	50	100	400	800	1600	100
w.t.	–	–	–	6.0	5.6	5.9	4.6	
Y166F	4.0	3.5	2.5	2.8	–	–	–	<1
blank	2.3	2.3	2.1	2.1	2.5	2.5	2.5	
	Fold dilution of purified enzyme[c]							Specific activity
	0	10	100	400	800			
w.t. (0.32 mg/ml)	–	5.5	5.5	3.9	3.0			40 000 U/mg[d]
Y166F (0.40 mg/ml)	3.7	2.8	2.8	–	–			250 U/mg[e]
blank	2.3	2.3	2.8	2.3	2.3			

Values shown are nmol of urogens synthesized per assay.
[a]Soluble fraction shown in Fig. 3.
[b]Determined from the highest dilution showing significant difference from the blank and estimating the relative amount of soluble enzyme in the extract (Fig. 2).
[c]Purified wild-type (WT) and Y166F mutant enzyme shown in Fig. 3.
[d]Calculated from the 400-fold dilution.
[e]Calculated from the 10-fold dilution.

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