First publ. in: Flavins and flavoproteins 1999 : proceedings of the Thirteenth International Symposium, Konstanz, Germany, August 29 - September 4, 1999, pp. 571-574

Mutation in an Hydrophobic Sequence Motif Common to N-Hydroxylating Enzymes

Oliver Seth, Liliana Smau, Wolfram Welte, Sandro Ghisla
Faculty of Biology, University of Konstanz, D-78457 Konstanz, Germany
Peter Macheroux
Institute for Plant Sciences, ETH Zürich, CH-8092 Zürich, Switzerland

Introduction

Lysine-N^6-Hydroxylase from E. coli (iucD) catalyses the first step in the biosynthesis of the siderophore aerobactin. Siderophores are chelators that release iron from insoluble hydroxy-compounds and make it available to bacteria, fungi and plants. In the reaction the ε-amino group is hydroxylated. The enzyme requires also FAD as a cofactor and NADPH. A similar reaction is carried out by a family of mammalian flavin-containing dimethylaniline monooxygenases. Their substrates are tertiary and secondary alkylamines.

Sequence alignment (Figure 1) of four siderophore biosynthetic enzymes from Escherichia coli (aerA, iucD), Pseudomonas aeruginosa (pvdA), Bordetella bronchiseptica (alcA) and Ustilage maydis (sid1) and about 30 sequences of flavin-containing mammalian monooxygenases revealed three dominant areas of similarity. Two of these regions of similarity were assigned to the FAD and the NADPH binding site. The third region of similarity was called the FATGY-motif (1). This similarity starts with a highly conserved aspartate and is followed by 8 hydrophobic amino acids. The core region consists of the sequence F-A-T-G-Y. It was suggested that this motif is part of a hydrophobic substrate binding site. To investigate the role of the highly conserved aspartate 325 in iucD two mutant-proteins were created.

The sequences were aligned using the programs MACAW and CLUSTALX. First column: protein names; last column: accession numbers from SWISS-PROT and PIR; the numbers before and after the sequence show the position of the displayed regions in their respective sequences. The last row gives the consensus sequence that is shared by the majority of the sequences. The grey shaded area illustrates absolutely conserved residues.

FATGY-MOTIF

alcA	330	ATDGLVL**ATGY**SHEI**P**AC	347	p:JC4556
iucD	323	ES**D**VVI**FATGY**RSAL**P**QI	340	sp:P11295
pvdA	345	TY**D**AVIL**ATGY**ERQLHRQ	362	p:A49892
sid1	433	RF**D**AVFLG**TG**FIRSPSKM	450	p:A47266
YHX6_YEAST	257	NI**D**YII**FATGY**YYSF**P**FI	274	sp:P38866
FMO1_MOUSE	321	PI**D**IIV**FATGY**TFAF**P**FL	338	sp:P50285
FMO1_RABIT	323	PI**D**VIV**FATGY**TFAF**P**SL	340	sp:P17636
FMO2_CAVPO	321	DI**D**VIV**FATGY**TFSF**P**FL	338	sp:P36366
FMO5_RABIT	321	DI**D**AVI**FATGY**SFSF**P**FL	338	sp:Q04799
FMO1_RAT	321	PI**D**VIV**FATGY**SFAF**P**FL	338	sp:P36365
FMO1_HUMAN	320	PI**D**IIV**FATGY**TFAF**P**FL	337	sp:Q01740
FMO3_HUMAN	320	GI**D**CVI**FATGY**SFAY**P**FL	337	sp:P31513

Consensus **DXXX**FATGY**XXXXP**

Figure 1

Multiple sequence alignment of N-hydroxylating siderophore biosynthetic enzymes and mammalian N-hydroxylating dimethylaniline monooxygenases

Aligned sequences: alcA - alcaligin biosynthesis enzyme (Bordetella bronchiseptica); iucD - L-Lysine N6-Hydroxylase (Escherichia coli); pvdA - L-ornithine N5-oxygenase (Pseudomonas aeruginosa); sid1 - L-ornithine N5-oxygenase (Ustilago maydis); YHX6 _YEAST - hypothetical 42.4 kD protein (Saccharomyces cerevisiae); FMO1_MOUSE - hepatic flavin-containing monooxygenase 1 (FMO 1) (Mus musculus) (mouse); FMO1_RABIT - hepatic flavin-containing monooxygenase 1 (FMO 1) (Oryctolagus cuniculus) (rabbit); FMO2_CAVPO - pulmonary flavin-containing monooxygenase 2 (FMO 2) (Cavia porcellus) (Guinea pig); FMO5_RABIT - hepatic flavin-containing monooxygenase 5 (FMO 5) (Oryctolagus cuniculus) (rabbit); FMO1_RAT - hepatic flavin-containing monooxygenase 1 (FMO 1) (Rattus norvegicus) (rat); FMO1_HUMAN - fetal hepatic flavin-containing monooxygenase 1 (FMO 1) (Homo sapiens) (human); FMO3_HUMAN - hepatic flavin-containing monooxygenase 3 (FMO 3) (Homo sapiens) (human); FMO4_HUMAN - hepatic flavin-containing monooxygenase 4 (FMO 4) (Homo sapiens) (human); FMO5_HUMAN - hepatic flavin-containing monooxygenase 5 (FMO 5) (Homo sapiens) (human).

Results and Discussion

The mutation was introduced by an inverse PCR strategy according to the manual of the QuickChange™ protocol (Stratagene). Two complementary oligonucleotides iucD-D325A-s and iucD-D325A-a were synthesized carrying the triplet gCC coding for an Ala at position 325. Additionally a silent mutation was introduced by the triplet acC coding for Threonin 331. This replacement resulted in an extra Age I restriction site for the convenient screening for mutants.
Typically, 75 mg of wt-enzyme can be purified from 1L cultures (grown at 37°C). With the two mutant proteins the yields were much lower and the fermentation temperature was found to be critical (Tabel 1).
The K_M values for the mutant proteins were determined under standard conditions (10 µM FAD; 300 µM NADPH; 1.5 mM Lys; 0.5 µM enzyme).

Tabel 1 **Kinetic characteristics of the D325-mutant proteins**

Protein	yields mg prot./L culture	specific activity mU/mg	K_M Lys µM	K_M FAD µM	K_M NADPH µM
wt-iucD	75 (30°C)	420	118	2	25
IucD-D325A	6.5 (20°C)	167	50±10	6±1	25±2.5
IucD-D325E	13 (30°C)	320	60±12	9.3±1.4	14±1.5

All mutant proteins were able to catalyse the hydroxylation of L-lysine. The K_M values for L-lysine were approx. 50% lower for the D325E and D325A mutant proteins in comparison to the wild-type enzyme. The K_M values for FAD were about three times higher in comparison to the wild-type protein. The K_M values for NADPH remained almost the same.

Conclusions

Both mutant proteins, D325E and D325A, were still able to catalyse the hydroxylation of L-lysine. The K_M values for lysine, FAD and NADPH were in the range of the wildtype protein. However the stability of the mutant enzyme seemed to be significantly lower in comparison to the wild-type enzyme. Usually 75 mg of wild-type enzyme could be purified from 1-litre culture (fermentation at 37°C). The yield dropped to 13 mg for the D325E mutant protein (fermentation at 30°C) and 6.5 mg for the D325A mutant protein (fermentation at 20°C).
These results indicate that Asp325 is not essential for catalysis but important for the stability of the enzyme.

References

1. Stehr, M., Diekmann, H., Smau, L., Seth, O., Ghisla, S., Singh, M. and Macheroux, P. (1998). *Trends Biochem Sci* **23(2)**, 56-57.

2. Schuler, G.D., Altschul, S.F. and Lipman, D.J. (1991). *Proteins* **9**, 180-190.

3. Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994). *Nucleic Acids Res.* **22**, 4673-4680.