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Oligomerization and Aggregation of Lysine N^6-Hydroxylase – an Enzyme of the Bacterial Aerobactin Biosynthesis

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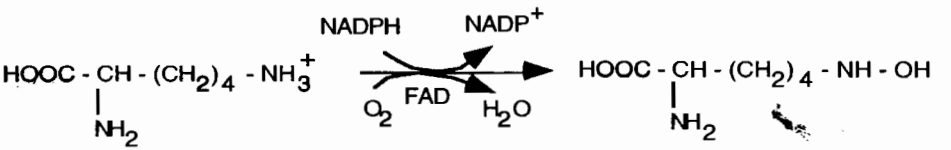
Introduction

Under low iron conditions many aerobic microorganisms synthesize highly efficient iron-scavenging low molecular weight compounds, the so-called siderophores. The formation of siderophores is regarded as a virulence factor. Therefore blocking the iron-supply of the bacteria is an interesting target for an antibacterial therapy (1).
Lysine N^6-hydroxylase (LH) is involved in the biosynthesis of aerobactin (2), a siderophore of the hydroxamate-type, and catalyzes the first step, i.e. the hydroxylation of L-lysine to N^6-hydroxylysine as shown below.



LH is an FAD containing hydroxylase which uses NADPH as a source of reducing equivalents. The enzyme was thus classified as an external FAD-dependent monooxygenase (EC: 1.1.4.13) (3). The enzyme used in the studies reported here was obtained from *E. coli* M15-0 and was cloned as a fusion protein with an N-terminal histidine-tag in the expression vector pQE30 (4). This led to very good yields of highly purified LH (75 mg protein per liter culture), obtained in a single affinity-chromatography step. The catalytical properties of the recombinant His-tagged protein are very similar to those reported for the wildtype (wt) enzyme. Under physiological conditions LH exists as a tetramer, consisting of four identical units with a subunit molecular weight of 50000 each. The three-dimensional structure of the enzyme is not yet known. Attempts in our laboratory to crystallize the protein failed because of the high tendency of LH to aggregate. Here we present a study aiming to understand the aggregation behaviour of LH.

Results and Discussion

The concentration dependence of the oligomerization of LH was investigated by means of analytical ultracentrifugation. At the highest LH-concentration studied (9.4 µM) only tetramers were observed in solution, whereas at lower LH-concentration (4.6 µM) also monomers were present, which were in equilibrium with tetramers. Further insight into the oligomerization was achieved by utilizing 8-anilino-1-naphthalene sulphonate (ANS) as a fluorescence probe. This fluorophore binds to proteins and becomes fluorescent upon binding. The stoichiometry of binding of ANS to LH was determined according to the method of Job (5). At a total concentration of 20 µM ANS and LH, 1.5 molecules ANS bind to one LH-molecule. When the total concentration of ANS + LH was lower (2 µM), the binding-ratio was 1:1 ANS/LH. These results indicate that at low LH-concentrations, where the monomer prevails, only one molecule ANS bind to LH. At higher concentrations, however, additional ANS binding sites are created by the oligomerization of the monomers to the tetramers.

Moreover, native PAGE showed that aggregates higher than the tetramer molecular weight (200 kD) were present (in the absence of DTT (dithiothreitol) or ß-ME (ß-mercaptoethanol)). These high aggregates disappeared upon addition of reducing agents. On the basis of these observations we concluded that the aggregation of LH is due to the formation of a covalent bond under oxidative conditions, i.e. intermolecular linkage of LH-tetramers via disulfide bridges. Hence, it was assumed that removal of the reactive cysteines would prevent formation of the higher aggregates and give rise to a stable protein. Therefore, we constructed single mutant proteins of the 5 cysteines of LH (C31, C51, C146, C158 and C166) beeing exchanged with serine or alanine. The results with the single mutants are summerized in Table 1. From previous work it was known, that three of the five cysteines of LH are accessible to 5,5'-Dithiobis-(2-Nitrobenzoic Acid)) (DTNB) (6). These cysteines are thought to be responsible for the formation of disulfide bridges. In order to determine which of the 5 cysteines are accessible, the mutants were treated in native form with an excess of DTNB. The mutant proteins C31S and C146S were shown to exhibit three exposed cysteines, while replacement of C51, C158 or C166 reduced the number of reactive cysteines to two. Hence, we have identified all three reactive cysteine residues in LH. These results are in agreement with the previous determination by Marrone (7) who established the accessibility of C51 and C158. Kinetically the three cysteines have different reactivities towards DTNB: C51 and C166 react fast, while C158 reacts ca. two times slower. Wild type LH (wt-LH) is inactivated by methyl methane-thiosulfonate (MMTS) (6). The reaction of MMTS with the single muteins revealed that only the C51S exchange had an effect. This protein is insensitive towards this reagent and hence C51 appears to be responsible for the inactivation. However, a direct participation of cysteine 51 in catalysis is doubtful since C51S-LH is fully active. Therefore, it is more likely that alkylation of C51 with MMTS exerts a sterical effect such that the substrate and/or cofactor can no longer bind to the enzyme.

Table 1: Properties of the cysteine-mutant proteins

LH	Yields (mg protein/L culture	Specific activity (mU/mg)	Reaction with DTNB		Inactivation with MMTS
			Nr. of detec. thiols	Nr. of exposed thiols	
Wild type	75	410	2.9	3	yes
Single mutants					
C31S	32	320	2.8	3	yes
C51S	29	290	1.9	2	no
C146S	15	165	2.8	3	yes
C158S	5.5	190	1.85	2	yes
C166A	11.2	250	1.9	2	yes
C166S	16	500	1.85	2	yes
Double mutants					
C31S/C51S	12	500	n.d.	n.d.	n.d.
C31S/C158S	1	520	n.d.	n.d.	n.d.
C51S/C158S	0.7	280	n.d.	n.d.	n.d.
Triple mutants					
C31S/C51S/C158S	16.5	380	n.d.	n.d.	n.d.
C31S/C51S/C166S	2	290	n.d.	n.d.	n.d.
C51S/C158S/C166	6.2	410	n.d.	n.d.	n.d.
Quadruple mutants					
C31S/C51S/C158S/ C166S	2.6	450	n.d.	n.d.	n.d.

In native PAGE all single mutant proteins showed the same higher aggregates in the absence of DTT as the wt-LH (Figure 1). Therefore, we assume that more than one of the reactive cysteines needs to be replaced in order to prevent aggregation. Consequently, we constructed multiple mutants as listed in Table 1. However, these double, triple and quadruple mutants showed the same aggregation behavior as the wt-protein as evidenced by gel electrophoresis.

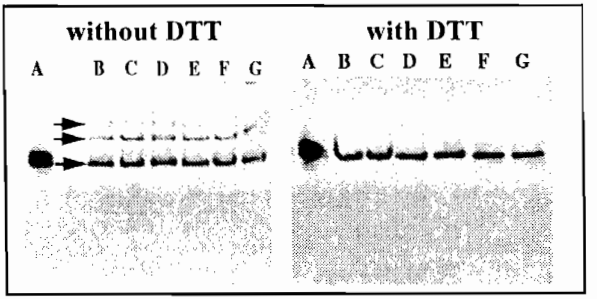


Figure 1: Native-PAGE of the single mutant proteins in the presence and abscence of DTT.
A: MCADH (M = 174.4 kDa), **B**: wt-LH; **C**:C31S; **D**: C51S; **E**: C146S; **F**:C158S;**G**:C166A.

In conclusion, the problem of LH-aggregation is not yet solved. The hypothesis that formation of intermolecular disulfide bridges is responsible for the aggregation of LH cannot be confirmed. Alternatively, the aggregation tendency of the His-tagged enzyme may be the result of cross-linking residual Ni-ions (8). In order to test this hypothesis a new expression system is beeing constructed, which allows expression of authentic, i.e. untagged LH.

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