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Site-directed mutagenesis of the FAD-binding histidine of 6-hydroxy-D-nicotine oxidase

Consequences on flavinylation and enzyme activity

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In 6-hydroxy-D-nicotine oxidase (6-HDNO) FAD is covalently bound to His^{71} of the polypeptide chain by an 8α-(N_3-histidyl)-riboflavin linkage. The FAD-binding histidine was exchanged by site-directed mutagenesis to either a Cys- or Tyr-residue, two amino acids known to be involved in covalent binding of FAD in other enzymes, or to a Ser-residue. None of the amino acid replacements for His^{71} allowed covalent FAD incorporation into the 6-HDNO polypeptide. Thus, the amino acid residues involved in covalent FAD-binding require a specific polypeptide surrounding in order for this modification to proceed and cannot be replaced with each other. Enzyme activity was completely abolished with Tyr in place of His^{71}. 6-HDNO activity with non-covalently bound FAD was found with 6-HDNO-Cys and to a lesser extent also with 6-HDNO-Ser. However, the K_m values for 6-HDNO-Cys and 6-HDNO-Ser were increased approximately 20-fold as compared to 6-HDNO-His. Both mutant enzymes, in contrast to the wild-type enzyme, needed additional FAD in the enzymatic assay (50 μM for 6-HDNO-Ser and 10 μM for 6-HDNO-Cys) for maximal enzyme activity.

6-Hydroxy-D-nicotine oxidase; Site-directed mutagenesis; Covalant flavinylation; Flavoenzyme; Covalent modification; Protein modification

1. INTRODUCTION

The prosthetic group of about a dozen flavoenzymes is covalently linked to the side chain of a particular amino acid. Involved in the protein modification by FAD are the imidazole of histidine, the SH-group of cysteine and the hydroxy-group of tyrosine [1]. Among the enzymes exhibiting an 8α-(N_3-histidyl)-riboflavin linkage, the most common covalent modification by FAD, is 6-hydroxy-D-nicotine oxidase (6-HDNO; EC 1.5.3.6) of *Arthrobacter oxidans* [2]. We showed recently that the covalent incorporation of FAD into the 6-HDNO polypeptide requires the presence of specific effectors represented by phosphopyruvate, 3-phosphoglycerate, glyceraldehyde-3-phosphate or glycerol-3-phosphate (G-3-P) [3]. Here we investigate how the replacement of the FAD-binding histidine in 6-HDNO by other amino acids known to be modified by flavins affects covalent flavinylation of the 6-HDNO polypeptide and enzyme activity.

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Abbreviations: G-3-P, glycerol-3-phosphate; 6-HDNO, 6-hydroxy-D-nicotine oxidase; IPTG, isopropyl-β-thiogalactoside

To this goal we replaced by site-directed mutagenesis the FAD-binding His^{71} of the 6-HDNO polypeptide with the amino acids cysteine, tyrosine or serine. The effects of these amino acid replacements on FAD attachment and enzyme activity are presented in this communication.

2. MATERIALS AND METHODS

2.1. *Bacterial strains, plasmids and growth conditions*

E. coli strain JM109 [4] was used as host for the expression plasmid pDB222 [5]. In vivo labeling with [^{14}C]FAD of the 6-HDNO polypeptide in the *E. coli* RR28rf strain transformed with plasmid DNA was as in [6].

Preparation of cell extracts, enzyme measurements and transformation of 6-HDNO apoenzyme into holoenzyme were as described previously [6].

Polyacrylamide gel electrophoresis (PAGE) was according to Laemmli [7] and Western blots were reacted with 6-HDNO-specific antiserum and stained with horseradish peroxidase conjugated second antibody as described by the manufacturer (Bio-Rad, Munich).

2.2. *Site-directed mutagenesis*

Site-directed mutagenesis was carried out according to Kunkel [8]. Oligonucleotides were synthesized with the aid of an automated nucleotide synthesizer by Dr Igloi (Institut für Biologie II, Freiburg, FRG). Verification of the introduced mutations was by DNA sequencing according to the dideoxynucleotide chain termination method [9].

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CGA TCC GGT GGC CAC AAT CCG AAT GGC
Arg Ser Gly Gly His Asn Pro Asn Gly      pDB222-wild-type

CGA TCC GGT GGC TGC AAT CCG AAT GGC
                **
               Cys                       pDB222-Cys

CGA TCC GGT GGC TCC AAT CCG AAT GGC
                **
               Ser                       pDB222-Ser

CGA TCC GGT GGC TAC AAT CCG AAT GGC
                **
               Tyr                       pDB222-Tyr
```

Fig.1. Site-directed mutagenesis of the FAD-binding His^{71} of 6-HDNO. Shown is the wild-type sequence surrounding the His^{71}. Mutagenic oligonucleotides were derived according to this sequence with the nucleotide exchanges and the resulting amino acid replacements as indicated.

3. RESULTS AND DISCUSSION

3.1. *Site-directed mutagenesis of the FAD-binding histidine residue*

The wild-type sequence containing the FAD-binding His^{71} and the oligonucleotides used for introduction of the mutations to serine, tyrosine and cysteine are presented in fig.1. After reconstitution of the 6-HDNO gene in pDB222, these constructs were designed pDB222-Ser, pDB222-Cys and pDB222-Tyr.

3.2. *Enzyme activity of the mutant 6-HDNO polypeptides*

In *E. coli* cells expressing 6-HDNO from recombinant plasmids, 6-HDNO activity in cell extracts can be increased by incubation with FAD and G-3-P. This increase in enzyme activity reflects the effector-dependent covalent incorporation of FAD into the apoenzyme [6]. For this reason 6-HDNO activity in extracts prepared from *E. coli* cells carrying different plasmids was measured before and after incubation with FAD and G-3-P. The observed results are presented in table 1. The assay for 6-HDNO activity was performed in 1 ml of 0.1 M glycine buffer, pH 9.0, 0.1 M NaCl and 0.5 μM 6-hydroxy-D-nicotine as substrate. Under these conditions, besides the wild-type enzyme, only the histidine to cysteine mutation exhibited enzyme activity. Incubation with FAD and G-3-P had no effect on the activity of the altered enzymes.

Table 1

Specific enzyme activity of wild-type and mutant enzymes expressed from recombinant plasmids in *E. coli* JM109 cells

Plasmids and phenotypes	6-HDNO activity ($nM \cdot s^{-1} \cdot mg^{-1}$)	6-HDNO activity ($nM \cdot s^{-1} \cdot mg^{-1}$) + FAD/G-3-P
pDB222.His (wild-type)	1.1	2.2
pDB222.Cys	0.67	0.67
pDB222.Ser	nm	nm
pDB222.Tyr	nm	nm

Enzyme activities were determined under assay conditions used for the wild-type enzyme (0.5 mM 6-hydroxynicotine and no FAD) [2]. nm, not measurable

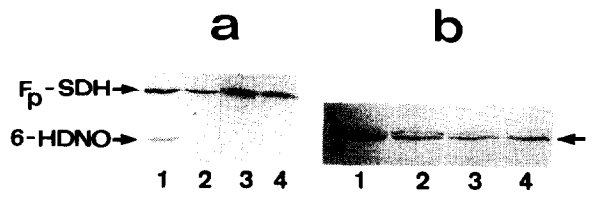

Fig.2. Expression of wild-type and mutated 6-HDNO in *E. coli* RR28rf cells. *E. coli* RR28rf cells transformed with plasmids carrying the wild-type or mutated 6-HDNO genes were grown in the presence of [^{14}C]riboflavin and cell extracts were analyzed for proteins with covalently incorporated flavins on SDS-PAGE followed by fluorography (a) and for 6-HDNO-synthesis by immunostaining of Western blots with 6-HDNO-specific antibody (b). (a) Extract from cells expressing (lanes): 6-HDNO-His (1), 6-HDNO-Cys (2), 6-HDNO-Ser (3), 6-HDNO-Tyr (4). Fp-SDH, flavoprotein subunit of succinate dehydrogenase; 6-HDNO, position of covalently ^{14}C-labeled 6-HDNO polypeptide. (b) 6-HDNO antiserum-mediated staining of polypeptides corresponding to 6-HDNO-His (1), 6-HDNO-Cys (2), 6-HDNO-Ser (3), and 6-HDNO-Tyr (4).

3.3. *[^{14}C]FAD incorporation into the 6-HDNO polypeptides*

Cell extracts from RR28rf cells grown in the presence of [^{14}C]riboflavin and expressing the wild-type and the mutant 6-HDNO genes were analyzed by SDS-PAGE for covalent FAD incorporation into the 6-HDNO polypeptide. The results of these experiments are presented in fig.2a.

One labeled polypeptide found in all cell extracts probably represents the flavoprotein subunit of succinate dehydrogenase [10]. A second labeled polypeptide representing 6-HDNO is found only in cell extracts derived from cells expressing wild-type enzyme. It is absent from extracts derived from cells expressing altered 6-HDNO. Thus, only the 6-HDNO polypeptide with His^{71} binds FAD covalently. The enzyme activity observed with 6-HDNO-Cys must therefore be accomplished with non-covalently bound FAD. Incubation of cell extracts containing the Ser-, Tyr- or Cys-6-HDNO polypeptide with [^{14}C]FAD and G-3-P did not result in covalent incorporation of the cofactor (not shown) in contrast to what is found with the 6-HDNO-His polypeptide [6].

The inability to detect covalently [^{14}C]FAD-labeled, altered 6-HDNO polypeptides did not result from an impaired biosynthesis of these proteins. Analysis of extracts from *E. coli* cells expressing wild-type and mutant polypeptides on Western blots by immunostaining with 6-HDNO-specific antibody revealed approximately equal amounts of each protein (fig.2b).

3.4. *FAD dependence of 6-HDNO activity of altered enzymes*

6-HDNO activity measured with cell extracts con-

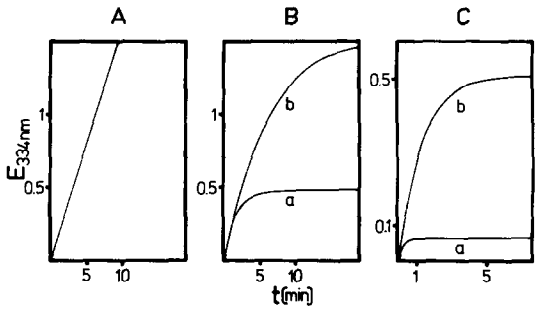

Fig.3. Time course of the 6-HDNO reaction performed with wild-type and mutant enzymes. 6-HDNO assays were performed as described in section 2. (A) Wild-type enzyme; (B) 6-HDNO-Cys, a, without addition of FAD, b, at 20 μM FAD; (C) 6-HDNO-Ser, a, without addition of FAD, b, at 50 μM FAD.

taining 6-HDNO-Cys or 6-HDNO-Ser and not supplemented with external FAD quickly plateaued (fig.3). With the altered enzymes an increase in [FAD] in the 6-HDNO assay increased the length of time during which enzyme activity could be measured (fig.3). That is, the period of linear increase in absorption at 334 nm, corresponding to the formation of the reaction product, was prolonged. This effect was not due to covalent FAD incorporation into the altered 6-HDNO polypeptide at the higher [FAD] nor was the effect observed with wild-type enzyme (not shown). Increased [FAD] in the reaction assay had, however, no significant effect on the reaction rate. This was a surprising finding, since we expected the altered enzymes to show perhaps a decreased affinity for FAD. In that case they would require higher [FAD] for saturation and full enzyme activity. That was, however, not the case. If the cofactor was only loosely associated with the mutant 6-HDNO polypeptides, it should have been possible to remove it by dialysis. When cell extracts containing 6-HDNO-Ser or 6-HDNO-Cys were extensively dialyzed against 100 mM phosphate buffer, pH 7.0, no decrease in the initially observed specific 6-HDNO activity was found. At 10 μM FAD final concentration in the reaction assays the 6-HDNO-Cys worked best. 6-HDNO-Ser required 50 μM FAD for optimal activity. Efforts are currently being made to elucidate the [FAD] effect on 6-HDNO-Cys and 6-HDNO-Ser activity by isolating the corresponding polypeptides.

From our results we may conclude the following. (i) Replacement of His^{71} with Cys, Ser or Tyr abolishes covalent binding of FAD to the apoenzyme. The amino acids Cys and Tyr used to replace His^{71} are involved in covalent binding of FAD in other enzymes. Thus, the specific features of the apo-6-HDNO polypeptide permit the establishment of a covalent bond to FAD only with the imidazole ring of histidine. (ii) Mutant 6-HDNOs with non-covalently bound FAD show enzyme activity. The extent of enzyme activity is dependent on the amino acid replacing His^{71}. 6-HDNO-Cys comes closest to the wild-type enzyme, whereas replacement of histidine with tyrosine abolishes enzyme activity. (iii) In contrast to the wild-type enzyme where covalent binding of FAD, and thus enzyme activity, can be modulated by effector molecules [6], no effect of these molecules on FAD-incorporation and enzyme activity of the altered 6-HDNO polypeptides was found.

Recently, similar results were presented in a short report on the site-directed mutagenesis of the FAD-binding histidine of fumarate reductase [11].

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