Expression and Flavinylation of *Arthrobacter oxydans* 6-Hydroxy-D-nicotine Oxidase in *Bacillus subtilis*

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6-Hydroxy-D-nicotine oxidase (6-HDNO) of *Arthrobacter oxydans*, an enzyme inducible by DL-nicotine, contains FAD covalently bound via an 8 α -N(3)His linkage. Expression of the gene encoding 6-HDNO and flavinylation of the protein were studied in *Bacillus subtilis*. In this heterologous system the following findings were made. 1. An enzymically active covalently flavinylated 6-HDNO of normal size can be expressed in *B. subtilis*. 2. The natural promoter of the 6-HDNO gene appeared inefficient in *B. subtilis*. The *B. subtilis* *sdh* promoter, when inserted upstream of the *A. oxydans* promoter, increased 6-HDNO expression > 50-fold. 3. Expression of the 6-HDNO gene from plasmids in *B. subtilis* was, independently of the promoter construct used, stimulated more than fivefold by DL-nicotine in the growth medium. It is concluded that flavinylation of 6-HDNO is possibly autocatalytic and mediated by factors generally found in bacterial cells.

INTRODUCTION

About two dozen different enzymes are known to contain a flavin covalently attached to a His, Tyr or Cys residue (Singer & McIntire, 1984). The mechanism(s) by which these covalent bonds are formed in cells is not known. Autocatalysis as well as a requirement for specific factor(s), not present in the final flavoprotein, have been proposed for the process (Decker, 1982). FAD linked via its 8 α -carbon to N-3 of His was first discovered in succinate dehydrogenase (SDH) (Salach *et al.*, 1972). The same type of covalently bound FAD is present in 6-hydroxy-D-nicotine oxidase (6-HDNO; EC 1.5.3.6) from *Arthrobacter oxydans* (Möhler *et al.*, 1972). 6-HDNO is a 48 kDa monomeric enzyme functioning in the nicotine degradative pathway of this bacterium (Brühmüller *et al.*, 1972). It is encoded on a large catabolic plasmid and enzyme synthesis is induced by DL-nicotine (Brandsch *et al.*, 1986; Gloger & Decker, 1969).

SDH, the analogous fumarate reductase, and 6-HDNO are at present the only proteins with 8 α -N(3)His FAD for which the structural gene has been isolated and sequenced (Wood *et al.*, 1984; Phillips *et al.*, 1987; Cole, 1982; Brandsch *et al.*, 1987*b*). With the cloned gene available, new approaches can be used to elucidate why the prosthetic group in particular flavoenzymes is covalently bound and how the bond is formed (cf. Cecchini *et al.*, 1988). One approach to these problems is to express the flavoprotein in a heterologous system. *Bacillus subtilis* SDH expressed from the cloned *sdh* operon in *Escherichia coli* lacks covalently bound flavin despite the fact that *E. coli* SDH in the same cell is flavinylated (Hederstedt *et al.*, 1987). The *B. subtilis* apoprotein synthesized in *E. coli* is correctly processed at the N-terminus and the cloned *sdh* operon encodes functional SDH when reintroduced into *B. subtilis*. These previous results indicate that a cell-specific factor is required or that in *E. coli* the flavinylation of the heterologous polypeptide is

Abbreviations: Ap, ampicillin; Cm, chloramphenicol; 6-HDNO, 6-hydroxy-D-nicotine oxidase; Km, kanamycin; PEP, phosphoenolpyruvate; SDH, succinate dehydrogenase.

blocked. In contrast to the *B. subtilis* SDH, *A. oxydans* 6-DHNO is flavinylated when expressed in *E. coli*, both *in vivo* and in a cell-free system (Brandsch & Bichler, 1985, 1986). This discrepancy made it interesting to determine whether FAD can be covalently bound to the 6-HDNO polypeptide in *B. subtilis*.

Bacillus and *Arthrobacter* species are of great industrial importance, e.g. as producers of enzymes and antibiotics. The two genera are evolutionarily only distantly related (Woese, 1987) but comprise Gram-positive aerobic bacteria commonly found in the soil. Comparatively little is known about gene organization and control in *Arthrobacter* species. These aspects were additional reasons to determine whether the 6-HDNO gene could be expressed and be regulated by nicotine in *B. subtilis*.

METHODS

Bacterial strains and plasmids. The strain of *A. oxydans* used was described by Eberwein *et al.* (1961).

B. subtilis 3G18 (*trpC2 ade met*) was obtained from G. Venema, University of Groningen, the Netherlands. Plasmids pDB121 (Brandsch *et al.*, 1986) and pDB222 (Brandsch *et al.*, 1987a) are both Ap^r and contain the *A. oxydans* 6-HDNO gene under the control of its natural promoter and the *E. coli* *tac* promoter, respectively. These plasmids were propagated in *E. coli* HB101 (F⁻ *hsdS20 supE44 ara-14 galK2 lacY1 proA2 rpsL20 xyl-5 mtl-1 recA13*) (Boyer & Roulland-Dussoix, 1969). *B. subtilis* plasmids pUB110 (Km^r) (Gryczan *et al.*, 1978) and pSDP4 (Km^r Cm^r) (Melin *et al.*, 1987) have been described before; pHB2 (Km^r) and pHB41 (Km^r) were constructed during this work.

General genetic techniques. Digestion of DNA with restriction endonucleases, agarose gel electrophoresis and ligation with T4 DNA ligase were done according to standard methods (Berger & Kimmel, 1987). DNA fragments separated in agarose gels were isolated by the use of GeneClean after the agarose had been dissolved in 4 M-NaI. *B. subtilis* plasmids were purified as described by Ish-Horowicz & Burke (1981) except that the cells were treated with 0.2 mg lysozyme ml⁻¹ at 37 °C for 30 min before alkaline sodium dodecyl sulphate (SDS) was added. Competent *B. subtilis* was prepared as described by Arwert & Venema (1973). Transformants were selected on plates of Tryptose Blood Agar Base (Difco) containing 5 µg Km or Cm ml⁻¹.

Construction of pHB2 and pHB41. The 6-HDNO gene from *A. oxydans* pAO1 was originally cloned in pBR322 as a 2.8 kbp *EcoRI*-*HindIII* fragment (Brandsch *et al.*, 1986). This recombinant plasmid, designated pDB121, and the *B. subtilis* cloning vector pUB110 were both digested with *EcoRI* and *Bam*HI. The 3.1 kbp fragment of pDB121 and the 3.7 kbp fragment of pUB110 were isolated from agarose gels and ligated. *B. subtilis* 3G18 was then transformed with the ligation mix and Km^r selected. Plasmid pHB2 (Fig. 1) was isolated from one of the transformants.

The plasmid pHB41 was constructed from plasmids pHB2 and pSDP4, a derivative of pPL603 (Williams *et al.*, 1981) in which the *cat-86* gene is transcribed from the *B. subtilis* *sdh* promoter. The vector part of pSDP4 including the *sdh* promoter, but only the latter half of *cat-86*, was isolated after removal of the 0.52 kbp *Pst*I-*Hind*III DNA fragment. The removed fragment was replaced by the 2.2 kbp *Pst*I-*Hind*III fragment of pHB2. The resulting plasmid pBH41, containing the *sdh* promoter in front of the 6-HDNO gene (Fig. 1), was isolated from one *B. subtilis* 3G18 Km^r Cm^s transformant.

Preparation of soluble cell extract. The bacteria were grown aerobically overnight at 30 °C in yeast-tryptone medium (YT; Berger & Kimmel, 1987) with and without added DL-nicotine (0.1%, v/v). The growth medium for plasmid-containing cells was further supplemented with 100 µg ampicillin ml⁻¹ or 5 µg kanamycin ml⁻¹, as appropriate. Cells were harvested by centrifugation at 4 °C, washed once with cold 0.1 M-sodium phosphate buffer pH 7.5 and lysed in the same buffer by sonication. *B. subtilis* cells were incubated at 20 °C for 10 min in buffer containing 0.1 mg lysozyme ml⁻¹ before sonication. The lysate was centrifuged at 12000 g for 10 min at 4 °C and the resulting supernatant was used as the soluble cell fraction.

Labelling with radioactive flavin. *In vivo* labelling with 20 µCi (740 kBq) D-[2-¹⁴C]riboflavin l⁻¹ in the growth medium has been described before (Hederstedt, 1983). For *in vitro* labelling with flavin, the cell extract was incubated with 10 µM-[¹⁴C]FAD and 8 mM-phosphoenolpyruvate (PEP) at 30 °C for 60 min.

Analytical methods. 6-HDNO activity was measured photometrically at 30 °C as described by Decker & Blegg (1965). Chloramphenicol acetyltransferase was assayed as described by Shaw (1975). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli (1970). Gels for autoradiography were treated with Amplify before they were exposed to X-ray film. Identification of 6-HDNO antigen by SDS-PAGE followed by immunoblotting was performed as before (Brandsch *et al.*, 1987a). Protein was determined by the Lowry method with serum albumin as the standard.

Fig. 1. Physical map of pHB2 and pHB41. Both plasmids are pUB110 derivatives. The fragment corresponding to *A. oxydans* DNA is indicated by a thicker line and the open reading frame of the 6-HDNO gene is marked by a box. The filled and open dots indicate the location of the *A. oxydans* natural promoter for the 6-HDNO gene and the *B. subtilis* *sdh* promoter respectively. Arrows show direction of transcription. Selected endonuclease restriction sites are indicated: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; Pv, *Pvu*II.

Materials. Restriction enzymes and T4 DNA ligase were obtained from Boehringer Mannheim; D-[2-¹⁴C]riboflavin (46 Ci mol⁻¹; 1702 GBq mol⁻¹) and Amplify were from Amersham; [¹⁴C]FAD was synthesized *in vitro* from D-[2-¹⁴C]riboflavin (Brandsch & Bichler, 1987); DL-nicotine and 6-hydroxy-D-nicotine were obtained as described previously (Decker *et al.*, 1961; Gloger & Decker, 1969). GeneClean was purchased as a kit from BIO 101 Inc. X-ray film was XR from Fuji.

RESULTS AND DISCUSSION

Expression of 6-HDNO activity in B. subtilis

To study expression of *A. oxydans* 6-HDNO in *B. subtilis*, the structural gene with flanking DNA was transferred to *B. subtilis* plasmid vectors. Two plasmids, both based on pUB110, were constructed as described in Methods. The first plasmid, pHB2, contains the 6-HDNO gene with its natural promoter (Fig. 1). The second construct, pHB41, has the *B. subtilis* *sdh* promoter region placed upstream and in tandem with the natural promoter of the 6-HDNO gene (Fig. 1).

Soluble cell extracts of *B. subtilis* harbouring pHB2 and pHB41, but not of cells containing pUB110 or pSDP4, showed 6-hydroxynicotine oxidase activity with specificity for the D-enantiomer. Extracts prepared from cells in the stationary phase of growth had higher specific enzyme activities than those of exponentially growing bacteria. Stationary phase cells were therefore used throughout this work.

Plasmid pHB41, with the *sdh* promoter placed in tandem with and in front of the natural promoter, resulted in >50-fold higher 6-HDNO activity compared to pHB2 (Table 1). This suggests that transcription, rather than translation, is limiting the expression of the protein from pHB2, assuming that the copy number is similar for pHB41 and pHB2. The promoter sequence of the *A. oxydans* 6-HDNO gene (Brandsch *et al.*, 1987b) and the consensus promoter sequence recognized by the major (sigma-43) *B. subtilis* RNA polymerase holoenzyme (Doi & Wang, 1986) are identical in the -35 region but show 3 mismatches out of 7 bases in the -10 region. The potential ribosome-binding sequence of the mRNA for the 6-HDNO gene and the 3'-end of the *B. subtilis* 16S rRNA has a calculated (Tinoco *et al.*, 1973) free energy of interaction of -58 kJ mol⁻¹ (-14 kcal mol⁻¹). This strength of interaction and also the 6 bp spacing between the ribosome-binding sequence and the TTG translational start codon are features commonly found for *B. subtilis* genes (Hager & Rabinowitz, 1985).

DL-Nicotine induces 6-HDNO in stationary-phase *A. oxydans* cells. The difference in activity between induced and non-induced bacteria is about 2000-fold. Expression of 6-HDNO activity in *B. subtilis* containing pHB2 or pHB41 was also stimulated by DL-nicotine (Table 1). In contrast and as observed before (Brandsch *et al.*, 1986), nicotine had no effect on the expression of 6-HDNO activity in *E. coli* harbouring pBD121, although this plasmid contains the same

Table 1. *Specific 6-HDNO activity in soluble cell extracts of different bacterial strains grown in the presence or absence of 0.1% (v/v) DL-nicotine*

Extracts from cells in stationary growth phase were used: all strains showed the highest 6-HDNO activity in this growth phase. Results are means $\pm$ standard deviation (number of determinations).

Strain	6-HDNO activity [mU (mg protein) ⁻¹]	
	Without nicotine	With nicotine
<i>A. oxydans</i> (pAO1)	0.1*	200 $\pm$ 12.7 (10)
<i>E. coli</i> HB101(pDB121)	13 $\pm$ 1.5 (10)	14 $\pm$ 2 (10)
<i>B. subtilis</i> 3G18(pHB2)	0.1*	0.4 $\pm$ 0.1 (3)
<i>B. subtilis</i> 3G18(pHB41)	6.5 $\pm$ 1.8 (3)	40 $\pm$ 3.8 (3)

* At the limit of detection.

Table 2. *Enhancement of 6-HDNO activity in soluble cell extracts of B. subtilis 3G18(pHB41) by incubation with FAD and PEP*

The same extracts of *B. subtilis* 3G18(pHB41) grown in the absence or presence of nicotine, and which showed 4 and 37 mU (mg protein)⁻¹ of 6-HDNO activity, respectively, were used several times in activation assays with FAD and PEP.

Extract	6-HDNO activity [mU (mg protein) ⁻¹]	
	+ FAD	+ FAD + PEP*
Without nicotine	4	12 $\pm$ 2 (5)
With nicotine	37	55 $\pm$ 4 (5)

* Results are means $\pm$ standard deviation (number of assays).

A. oxydans DNA fragment as pHB2. How nicotine can influence the expression of 6-HDNO in *B. subtilis* is not known. However, the effect was not specific since the expression of chloramphenicol acetyltransferase from the *cat-86* gene transcribed from the *sdh* promoter in pSDP4 could also be stimulated (about six-fold) by nicotine.

Flavinylation of 6-HDNO

B. subtilis 3G18(pHB41) contained 6-HDNO polypeptide of the same size as authentic 6-HDNO (Fig. 2). The increased 6-HDNO activity measured in extracts from cells grown in the presence of nicotine compared to cells grown without this alkaloid (Table 1) was accompanied by a corresponding increase in 6-HDNO protein (Fig. 2, lanes 2 and 3).

In nicotine-induced *A. oxydans* and also in *E. coli* HB101 harbouring pDB121 or the expression plasmid pDB222, a proportion of the 6-HDNO protein is in its apoenzyme form. Such apoenzyme can be flavinylated *in vitro* by incubation of soluble cell extracts with FAD and PEP (Nagursky *et al.*, 1988). It is not understood why a fraction of the 6-HDNO protein exists as apoenzyme. The relative proportion of apoenzyme to holoenzyme is not related to the rate of protein synthesis, i.e. it does not result from a limiting supply of flavin. The formation of holoenzyme during the incubation can be monitored as an increase in enzyme activity and by incorporation of [¹⁴C]FAD into the protein. A fraction of 6-HDNO expressed in *B. subtilis* 3G18(pHB41) was similarly in the form of apoenzyme, since incubation of the cell extract with FAD and PEP resulted in increased 6-HDNO activity (Table 2).

B. subtilis, but not *E. coli* cells, take up riboflavin efficiently from the growth medium and use it for FMN and FAD synthesis (Cecchini *et al.*, 1979). This property was exploited to confirm that the 6-HDNO synthesized from the *A. oxydans* gene in *B. subtilis* contains covalently bound flavin. Soluble cell extract was prepared from *B. subtilis* 3G18(pHB41) grown in the presence of [¹⁴C]riboflavin. The cell extract was then incubated with [¹⁴C]FAD and PEP to further increase

Fig. 2

Fig. 3

Fig. 2. Analysis of 6-HDNO antigens by Western blotting. Soluble cell extracts, each containing 50 μ g protein, were run in SDS-PAGE (10%, w/v, acrylamide), electroblotted to nitrocellulose membrane and stained immunochemically using 6-HDNO-specific antiserum from rabbit. Lane 1, *E. coli* HB101(pDB222); lane 2, *B. subtilis* 3G18(pHB41) grown with nicotine; lane 3, *B. subtilis* 3G18(pHB41) grown without nicotine; lane 4, *B. subtilis* 3G18(pSDP4) (negative control). The arrow indicates the position of 48 kDa 6-HDNO polypeptide. Smaller-sized antigens seen in lane 1 are proteolytic degradation products of 6-HDNO.

Fig. 3. Covalently bound flavin in cell extracts. An autoradiograph of a SDS-polyacrylamide (10%, w/v, acrylamide) gel is shown. Extract of *B. subtilis* 3G18(pHB41) grown in the presence of DL-nicotine and [14 C]riboflavin (lane 2) and then incubated *in vitro* with [14 C]FAD and PEP (lane 1). Extract of *E. coli* HB101(pDB222) *in vitro* incubated with [14 C]FAD and PEP (lane 3). About 50 μ g of protein was loaded on each lane.

radioactive labelling. The extracts were finally subjected to SDS-PAGE. Only covalently bound flavin is retained on protein after electrophoresis in the presence of SDS (Hederstedt, 1983). An autoradiograph of the resulting gel is shown in Fig. 3. Two radioactive polypeptides can be seen. The 65 kDa protein is the flavoprotein subunit of SDH. The 48 kDa band corresponds to 6-HDNO. This band was not present on autoradiographs of gels containing similarly prepared extracts from *B. subtilis* not expressing 6-HDNO. We conclude that *A. oxydans* 6-HDNO is flavinylated in *B. subtilis*.

CONCLUSION

A. oxydans 6-HDNO seems different with regard to the mechanism of flavinylation, when compared to the flavoprotein subunits of SDH and fumarate reductase. The 6-HDNO protein can be flavinylated in different organisms and it can also be flavinylated *in vitro* in the presence of FAD and PEP (Brandsch & Bichler, 1987; Nagursky *et al.*, 1988). The flavoprotein of *B. subtilis* SDH, on the other hand, is not flavinylated in *E. coli* *in vivo* (Hederstedt *et al.*, 1987) or *in vitro* even if *B. subtilis* extract is added (unpublished data). *E. coli* fumarate reductase could not be flavinylated when expressed *in vitro* in an *E. coli* coupled transcription-translation system

(Cecchini *et al.*, 1985). Furthermore, flavinylation of *B. subtilis* SDH is very sensitive to structural changes: amino acid substitutions in the flavoprotein far from the flavin-binding His residue can affect the attachment of flavin (Maguire *et al.*, 1986). 6-HDNO apparently can be flavinylated despite gross changes in the polypeptide, such as deletions and fusions (Brandsch *et al.*, 1987a). FAD is bound to a His in the N-terminal part of the flavoprotein in 6-HDNO, SDH and fumarate reductase. The primary sequence around this His in 6-HDNO shows little similarity compared with those in SDH and fumarate reductase. In the flavoprotein of SDH and fumarate reductase, but not in 6-HDNO, the FAD-His residue is preceded by a sequence typical for nucleotide-binding proteins (Brandsch *et al.*, 1987c). All these data together indicate that the mechanism for flavinylation of 6-HDNO is different from that of SDH and fumarate reductase. The flavinylation of the *A. oxydans* protein could be autocatalytic and mediated by universal cell factors, such as PEP.

Note added in proof. A mutation has been found in the -10 region of the *sdh* promoter in pSDP4 used for construction of pHB41. The mutation, a T to C change at nucleotide position 163, decreases expression about fourfold.

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