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Covalent flavinylation of 6-hydroxy-D-nicotine oxidase involves an energy-requiring process

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E. coli cells harbouring the recombinant plasmid pDB222 with the 6-HDNO gene under the control of the tac-promotor were induced with IPTG to synthesize a high amount of 6-HDNO protein. Part of this protein was present as 6-HDNO apoenzyme. The proportion of 6-HDNO apoenzyme formed could be increased when the induction of 6-HDNO synthesis by IPTG was performed in the presence of the inhibitor diphenyleneiodonium. The 6-HDNO apoenzyme thus formed could be transformed into enzymatically active holoenzyme in the presence of FAD by a process requiring an energy-generating system consisting of ATP, phospho*enol*pyruvate and pyruvate kinase. This finding suggests that an enzymatic step(s) is (are) involved in the covalent flavinylation of 6-HDNO.

6-Hydroxy-D-nicotine oxidase; Covalent flavinylation; Flavoenzyme; FAD

1. INTRODUCTION

One unsolved problem in the biosynthesis of flavoenzymes with covalently attached flavin [1] is the mechanism, enzymatic or not, of flavin attachment. 6-Hydroxy-D-nicotine oxidase, a bacterial enzyme involved in the catabolism of nicotine by *Arthrobacter oxidans* contains FAD bound via an 8α-N3(His) linkage to the polypeptide chain [2]. We cloned the corresponding gene in *Escherichia coli* and showed that in this heterologous system 6-HDNO was expressed as a covalently flavinylated polypeptide [3]. We were able to confirm previous reports [4] showing that covalent flavinylation of 6-HDNO can take place cotranslationally [5]. Here we show that under certain conditions in a genetically engineered, 6-HDNO overproducing *E. coli* strain 6-HDNO apoenzyme was synthesized. Conditions required for the transformation of this apoenzyme into enzymatically active holoenzyme are presented in this paper.

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Abbreviations: 6-HDNO, 6-hydroxy-D-nicotine oxidase; IPTG, isopropyl-β-D-thiogalactopyranoside; DPI, diphenyleneiodonium; HPLC, high-performance liquid chromatography; CCCP, carbonylcyanide chlorophenylhydrazone; PEP, phospho*enol*pyruvate; PK, pyruvate kinase; Cm, chloramphenicol

2. MATERIALS AND METHODS

DPI and 8-desmethyl-FAD were kind gifts from Dr A.R. Cross, Bristol and Dr A. Bacher, Munich, respectively. [^{14}C]Riboflavin was purchased from Amersham Buchler, Braunschweig. [^{14}C]FAD was synthesized from [^{14}C]riboflavin with the aid of a FAD synthetase preparation (a kind gift from Dr Bacher, Munich) and the [^{14}C]FAD formed was HPLC purified after acetone precipitation of the protein. ATP, PEP and PK were purchased from Boehringer, Mannheim. 6-HDNO activity was measured photometrically [6].

Expression of 6-HDNO in *E. coli* carrying the

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recombinant plasmid pDB222 [7] was induced during log phase growth of the bacterial culture by addition of 200 μM IPTG in the presence or absence of 5 μM DPI. Growth was continued for 150 min and the bacterial cells were harvested by centrifugation. Cells from 10 ml bacterial culture were resuspended in 2 ml phosphate buffer, pH 7.0 and lysed by sonication. Cell debris was removed by centrifugation at 12000 × g and the resulting S-12 supernatant was dialyzed overnight against phosphate buffer, pH 7.0, in order to remove free DPI and low molecular mass substances. From this cell extract a high-speed supernatant, S-150, was obtained by centrifugation at 150000 × g at 4°C for 2.5 h. The S-12 and S-150 were used in assays for the generation of 6-HDNO activity from 6-HDNO apoenzyme. A 6-HDNO activation assay consisted of 200 μl cell extract containing 5 μM FAD or 8-desmethyl-FAD and supplemented with an energy mixture (2.5 mM ATP, 8 mM PEP, 10 ng PK, final concentration per assay) where appropriate. Assays were incubated at 30°C, 30 μl samples were withdrawn at various intervals and 6-HDNO activity measured photometrically. Generation of ATP in cell extracts was determined according to [8].

[^{14}C]FAD incorporation into Cl_3AcOH-precipitable material was determined in 50 μl cell extract obtained from DPI-treated cells. [^{14}C]FAD was added to a final concentration of 5 μM. Samples were supplemented with the energy-generating system and cold FAD (20 μM final concentration) where appropriate and they were incubated at 30°C for 90 min. Protein was precipitated with 10% Cl_3AcOH in the cold, the protein pellet was resuspended in 100 μl 10 mM phosphate buffer and reprecipitated twice. The washed protein pellet was resuspended in 50 μl phosphate buffer, applied to a 3 MM cellulose filter paper disc and dried. The Cl_3AcOH-precipitable radioactivity was measured in a liquid scintillation counter.

3. RESULTS AND DISCUSSION

Induction of *E. coli* cells harbouring pDB222 with IPTG in the presence of DPI resulted in an S-12 with lower 6-HDNO activity compared to an S-12 where DPI was omitted during induction (fig.1, ○, ◇). Incubation of these cell extracts with

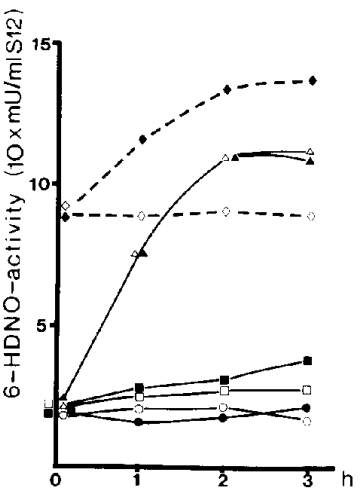

Fig.1. FAD-dependent 6-HDNO activity. Solid lines, S-12 from DPI-treated cells; broken lines, S-12 from DPI-untreated cells. (○) − FAD; (▲) + FAD; (●) + 8-desmethyl-FAD or FMN; (△) + FAD + Cm; (□) on ice; (■) + 0.01% SDS. Incubation conditions as described in section 2.

FAD resulted in both cases in an increase in 6-HDNO activity (fig.1, ▲, ◆). Apparently the 6-HDNO apoenzyme was present in both cell extracts but with more in the DPI-treated S-12 since it allowed a higher increase in 6-HDNO activity. We took this as an indication that DPI inhibits the binding of FAD to the active centre of the enzyme, possibly by competition with FAD or by modification of the protein conformation [8]. 8-Desmethyl-FAD, lacking the methyl group involved in the covalent attachment of FAD to the His residue of the polypeptide chain, or FMN cannot replace FAD in the generation of 6-HDNO activity (fig.1, ●). Thus, the observed increase in 6-HDNO activity was FAD-dependent and probably reflected FAD binding to the 6-HDNO apoenzyme synthesized under the induction conditions used.

The increase in 6-HDNO activity was not due to de novo synthesis of 6-HDNO during the incubation period, since addition of 15 μg/ml Cm did not inhibit the generation of 6-HDNO activity (fig.1, △). Also, equal amounts of material were found in cell extracts from DPI-treated or untreated cells that could be identified with the 6-HDNO antibody on Western blots obtained from denaturing polyacrylamide gels (not shown).

The increase in 6-HDNO activity could be inhibited, however, by 0.01% SDS (fig.1, ■), suggesting that a particulate fraction is required for this reaction to take place. No increase in 6-HDNO activity was found when FAD-supplemented S-12 was kept on ice (fig.1, □) and the rate of increase in 6-HDNO activity was temperature-dependent (not shown) as expected for an enzyme-catalyzed reaction. Since S-12 obtained from DPI-treated cells apparently contained a larger fraction of 6-HDNO apoenzyme, data obtained with these extracts are presented in the following. Similar results, however, were obtained with S-12 from untreated cells.

When the dialyzed S-12 was ultracentrifuged, the resulting S-150 no longer showed an increase in 6-HDNO activity on the addition of FAD alone (fig.2,○). It was found that generation of 6-HDNO activity could be restored in S-150 by the addition of an energy-generating system consisting of ATP, PEP and PK (fig.2, ▲). No increase in 6-HDNO activity was found by adding the energy-generating system in the absence of FAD (fig.2, △). Apparently, membrane fragments still capable of oxidative phosphorylation were removed from S-150 by centrifugation. This assumption was confirmed by the finding that the uncoupler of oxidative phosphorylation, CCCP, prevented the increase of 6-HDNO activity in FAD-supplemented S-12 (fig.2, ■). Measurement of ATP generation in S-12 by a coupled enzymatic assay [8] revealed that 96 pmol ATP/ml S-12 were present. No ATP production could be measured in S-150. The effect of low concentrations of SDS on the increase in 6-HDNO activity could thus be explained by the disassembly in its presence of the membrane structures carrying the enzymes of the respiratory chain.

In the presence of the energy-generating system the increase in 6-HDNO activity in S-150 is faster and reaches higher values as in the case of the S-12 extract supplemented only with FAD. However, if the system is supplemented with energy in addition to FAD, the same level of enzyme activity is also

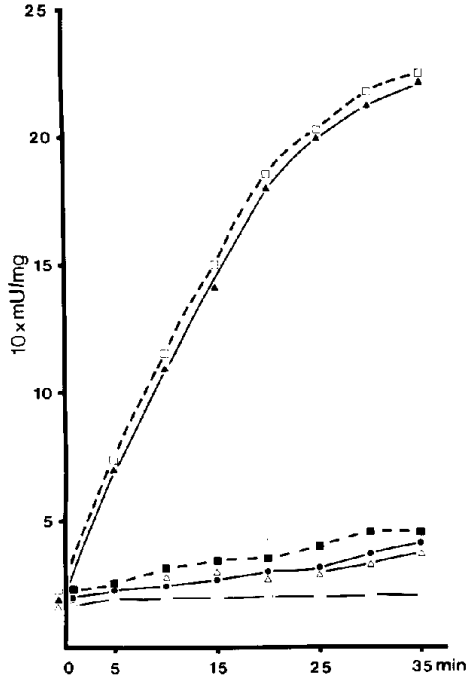

Fig.2. Energy-dependent 6-HDNO activity. Solid lines, S-150: (○) − FAD; (●) + FAD; (△) − FAD + energy-generating system; (▲) + FAD + energy-generating system. Broken lines, S-12: (■) + FAD + CCCP; (□) + FAD + energy-generating system.

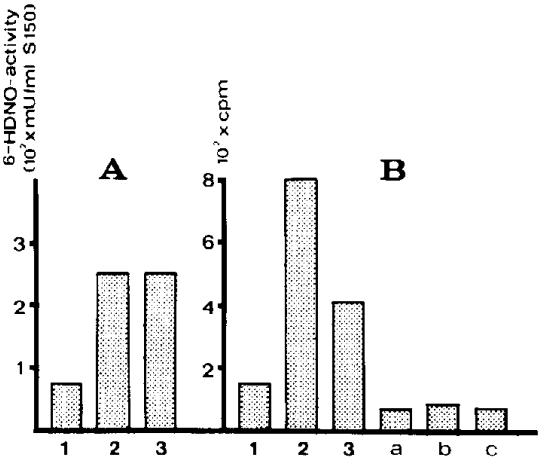

Fig.3. Correlation between increase in 6-HDNO activity and covalent [^{14}C]FAD incorporation. All samples contained [^{14}C]FAD at 5 μM. (A) Enzyme activity of 30 μl S-12 from DPI-treated cells containing the following additions: 1, [^{14}C]FAD; 2, [^{14}C]FAD + energy; 3, as 2 + 20 μM cold FAD. (B) Cl_3AcOH-precipitable [^{14}C]FAD from samples shown in A (lanes 1–3). Lanes a–c: background [^{14}C]FAD incorporation into S-12 from *E. coli* cells harbouring pBR322, same addition as in lanes 1–3, respectively.

reached in S-12 (fig.2, □). Purified 6-HDNO incubated under the same conditions showed no increase in enzyme activity with or without added cell extract (not shown). Thus, the increase in enzyme activity was due to the supplementation of 6-HDNO apoenzyme rather than to a factor-mediated activation of 6-HDNO holoenzyme. If the measured increase in 6-HDNO activity is indeed the result of covalent incorporation of FAD into 6-HDNO, then this increase in enzyme activity should be paralleled by an increase in Cl_3AcOH-precipitable [^{14}C]FAD radioactivity in the corresponding samples. This is indeed the case as is shown in fig.3.

As can be seen from fig.3A, addition of [^{14}C]FAD alone or [^{14}C]FAD plus energy led to the expected increase in 6-HDNO activity (fig.3A,1,2). Addition of cold FAD to 20 μM as a competitor for [^{14}C]FAD did not affect 6-HDNO activity (fig.3A,3). Cl_3AcOH-precipitable radioactivity from corresponding samples is presented in fig.3B. As background control an S-12 from *E. coli* cells harbouring pBR322 was used (fig.3B,a–c). From the results presented in fig.3, it becomes obvious that the increase in enzyme activity is closely followed by an increase in covalently bound [^{14}C]FAD. The effect is specific, since cold FAD competed with [^{14}C]FAD, resulting in a reduction of Cl_3AcOH-precipitable radioactivity (fig.3B, 2). The amount of cpm incorporated corresponded to the expected amount of 6-HDNO apoenzyme transformed by FAD attachment into enzymatically active holoenzyme. These findings suggest that the observed energy-dependent increase in 6-HDNO activity indeed represents covalent FAD binding to the 6-HDNO apoenzyme.

In conclusion, we have demonstrated that under conditions of high 6-HDNO synthesis, 6-HDNO apoenzyme was formed. The transformation of this apoenzyme into holoenzyme, i.e. the covalent binding of FAD to the apoenzyme, is an energy-requiring process. This explains in our opinion why, until now, only cotranslational flavinylation of 6-HDNO was found. Our findings strongly suggest the involvement of some enzymatic step(s) in the covalent flavinylation of 6-HDNO. The nature of this reaction and the hypothetical enzyme(s) involved are currently under investigation.

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