In vivo and in vitro expression of the 6-hydroxy-D-nicotine oxidase gene of *Arthrobacter oxidans*, cloned into *Escherichia coli*, as an enzymatically active, covalently flavinylated polypeptide

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Received 12 September 1985

The 6-hydroxy-D-nicotine oxidase gene of *Arthrobacter oxidans* was cloned into *E.coli* with the aid of the expression vector pKK223-3. This enzyme, as well as the *E.coli* enzymes succinate dehydrogenase and fumarate reductase, bears the cofactor FAD covalently attached to the polypeptide through a His-N_3-8α-linkage. The amino acid sequence surrounding the histidine residue involved in FAD binding in 6-hydroxy-D-nicotine oxidase and the two *E.coli* enzymes, however, show no homology. Nevertheless, 6-hydroxy-D-nicotine oxidase is expressed in *E.coli* in vivo and in an *E.coli*-derived coupled transcription-translation system as a covalently flavinylated, enzymatically active polypeptide.

(Arthrobacter oxidans) *6-Hydroxy-D-nicotine oxidase* *Covalent flavinylation* *Coupled transcription-translation*

1. INTRODUCTION

Several flavoenzymes of prokaryotic as well as of eukaryotic origin are known to contain the flavin moiety covalently bound to the enzyme [1]. The mechanism of flavin attachment, enzyme catalyzed or nonenzymatic, is still a matter of dispute [2] and the functional sigificance of this type of binding is not obvious [1]. In the *E. coli* enzymes SDH and FR, 2 genetically closely related proteins [3], the covalent bond is formed between the 8-methyl group of the isoalloxazine ring of FAD and the N_3 of histidine [4]. In 6-HDNO from *Arthrobacter oxidans* we have the same type of binding but the amino acid sequence of the FAD-binding peptide is different [5]. The question of whether the covalent binding of FAD is dependent on the recognition by a specific enzyme of a certain amino acid sequence surrounding the histidine residue to be flavinylated was approached by cloning the 6-HDNO gene into *E. coli* [6] and an attempt to express an enzymatically active, covalently flavinylated polypeptide. Here we show, to our knowledge for the first time, that an enzyme with an amino acid sequence of the flavin peptide different from that of the *E. coli* enzymes SDH and FR is made in vivo and in an in vitro coupled transcription-translation system as an enzymatically active, covalently flavinylated polypeptide.

Abbreviations: 6-HDNO, 6-hydroxy-D-nicotine oxidase; SDH, succinate dehydrogenase; FR, fumarate reductase; DNBD, dinucleotide-binding domain; kb, kilobases

2. MATERIALS AND METHODS

[^{14}C]Riboflavin was purchased from Amersham Buchler, Braunschweig, [^{14}C]FAD was prepared as described by Brühmüller and Decker [7]. Protein A-Sepharose was from Pharmacia, Freiburg i.Br.; restriction endonucleases were obtained from Boehringer, Mannheim. The 6-HDNO gene was cloned as a 2.8 kb *Eco*RI-*Hin*dIII DNA restriction

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

fragment derived from pA01 [6] and inserted into the corresponding restriction sites of pKK223-3 [8] giving rise to pDB221. pKK223-3 is a derivative of pBR322 carrying the *tac* promoter in front of a polylinker sequence. Expression of 6-HDNO in pDB221 most probably starts from the *tac* promoter (not shown). *E. coli* HB101 [9] was used as host strain. pDB 221 and pBR322 DNA was prepared and purified on CsCl gradients according to [9].

[^{14}C]Riboflavin labeling of *A. oxidans* and *E. coli* whole cells was done according to Hederstedt [10]. Cell-free extracts of labelled cells were precipitated with 6-HDNO-specific antiserum coupled to protein A-Sepharose [11]. The precipitated peptides were separated on 7.5% polyacrylamide gels, fixed, stained and prepared for fluorography. 6-HDNO activity in transformed *E. coli* HB101 cells was determined spectrophotometrically [12].

pDB221 and pBR322 DNA was used to prime an *E. coli*-derived in vitro coupled transcription-translation system as described by Müller and Blobel [13]. In vitro synthesized polypeptides were labelled with [^{35}S]methionine, precipitated with 6-HDNO antiserum and analysed on 10–15% polyacrylamide gradient gels, followed by fluorography. 6-HDNO activity synthesized in the coupled transcription-translation system was immunoprecipitated from ten 25-μl assays with protein A-Sepharose-bound antiserum. The bound 6-HDNO activity was determined by luminol-mediated chemiluminescence as described by Hinkkanen et al. [14]. [^{14}C]FAD incorporation into in vitro-made polypeptides was measured on the immunoprecipitates obtained as described above, synthesized in the presence of 1 μM [^{14}C]FAD. The immunocomplex was dissociated by boiling in PAGE sample buffer and the protein-bound radioactivity determined in a liquid scintillation counter.

3. RESULTS AND DISCUSSION

In pDB221-transformed *E. coli* HB101 cells the specific 6-HDNO activity is about 60 mU as compared to approx. 80 mU/mg in DL-nicotine-induced *A. oxidans* cells. Is this 6-HDNO synthesized in *E. coli* HB101 cells also covalently flavinylated? To answer this question we labelled *E. coli* cells harbouring pDB221 or pBR322 and *A. oxidans* cells with the FAD precursor [^{14}C]riboflavin and precipitated cell-free extracts obtained from these cells with 6-HDNO-specific antiserum. Although *E. coli* cells take up [^{14}C]riboflavin very poorly, they were sufficiently labelled to identify by immunoprecipitation one labelled polypeptide in cells harbouring pDB221, but not pBR322 (fig.1, lanes 2 and 3). From *A. oxidans* cells 2 labelled polypeptides were precipitated (fig.1, lane 1); the major band of 48 kDa corresponds to 6-HDNO [15] and a second one of approx. 70 kDa. The labelled polypeptide identified in *E. coli* cells has an apparent molecular mass of 52 kDa and, thus is about 4 kDa larger than the authentic 6-HDNO made by *A. oxidans*. The significance of the coprecipitated 70 kDa polypeptide in *A. oxidans* in relation to 6-HDNO as well as the expression of the 6-HDNO gene in *E. coli* as a larger polypeptide are currently under investigation. The immunoprecipitated polypeptides are covalently

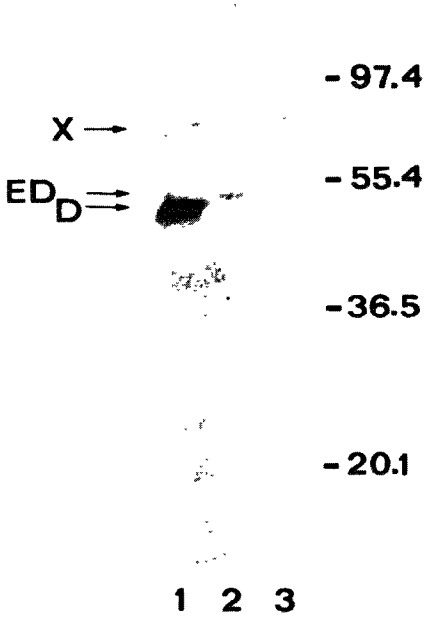

Fig.1. In vivo [^{14}C]riboflavin-labelled polypeptides precipitated with 6-HDNO-specific antiserum from: (1) *A. oxidans* cells, (2) *E. coli* HB101 cells harbouring the recombinant plasmid pDB221, (3) *E. coli* HB101 cells harbouring pBR322.D, 6-HDNO from *A. oxidans;* ED, labelled polypeptide precipitated from *E. coli* cells; X, unidentified coprecipitated labelled polypeptide.

flavinylated since fixing of the polyacrylamide gels in acetic acid and treatment for fluorography removes noncovalently bound FAD [10].

When the [^{35}S]methionine-labelled polypeptides synthesized in an *E. coli* coupled transcription-translation system primed with pDB221 were compared to those synthesized with pBR322, one major additional polypeptide of approx. 52 kDa was synthesized in the pDB221-primed assays (fig.2A). No polypeptide was precipitated with 6-HDNO-specific antiserum from pBR322-primed assays (fig.2B, lane 1), but from pDB221 assays the 52 kDa polypeptide was recovered (fig.2B, lane 2). The coprecipitated low molecular mass material probably represents run-off products. Antibody-bound 6-HDNO is enzymatically active [14]. Immunoprecipitated from in vitro assays and bound to protein A-Sepharose it can be used directly for the determination of enzyme activity. Only

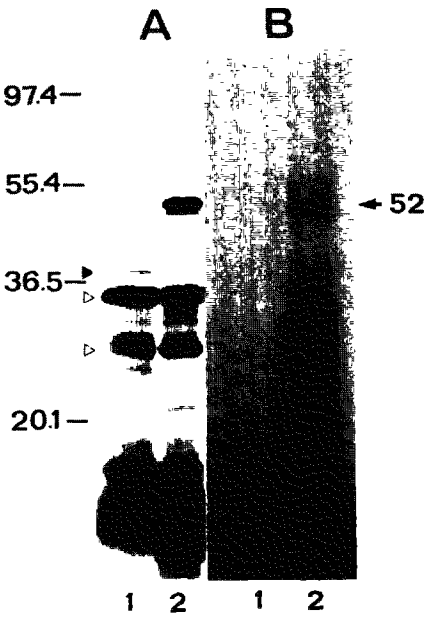

Fig.2. [^{35}S]Methionine-labelled polypeptides synthesized in an *E. coli*-derived coupled transcription-translation system and analyzed by PAGE. (A) Total labelled polypeptides: (1) pBR322-primed system, (2) pDB221-primed system. (B) Polypeptides precipitated with 6-HDNO-specific antiserum from pBR322 (1)- and pDB221 (2)-primed systems. (►) tet-gene product, (▷) β-lactamase gene product. Arrow indicates 52 kDa polypeptide. Molecular mass standards are indicated in kDa.

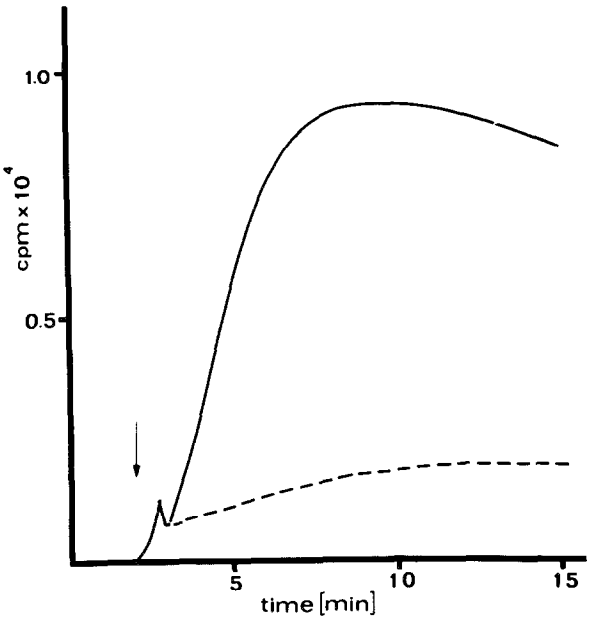

Fig.3. Luminometric determination of 6-HDNO activity in an *E. coli*-derived coupled transcription-translation assay. (——) pDB221-primed assays, (– – –) pBR322-primed assays. Arrow indicates start of reaction.

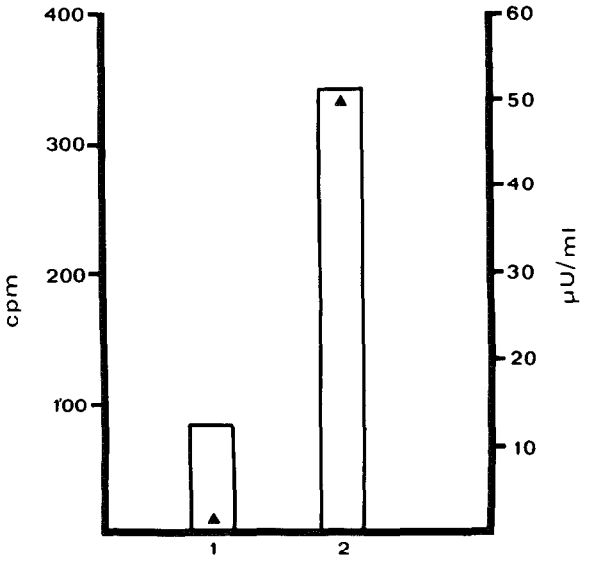

Fig.4. [^{14}C]FAD binding and 6-HDNO activity in *E. coli*-derived coupled transcription-translation assays. (1) Assays primed with pBR322, (2) assays primed with pDB221. (▲) Enzyme activity, (bars) bound [^{14}C]FAD.

	I DNBD	II covalent attachment side
SDH [3]	Gly-X-Gly-X-X-Gly	.....Lys-Val-Phe-Pro-Thr-Arg-Ser-His-Thr-Val-Ser-Ala
FR [17]	Gly-X-Gly-X-X-Gly	.....Lys-Val-Tyr-Pro-Met-Arg-Ser-His-Thr-Val-Ala-Ala
6-HDNO [5]	?	Ser-Gly-Gly-Asn-Asn-Pro-Asp-His-Tyr-Gln-Pro-Ala
OR [16]	Xh-Xh-Gly-X-Gly	.....Lys-Val-His-Pro-Asn-Ser-Val-His-Ile-Cys-Ala-Val

Fig.5. Comparison of the FAD-binding domains of different enzymes.

pDB221-primed transcription-translation assays produced 6-HDNO activity (fig.3). When [^{14}C]FAD was added to these assays, protein-bound label was coprecipitated with 6-HDNO activity by 6-HDNO antiserum (fig.4). This means that not only in vivo *E. coli* HB101 cells harbouring pDB221, but also in the in vitro transcription-translation system, 6-HDNO was made as an enzymatically active, covalently flavinylated polypeptide.

Our results are the first indication that it is possible to express in a heterologous system the same covalently flavinylated enzyme as in the organism of origin. A comparison of the amino acid sequences of the FAD-binding peptide of 6-HDNO and the corresponding domains of SDH and FR from *E. coli* shows no obvious homology, with the exception of the common His residue involved in the covalent FAD binding (fig.5). In general, no amino acid sequence homology was found between the FAD peptides of various origin [1]. On the other hand, NADPH-dependent cytochrome P-450 oxidoreductase, cloned into *E. coli,* was shown to possess strong homologies in the FAD-binding domain to SDH and FR [16], yet carries the cofactor noncovalently bound. It differs, however, in the DNBD from SDH and FR ([18], table 1). These findings argue, in our opinion, against an enzymatic mechanism of covalent flavinylation. We consider, however, as a prerequisite for such a type of binding the presence of the DNBD Gly-X-Gly-X-X-Gly- at the N-terminal part of the protein. The cofactor first bound to this domain is later on attached cotranslationally [1], covalently or noncovalently, to the protein, depending on the presence of a histidine residue placed in the right position [1]. The prediction of this hypothesis is that 6-HDNO, as well as all other enzymes with covalently bound FAD, should exhibit at their N-terminal part the dinucleotide-binding consensus sequence mentioned above. The establishment of the nucleotide sequence of the 6-HDNO gene, currently in progress, will provide an answer to this assumption.

ACKNOWLEDGEMENTS

We wish to thank Professor K. Decker for valuable suggestions and for critical reading of the manuscript. This work was supported by a grant from the Deutsche Forschungsgemeinschaft, Bonn, through SFB 206.

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