Riboflavin 5′-Hydroxymethyl Oxidation

MOLECULAR CLONING, EXPRESSION, AND GLYCOPROTEIN NATURE OF THE 5′-ALDEHYDE-FORMING ENZYME FROM SCHIZOPHYLLUM COMMUNE

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(Received for publication, April 21, 1997)

Vitamin B₂-aldehyde-forming enzyme catalyzes oxidation of the 5′-hydroxymethyl of riboflavin to the formyl group. We have purified the enzyme from the culture media of Schizophyllum commune (ATCC 38719) by modifying the procedure of Tachibana and Oka (Tachibana, S., and Oka, M. (1981) J. Biol. Chem. 256, 6682–6685) for cell-free extract. By SDS-polyacrylamide gel electrophoresis, the enzyme appears to be 78 kDa. The enzyme has a blocked amino terminus, so fragments were obtained by cleaving the purified enzyme with lysyl endopeptidase. Selected peptides were sequenced from their amino termini. We have isolated a full-length cDNA clone using a DNA hybridization probe amplified by polymerase chain reaction with two degenerate oligonucleotide primers, the design of which was based on one of the partial amino acid sequences. From the cDNA clone, it is evident that the enzyme has a Ser/Thr-rich fragment near the COOH-terminal Asp. The enzyme was determined to be a glycoprotein; however, O-deglucosylation only slightly affects activity. Computer searches showed that the B₂-aldehyde-forming enzyme has little homology with other proteins, but domain motifs may reflect N-myristoylation of a dehydrogenase with a signature similar to 4Pe-4S ferredoxins. The enzyme cDNA was subcloned into a Pichia expression vector pPIC9K to produce a recombinant protein which exhibited B₂-aldehyde-forming enzyme activity.

The metabolism of riboflavin is better understood in terms of enzymes responsible for the biosynthetic conversion of this vitamin to its functional coenzymes than as concerns enzymes catalyzing catabolic degradation of the vitamin (1). Complete purification of flavokinase (2), which catalyzes formation of FMN, and FAD synthetase (3), have led to significant understanding of flavoenzyme formation and its kinetic control (4); however, not one of the enzymes responsible for degradation of riboflavin had been sequenced or obtained in sufficient quantity to allow suitable molecular characterization.

The considerable extent to which riboflavin is apparently catabolized in the human was reflected by early balance experiments in adult men (5). Although diverse flavin catabolites have been described in nature (6), little has been done to elucidate the enzyme systems responsible for their formation. Certain bacteria have been found able to degrade the flavin isosalloxazine ring (7, 8) as well as d-riboflavin chain (9, 10) and its analogues (11). Oxidations of flavin 7- and 8-methyl functions lead to the corresponding hydroxymethyl compounds found in urine (12–14) and milk (15, 16) and to 7α-hydroxymethyl-riboflavin as the main catabolite in human plasma (17). The enzyme system responsible for forming so-called “schizoflavins,” which are the aldehyde and carboxylic acid derived from oxidation of the terminal 5′-hydroxymethyl of the riboflavin side chain (18), was purified from the fungus Schizophyllum commune by Tachibana and Oka (19, 20). Our laboratory first ascertained the rather narrow flavin specificity of the S. commune riboflavin 5′-hydroxymethyl oxidase (21). However, the trace of protein obtained in previous studies was insufficient to allow characterization other than indirect indications that a conventional alcohol pyridine-nucleotide dehydrogenase or even an azide-sensitive cytochrome P450-dependent system did not seem to be involved (20).

As will be described in this paper, we have now succeeded in modifying the original purification to obtain enzyme from which peptide fragments were derived to ultimately obtain a complete cDNA for reflecting the primary sequence of the enzyme, further to express its activity in Pichia pastoris, and additionally to determine that deglycosylation has little effect on the overall activity.

EXPERIMENTAL PROCEDURES

Growth of Microorganism—S. commune (ATCC 38719) was obtained from the American Type Culture Collection and maintained on YM (Difco) agar plates. Seed cultures were prepared by cultivation in a YM medium with shaking on a reciprocal shaker for 3 days at 30 °C. Large scale cultures (4 liters) were prepared by inoculation with 0.2 liter of seed culture with shaking as before. Mycelia were separated from culture media by filtration onto cheesecloth. The resulting culture medium was used for enzyme purification.

Enzyme Assay—B₂-aldehyde-forming enzyme activity was assayed by the method described by Tachibana and Oka (19). The rate of dichlorophenol indophenol reduction was monitored by the decrease in absorbance at 600 nm using a Milton Roy spectrophotometer at 25 °C. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of dichlorophenol indophenol/min at 25 °C as calculated from its molar extinction coefficient (7.6 × 10³ at pH 5.5). Protein was determined by the method of Bradford (22) using bovine serum albumin as standard.

Purification of B₂-aldehyde-forming Enzyme—Four liters of culture media of S. commune was brought to 50% saturation by addition with stirring of solid ammonium sulfate at 4 °C. The resultant precipitate was removed by centrifugation for 30 min at 25,000 × g. The supernatant fluid at 4 °C was then brought to 90% saturation by further addition with stirring of solid ammonium sulfate. After standing overnight the resultant precipitate was collected by centrifugation for 30 min at 25,000 × g. The precipitate was resuspended and dialyzed overnight against 50 mM sodium acetate buffer, pH 5.0. The enzyme solution was then applied to a DEAE-Sepharose column (4 × 40 cm) equilibrated with the pH 5 acetate buffer. After removal of the unbound material, the column was eluted with 1 liter of 0–1 M linear NaCl...
performed in 0.1 mg/ml of sheared, denatured, herring sperm DNA. The final wash was derived from one peptide were synthesized: 5
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nondenatured gel was resolved on SDS-PAGE 1 and transferred to a purified poly(A)
(512-fold degeneracy). These degenerate primers were used in the po-

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phoresis; bp, base pair(s); RACE, rapid amplification of cDNA ends; bp, base pair(s).

Affinity chromatography with flavin immobilized gel has also been used for the final purification step. Flavin immobilized gel was synthe-
sized by the method described by Kasai et al. (23). The partially purified enzyme from the DEAE-Sepharose column was subjected to an affinity column (1 \times 10 cm) equilibrated with the pH 5 acetate buffer. The column was washed with the buffer until the washes were nearly free of 280-nm-absorbing material. The desired protein was then eluted with the buffer containing 0.1 mM riboflavin. The fractions containing the enzyme activity were excised and homogenized with minimal volume of the pH 5 acetate buffer.

For the purpose of partial amino acid sequencing, the enzyme solution was concentrated by ultrafiltration to a total volume of 1 ml, applied to a nondenaturing gel, and electrophoresed at 4 °C. The loca-
tion of the enzyme activity on the gel was determined as described by Tachibana and Oka (19). The protein band containing the enzyme activity was excised and homogenized with minimal volume of the pH 5 acetate buffer.

Affinity chromatography with flavin immobilized gel was also used for the purification, we have chosen the DEAE-Sepharose column as described under “Experimental Proce-
sures.”

RESULTS AND DISCUSSION

Enzyme Purification and Sequencing—In the previous method for the purification of B₂-aldehyde-forming enzyme from S. commune, only a trace of purified enzyme was obtained from very large amounts of mycelia because of very low specific activity in the cell-free extract (19). We have found that the culture medium of S. commune also contains the enzyme with a specific activity about 15-fold higher than that of cell-free extract. So it is possible to obtain pure enzyme from the culture media in greater yield and in much shorter time. To facilitate the purification, we have chosen the S. commune (ATCC 38719) strain, which secreted very small amounts of polysaccharide to the media (26). Table I summarizes the partial purification of B₂-aldehyde-forming enzyme from the S. commune culture me-

### Table I

| Purification step | Volume (ml) | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Yield (\%) |
|------------------|-------------|--------------------|-----------------------|----------------------------|------------|
| Culture supernatant | 4000 | 852.0 | 3.25 | 0.0038 | 100 |
| (NH₄)₂SO₄ | 200 | 53.9 | 2.81 | 0.052 | 86.5 |
| DEAE-Sepharose chromatography | 14 | 5.6 | 1.90 | 0.34 | 58.5 |

**FIG. 1. SDS-polyacrylamide gel electrophoresis of S. commune B₂-aldehyde-forming enzyme.** Lane a, 20 µg of protein obtained from the DEAE-Sepharose column as described under “Experimental Proce-
sures.” Lane b, 5 µg of the purified enzyme obtained from the nonde-
natured gel.

chased from Boehringer Mannheim. Total RNA for the 5'-RACE exper-
iments was isolated from 1 g of lyophilized mycelia as described above. Primers for the 5'-RACE experiments were GSP1 (AAAGTCGAAAGC- CTCGGTTCA) and GSP2 (AGGGGAGAGGATAGAAAG); both were de-
signed with the cDNA sequence as shown in Fig. 3. The 5'-RACE products were separated by agarose (1%) gel electrophoresis. Each product was then purified from the agarose gel using a GeneClean II kit from BIO 101, Inc. (Vista, CA) and subcloned using a TA cloning kit (Invitrogen), and subclones containing the 5'-RACE products were sequenced.

**DNA Sequencing**—A Sequenase kit (U. S. Biochemical Corp.) was used for plasmid sequencing. Sequence analysis was performed using the sequence analysis software package (GCC) of the University of Wisconsin Genetics Computer Group (24) and other tools via the World Wide Web.

**Expression of B₂-aldehyde-forming Enzyme in P. pastoris**—A Pichia expression kit (Invitrogen) was used for functional expression of the enzyme in P. pastoris. The pPIC9K vector (Invitrogen) was selected to construct the expression plasmid. P. pastoris strain KM71 was main-
tained and transformed as described in the manual version F of the Pichia expression kit. His⁺ transformants in KM71 were purified on regeneration dextrose plates (Invitrogen) without histidine. Gene integration was detected using a rapid DNA dot blot technique (25). To express the enzyme, a single colony of multiple integration transformants was inoculated into 25 ml of pH 6-buffered complex methanol media (Invitrogen). After growing at 30 °C with shaking (250 rpm) for 18 h, cells were harvested by centrifuging and transferred to 250 ml of pH 6-buffered complex methanol media (Invitrogen) with 1% casamino acids. The culture was then returned to the incubator to continue growth for 72 h.

**Deglycosylation of B₂-aldehyde-forming Enzyme**—An enzymatic de-
glycosylation kit (Bio-Rad) was used to enzymatically cleave all possible N- and O-linked oligosaccharides from the B₂-aldehyde-forming en-
zyme. Partially purified enzyme from the affinity purification step was used in deglycosylation reactions using a denaturing protocol described by the manufacturer. The effect of deglycosylation with only O-glycosi-
dase on the B₂-aldehyde-forming enzyme activity was examined using a nondenaturing protocol as described by the manufacturer.

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1 The abbreviations used are: PAGE, polyacrylamide gel electro-
phoresis; bp, base pair(s); RACE, rapid amplification of cDNA ends; bp, base pair(s).

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**TABLE I**

Partial purification of B₂-aldehyde-forming enzyme from S. commune culture media

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natured gel.
dia. After the DEAE-Sepharose chromatography step, the enzyme was purified about 90-fold based on the activity of the culture supernatant. Analysis of the partially purified enzyme by SDS-PAGE showed that there are five major bands on the gel (Fig. 1). In the previous method (19), Sephadex G-100 chromatography was used for the final purification. However, a substantial loss in yield in this step has been found. For the purpose of partial amino acid sequencing, we have separated the partially purified enzyme on a nondenaturing gel. The final product of this purification showed a single protein with an apparent molecular mass of 78 kDa on SDS-PAGE (Fig. 1). But this also resulted in a significant activity loss. A potential procedure for the final purification step is to use flavinyl-
agaroses as bioselective affinity materials as described by Merrill and McCormick (27). This technique has been successfully used for the purification of proteins that bind riboflavin, such as flavokinase from rat liver (27, 28) or from small intestine (23). The elution pattern for affinity chromatography of the B₂-aldehyde-forming enzyme is shown in Fig. 2. The addition of 0.1 mM riboflavin released the enzyme in 20% yield. The rest of the enzyme activity was eluted with 0.1% Tween 20, suggesting additional binding to the matrix.

The purified enzyme obtained from the nondenaturing gel was transferred to a polyvinylidene difluoride membrane for amino acid sequencing. Attempts to directly sequence the polypeptide demonstrated that it had a blocked amino terminus. Therefore, the purified enzyme was cleaved with lysyl endopeptidase. Peptide fragments were separated by high performance liquid chromatography, and selected peptides were sequenced. Two resultant amino acid sequences (underlined) are shown in Fig. 3.

cDNA Sequencing—A 78-bp probe was amplified from *S. commune* cDNA with two degenerate primers as described under “Experimental Procedures” and was used for screening the *S. commune* cDNA library. One phage that showed the strongest hybridization to the probe was converted into pBlue-script SK(-) (Stratagene) by in vivo excision. This recombinant plasmid, designated pBRAF, was then sequenced (Fig. 3). The whole sequence contained 1080 bp with a 19-mer poly(A) tail at its 3’ end. To obtain the complete coding region for the enzyme, we employed the 5’-RACE procedure. No more nucleotide sequence at the 5’ end of the B₂-aldehyde-forming enzyme cDNA was found from the two major 5’-RACE products. An open reading frame in the cDNA was identified with ATG as putative start codon 304 bp from the beginning of the insert. This open reading frame encodes a polypeptide matching the amino acid sequences of the peptides previously determined (underlined). The calculated coding sequence and predicted amino acid sequence (single-letter amino acid code) of the *S. commune* vitamin B₂-aldehyde-forming enzyme. The open reading frame, defined by assigning the initiation codon ATG (at position 304), is in frame with the amino acid sequences of the peptides previously determined (underlined). The translation stop code (TGA) is shown with an asterisk.
molecular weight and pI value of the encoded polypeptide are 20,384 and 3.6, respectively. Computer searches with known sequences showed that this sequence has little similarity to other proteins. However, searches on the PRINTS data base (29) via World Wide Web exhibited three motif fragments in the predicted amino acid sequence. The fragment from position 41 to 52 matches to a 4FE4SFRDOXIN motif, which is a two-element fingerprint that provides a signature for 4Fe-4S ferredoxins; fragment 61–71 matches to a BCCTRLSENSOR motif, which is a four-element fingerprint that provides a signature for the bacterial sensor proteins; fragment (186–197) is similar to a GLFDHDRGNASE motif, which is a four-element fingerprint that provides a signature for glutamate, leucine, and phenylalanine dehydrogenases. These latter are NAD- and/or NADP-dependent enzymes (30–32). However, Tachibana and Oka (20) have demonstrated that neither NAD nor NADP function as coenzyme for B2-aldehyde-forming enzyme, although binding of NADPH stimulates aldehyde-forming activity under aerobic conditions. ScanProsite searches showed that there were three potential N-myristoylation sites near the NH2 terminus (fragments 1–5, 20–25, and 24–29). These results suggest that the NH2-terminal block is due to the N-myristoylation.

In the predicted amino acid sequence, there is a Ser/Thr-rich fragment (98–176) near the COOH-terminal Asp residue. BLAST searches with this fragment as a query sequence revealed that many glycoproteins have similar Ser/Thr-rich sequences near their COOH termini. One of these glycoproteins, acid phosphatase from Leishmania mexicana, has been found to have its Ser/Thr-rich domains serve as targets for O-linked modification of phosphoserines by mannooiglucosacharides and phosphoglycans (33). It has been found that the calculated molecular mass of the encoded polypeptide (20.4 kDa) is much less than that (78 kDa) revealed by SDS-PAGE (cf. Fig. 1). Because no more nucleotide sequence at the 5' end of the B2-aldehyde-forming enzyme cDNA was found using the 5'-RACE procedure, it appeared that B2-aldehyde-forming enzyme is also a glycoprotein with saccharides O-linked to its Ser/Thr-rich domain. No consensus Asn-X-Thr/Ser sequence for covalent attachment of N-linked glycans was found in the predicted amino acid sequence. Deglycosylation substantiated that B2-aldehyde-forming enzyme is a glycoprotein with an expected polypeptide size of approximately 20 kDa (Fig. 4). The removal of O-linked oligosaccharides from B2-aldehyde-forming enzyme has only a slight effect on the enzyme activity (data not shown). This implies that O-linked oligosaccharides are not essential for the catalysis.

Expression of B2-aldehyde-forming Enzyme Activity—The methylotrophic yeast P. pastoris has been developed as an efficient system for high-level production of foreign proteins (34, 35). We therefore chose the P. pastoris expression system (Invitrogen) to express the B2-aldehyde-forming enzyme activity. Because the enzyme is glycosylated and then secreted by the fungus, we have selected a vector pPIC9K (Invitrogen) that has an α-factor signal sequence to secrete the protein. A plasmid, derived from pPIC9K and designated pPICRAF9K, for the expression of the B2-aldehyde-forming enzyme was constructed. In plasmid pPICRAF9K, polymerase chain reaction was used to generate an EcoRI site before the first ATG and a NotI site after the poly(A) end in the B2-aldehyde-forming enzyme cDNA. After digestion with EcoRI/NotI, the polymerase chain reaction product was cloned into the pPIC9K vector, which had also been digested with EcoRI/NotI. DNA sequencing showed that the inserted fragment (304–1080 in Fig. 3) is in frame with the secretion signal open reading frame of protein from a pPICRAF9K transformant (filled circles) and a control transformant with pPIC9K (open circles). B2-aldehyde-forming enzyme activity from a pPICRAF9K transformant (filled circles) and a control transformant with pPIC9K (open triangles) was used to generate an EcoRI site before the first ATG and a NotI site after the poly(A) end in the B2-aldehyde-forming enzyme cDNA. After digestion with EcoRI/NotI, the polymerase chain reaction product was subcloned into the pPIC9K vector, which had also been digested with EcoRI/NotI. DNA sequencing showed that the inserted fragment (304–1080 in Fig. 3) is in frame with the secretion signal open reading frame. pPICRAF9K was linearized with Bpu1102I, and was then transformed to P. pastoris strain KM71 as described by the manufacturer.

Ninety-one His+ transformants were tested for gene integration into the Pichia genome using a rapid DNA dot-blot technique (25). There were seven transformants that exhibited strong hybridization signals. These transformants were chosen to produce B2-aldehyde-forming enzyme in the induced cultures. For the examination of the enzyme activity, culture media were concentrated to 10-fold using a centrifugal filter device (Millipore). The enzyme activity could be detected in all the seven concentrated cultures. Fig. 5 showed the time courses of the enzyme activity (A) and protein concentration (B) for a pPICRAF9K transformant and a control transformant with pPIC9K. These results confirmed that the cloned 1080 bp cDNA represents the gene for the B2-aldehyde-forming enzyme from S. commune. Unfortunately, the enzyme was secreted in a
very low expression level, as shown in Fig. 5. Analysis by SDS-PAGE showed that the cell pellets of the pPICRAF9K transformant contained recombinant protein (data not shown). This implies that the recombinant protein may not be processed correctly and, hence, fails to be secreted.

Overall, the present work has led to the first-time cloning, sequencing, and expression of the cDNA encoding the enzyme that uniquely oxidizes the \(5'\)-hydroxymethyl terminus of riboflavin. That the enzyme is \(O\)-glycosylated in a manner that does not significantly affect its activity may relate to its biologic function, which could conceivably be to remove a nutrient that is essential for other organisms. Hence, as with penicillin or streptomycin production by fungi that can decrease competition for nutrients in their environment, this may be an example of the production of an enzyme for such a purpose.

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