MioC Is an FMN-binding Protein That Is Essential for Escherichia coli Biotin Synthase Activity in Vitro*

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Biotin synthase (BioB) catalyzes the final step of the biotin biosynthetic pathway, where a sulfur atom is inserted between the unactivated methyl and C-6 methylene carbon atoms of dethiobiotin (1) (Fig. 1). Catalytic activity has not yet been consistently observed for this reaction in vitro (2–4). The activity measured in vitro requires a complex mixture of small molecules and accessory proteins (3, 5–7). Cysteine is the initial source of the sulfur atom for the reaction, and Fe²⁺, dithiothreitol, NADPH, S-adenosylmethionine, and one of the amino acids asparagine, aspartate, glutamine, or serine are required (7). At least three other proteins are required for biotin synthase activity. Two have been identified as flavodoxin and ferredoxin (flavodoxin) NADP⁺ reductase. We now report the identification of MioC as a third essential protein, together with its cloning, purification, and characterization. Purified MioC has a UV-visible spectrum characteristic of a flavoprotein and contains flavin mononucleotide. The presence of flavin mononucleotide and the primary sequence similarity to flavodoxin suggest that MioC may function as an electron transport protein. The role of MioC in the biotin synthase reaction is discussed, and the structure and function of MioC is compared with that of flavodoxin.

Biotin synthase is required for the conversion of dethiobiotin to biotin and requires a number of accessory proteins and small molecule cofactors for activity in vitro. We have previously identified two of these proteins as flavodoxin and ferredoxin (flavodoxin) NADP⁺ reductase. We now report the identification of MioC as a third essential protein, together with its cloning, purification, and characterization. Purified MioC has a UV-visible spectrum characteristic of a flavoprotein and contains flavin mononucleotide. The presence of flavin mononucleotide and the primary sequence similarity to flavodoxin suggest that MioC may function as an electron transport protein. The role of MioC in the biotin synthase reaction is discussed, and the structure and function of MioC is compared with that of flavodoxin.

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* The abbreviation used are: Fe-S cluster, iron-sulfur cluster; TPP, thiamine pyrophosphate; PAGE, polyacrylamide gel electrophoresis; AE, activating enzyme; HPLC, high pressure liquid chromatography.

Materials and Methods

Chemicals—All chemicals were of reagent grade and unless otherwise stated were obtained from either Sigma or Fluka. Restriction enzymes and molecular biology reagents were from Promega, and oligonucleotides were from Genosys. [¹⁴C]Dethiobiotin (57.2 mCi/mmol) was custom synthesized by Isotopchem (Ganagobie, France). Recombinant flavodoxin was purified from Escherichia coli (16).

Initial Purification and Sequencing of MioC—MioC was first purified from an extract of E. coli BM4062 that contained the plasmid pB30A15/9 (see Ref. 7 for details of bacterial strains, plasmids, cell growth, and preparation of extracts). The amino acid sequence was determined with a model 477A Microsequencer (Applied Biosystems) after cutting the protein band from a polyvinylidene difluoride membrane (blotting an SDS-PAGE gel) stained with Amido Black (17).

Cloning, Overexpression, and Purification of MioC—Standard methods for the manipulation of DNA were used (18). MioC was initially cloned from an E. coli Kohara gene bank. The mioC gene was identified by hybridization against a specific oligonucleotide formulated from the mioC DNA sequence (19) and then cloned to form plasmid pMO14-1. The mioC gene was then amplified by polymerase chain reaction from pMO1-4 and ligated into the pGEM-T vector. The primers used were: forward, 5’-CCATGCGCATATGCTCTCTGG-3’; reverse, 5’-GGATCTTTATTTGATGAAATTAACCCAGATC-3’. A 0.4-kilo base fragment encoding the mioC gene was then excised from the pGEM-T vector with Ncol and BamHI and subcloned into the pET 24d(+) vector (Novagen) to generate an expression vector for MioC, pMIO.

E. coli BL21(DE3) cells were transformed with pMIO and grown at 37 °C in 2TY medium containing 30 μg/ml kanamycin. When the temperature was at 600 mV (Dₗ₀) reached 1.0, isopropyl-β-D-thiogalactoside was added to a final concentration of 0.1 mM. After 2.5 h the cells were harvested by centrifugation at 4 °C and stored at −80 °C.

Purification of MioC was carried out at 4 °C. Frozen cells containing pMIO (~100 g of wet weight) were thawed and resuspended in 300 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM dithiothreitol. Chicken egg white lysozyme was added (0.5 mg/ml), the mixture was stirred for 15 min, and Triton X-100 was added to 1.0% (w/v). After a further 15 min, the cell debris was removed by centrifugation for 30 min at 15,000 × g. The resulting cell-free supernatant was loaded onto a 285.0 ml Q-Sepharose HP column that had been equilibrated with 50 mM Tris-HCl buffer, pH 7.5 (buffer A). Proteins were eluted with a linear gradient of NaCl from 0 to 600 mM in buffer A over 10 column
We have now identified the third essential protein as MioC, which we purified on the basis of its activity in a biotin synthase assay (7). When partially purified biotin synthase, a flavodoxin (flavodoxin) NADP⁺ reductase and pure flavodoxin were incubated in a final volume of 250 μl at pH 7.5 with Fe²⁺-glutamate (50 nmol), NADPH (25 nmol), thiamine pyrophosphate (25 nmol), S-adenosylmethioneine (23 nmol), asparagine (3.75 μmol), dithiothreitol (250 nmol), Hepes buffer (25 μmol), and [¹⁴C]dethiobiotin (0.1 μCi, 1.95 nmol) plus cytosine (83 nmol). After incubation at 37°C for 1 h, the reaction was stopped by the addition of 250 μl of 12.5% (w/v) trichloroacetic acid. [¹⁴C]Dethiobiotin and [¹⁴C]biotin were then purified before analysis by TLC or HPLC (7).

**RESULTS**

**Evidence That the MioC Protein Is Required for Biotin Synthase Activity in Vitro**—We have shown previously that a number of low molecular weight compounds plus at least flavodoxin, ferredoxin (flavodoxin) NADP⁺ reductase, and a third protein are essential for biotin synthase activity in vitro (6, 7). We have now identified the third essential protein as MioC, which we purified on the basis of its activity in a biotin synthase assay (7). When partially purified biotin synthase, flavodoxin, and ferredoxin (flavodoxin) NADP⁺ reductase fractions prepared by ion exchange chromatography on a Q Sepharose Fast Flow column plus the essential low molecular weight compounds were incubated with dethiobiotin, no biotin was formed. Addition of MioC, either as a partially purified fraction or as the purified protein (Fig. 2), resulted in the formation of biotin (Fig. 3).

We initially purified MioC, based on its activity in the biotin synthase reaction, from 36.0 g of cells using a multi-step chromatographic procedure (Q Sepharose Fast Flow, HiLoad Q Sepharose HP, Sephacryl S-100 HR, HiLoad 26/10 Phenyl Sepharose HP, Phenyl Superose HR5/5, heat treatment at 100°C for 3 min, and further chromatography on Sepharose Blue Hi-Trap and Mono Q HR 5/5); details of this purification are not given, because a more elegant purification scheme was designed after the protein was identified and cloned: see "Materials and Methods" and obtained one fraction that contained pure MioC (fraction 25) as judged by SDS-PAGE analysis with silver staining (Fig. 2). Protein from a gel similar to that shown in Fig. 2 was blotted onto a polyvinylidene difluoride membrane, and the band at ~14 kDa was sequenced. The amino acid sequence was Ala-Asp-Ile-Thr-Leu-Ile-Ser-Gly-Ser-Thr, which corresponded to amino acids 2–11 of E. coli MioC.

Activity in the in vitro biotin synthase assay described above was observed when fraction 25, which contained MioC, was included (Fig. 3). Although MioC was essential for biotin synthase activity in vitro, we calculated that the catalytic center activity of the enzyme was less than 1. This is consistent with our earlier results and those from other groups (2, 3, 4, 7). MioC alone could not replace either the flavodoxin or the ferredoxin (flavodoxin) NADP⁺ reductase fractions, or both, in the assay.

**Overexpression and Purification of MioC**—Expression of MioC to ~15% of the total soluble protein, as judged by SDS-PAGE analysis, was achieved by the use of plasmid pMIO. Development of an efficient expression system allowed the purification to be simplified to a two-column procedure that yielded relatively large quantities of protein, typically 200 mg from 100 g of cells (see "Materials and Methods"). The initial chromatography step in the purification was a Q Sepharose column. Analysis of the fractions by SDS-PAGE revealed that MioC eluted in two peaks. The first peak was colorless and was assumed to be the apoprotein. This was confirmed by amino acid sequencing. The second peak was bright yellow, which was consistent with the protein containing the bound cofactor. The
**MioC Is Essential for Biotin Synthase Activity in Vitro**

Biotin synthase activity was measured by the conversion of [14C]dethiobiotin to [14C]biotin. Substrate and product were separated by TLC and detected by autoradiography. Lane 1, biotin standard; lane 2, assay containing all standard components except for a fraction containing MioC; lane 3, assay containing all standard components plus the preparation of MioC from the first Q Sepharose Fast Flow chromatography fraction 25 from the final Mono Q column (see Fig. 2).

The supernatant from the MioC extraction gave a retention time of 11.89 and 14.47 min, respectively. The flavin cofactor was confirmed by HPLC with retention times of 11.89 and 14.47 min, respectively. The calculated mass of FMN is 456.3 Da, and that of FAD is 783.5 Da, so the spectrum showed a peak at 455.24 Da (Fig. 5). From this spectrum it was not possible to discriminate between FMN and FAD. However, electrospray ionization mass spectrometry of MioC under negative ion conditions showed a peak at 455.24 Da (Fig. 5). The calculated mass of FMN is 456.3 Da, and that of FAD is 783.5 Da, so the spectrum was consistent with FMN being the flavin cofactor bound to MioC.

Denaturation, by the addition of 5.0% trichloroacetic acid to MioC, permitted the flavin cofactor to be released from the protein, indicating that the flavin was noncovalently bound to the enzyme. FAD and FMN standards could be separated by HPLC with retention times of 11.89 and 14.47 min, respectively. The supernatant from the MioC extraction gave a retention time of 14.52 min, again confirming FMN as the flavin cofactor.

**Molecular Mass Determination**—The subunit molecular mass of MioC predicted from the DNA sequence is 15,676 Da. Gel filtration chromatography indicated that the apparent native molecular mass of MioC was 31,500 Da when 100 mM Tris-HCl, pH 7.5, was used as the running buffer, which may be explained if MioC exists as a dimer under these conditions. The gel filtration experiments were repeated together with flavodoxin, whose subunit molecular mass is 19,606 Da and with a range of NaCl concentrations in the running buffers. As the NaCl concentration was increased, both MioC and flavodoxin eluted later from the column. At 1.0 M NaCl the elution volume corresponded approximately to that of the monomer for both proteins (Table I). The apo form of MioC behaved as for the holoenzyme. The elution volumes of MioC and flavodoxin were not further affected when 2.0 M NaCl was added to the running buffer.

On native gel electrophoresis MioC ran with a lower apparent molecular mass than trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) standards and with a lower apparent molecular mass than expected for the monomer (Fig. 6). This may be due to the low pI value of MioC (the calculated pI for the MioC protein sequence is 4.3), or MioC may be tightly folded.

**DISCUSSION**

Biotin synthase, anaerobic ribonucleotide reductase activating enzyme (AE), and pyruvate formate lyase AE all utilize flavodoxin, ferredoxin (flavodoxin) NADP+ reductase, NADPH, and S-adenosylmethionine and contain an Fe-S cluster. In contrast to biotin synthase, the 5'-deoxyadenosyl radical formed by the reductive cleavage of S-adenosylmethionine by pyruvate formate lyase AE and anaerobic ribonucleotide reductase AE abstracts a hydrogen atom to give a protein-based radical, which then reacts with the substrate (20, 21). The requirement for further additional proteins for activity also distinguishes biotin synthase from the pyruvate formate lyase and anaerobic ribonucleotide reductase enzyme systems. Unlike biotin synthase, both anaerobic ribonucleotide reductase AE and pyruvate formate lyase AE are catalytic in vitro and have fully defined assay systems.

The mioC gene is located next to oriC on the *E. coli* chromosome and although several investigations have tried to establish a role for mioC transcription in chromosome replication (22–24), the results were inconclusive (25, 26). To date, no function for MioC has been established. We have now shown that MioC is essential for *E. coli* biotin synthase activity in vitro.

**FIG. 4.** UV-visible spectrum of holo-MioC.

**FIG. 5.** Electrospray ionization mass spectrometry of MioC. The low molecular weight region shows a peak with a mass corresponding to that of FMN.

**TABLE I**

| Sample | Molecular mass (Da) |
|--------|-------------------|
|        | 100 mM Tris-HCl | 100 mM Tris-HCl + 0.25 M NaCl | 100 mM Tris-HCl + 1.0 M NaCl |
| MioC   | 31,500           | 22,900                        | 19,600                        |
| Flavodoxin | 37,000           | 24,800                        | 22,000                        |

**FIG. 3.** MioC is required for biotin synthase activity in vitro. Biotin synthase activity was measured by the conversion of [14C]dethiobiotin to [14C]biotin. Substrate and product were separated by TLC and detected by autoradiography. Lane 1, biotin standard; lane 2, assay containing all standard components except for a fraction containing MioC; lane 3, assay containing all standard components plus the preparation of MioC from the first Q Sepharose Fast Flow chromatography fraction 25 from the final Mono Q column (see Fig. 2).
When highly purified biotin synthase was incubated with photo-reduced deazaflavin in the absence of any other proteins, it was active (27). This suggests that photo-reduced deazaflavin can replace both the NADPH, flavodoxin, and ferredoxin (flavodoxin) NADP⁺ reductase electron transport system and MioC. In addition, sequence comparison studies predicted that MioC had a similar structure to flavodoxin and a flavin-binding motif (28), and we have provided experimental evidence that it binds FMN. Based on these observations, we propose that MioC is part of the electron transport system for biotin synthase. Our results can most easily be explained by MioC transferring electrons from flavodoxin to the Fe-S cluster of biotin synthase. Although the NADPH → ferredoxin (flavodoxin) NADP⁺ reductase → flavodoxin electron transport pathway has recently been verified by redox potentiometry (8), the reduction potential of the Fe-S cluster of biotin synthase remains unknown, and hence the final electron transfer step from flavodoxin to biotin synthase has not been confirmed.

Biotin synthase has not yet been shown to catalyze the conversion of dethiobiotin to biotin in vitro. The Fe-S cluster of biotin synthase provides the sulfur atom for the dethiobiotin to biotin conversion (4, 12), so that Fe-S cluster forming or reforming enzymes may also be required for activity. The absence of these and MioC may be partly responsible for the very low catalytic center activity of the enzyme in the assay system with photo-reduced deazaflavin and highly purified biotin synthase (between 0.04 and 0.08/h, calculated from the results in Ref. 28).

The x-ray crystal structure of E. coli flavodoxin (Protein Data Bank file code 1ag9) shows that it crystallizes with two molecules in the asymmetric unit. Calcium, sodium, and chloride ions form salt bridges between the two molecules. Determination of the native molecular mass of MioC by gel filtration chromatography in low salt conditions showed MioC to be a dimer, but at relatively high salt concentrations (1.0 M NaCl) it behaved as a monomer, which is consistent with the disruption of intermolecular salt bridges.

During the original purification of MioC, where its activity in the biotin synthase reaction was measured, the addition of TPP to the buffers helped to maintain its activity. MioC has no TPP binding site, and following purification, TPP had no effect on its activity in the assay. We have no explanation for the positive effect of TPP during purification.

In conclusion, we have shown that MioC is required for biotin synthase activity in vitro. We suggest that MioC may have an electron transport role based on its predicted similarity to flavodoxin and its binding of FMN, and the fact that it can be replaced in the biotin synthase assay in vitro by photo-reduced deazaflavin. Despite inclusion of MioC in the biotin synthase assay, catalytic activity was still not observed. Unknown factors, such as Fe-S cluster forming enzymes, may have been missing. Further experiments are required to confirm the role of MioC in vivo and as part of a catalytic system for the formation of biotin.

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Fig. 6. Native PAGE of MioC. Lane 1, trypsin inhibitor (20.1 kDa); lane 2, a-lactalbumin (14.4 kDa); lane 3, MioC (15.7 kDa).