The Aspergillus nidulans cnxF Gene and Its Involvement in Molybdopterin Biosynthesis

MOLECULAR CHARACTERIZATION AND ANALYSIS OF IN VIVO GENERATED MUTANTS*

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The product of the Aspergillus nidulans cnxF gene was found by biochemical analysis of cnxF mutants to be involved in the conversion of precursor Z to molybdopterin. Mutants cnxF1242 and cnxF8 accumulate precursor Z, while the level of molybdopterin is undetectable. The DNA sequence of the cnxF gene was determined, and the inferred protein of 560 amino acids was found to contain a central region (residues 157 to 396) similar in sequence to the prokaryotic proteins MoeB, which is thought to encode molybdopterin synthase sulfurylase, ThlF, required for thiamine biosynthesis, and HesA, involved in heterocyst formation, as well as eukaryotic ubiquitin-activating protein E1. Based on these similarities, a possible mechanism of action is discussed. Sequence comparisons indicate the presence of one and possibly two nucleotide binding motifs, Gly-X-Gly-X-Gly, as well as two metal binding Cys-X-Cys motifs in this central region of similarity to other proteins. Alteration of this central region of similarity to molybdopterin synthase sulfurylase, indicating that these seven amino acids are essential and that this domain is crucial for function. Of these seven, the cnxF1285 mutation results in the replacement of Gly-178, the last glycine residue of the N-proximal Gly-X-Gly-X-Gly motif, indicating that this motif is essential. Mutation of the conserved Arg-208, also probably involved in nucleotide binding, leads to a loss-of-function phenotype in cnxF200. Alteration of Cys-263, the only conserved Cys residue (apart from the metal binding motifs), in cnxF472 suggests this residue as a candidate for thioester formation between molybdopterin synthase and the sulfurylase. Substitution of Gly-160 in two independently isolated mutants, cnxF21 and cnxF243, results in temperature-sensitive phenotypes and indicates that this residue is important in protein conformation. The C-terminal CnxF stretch (residues 397–560) shows substantial sequence conservation to a yeast hypothetcal protein, Yhr1, such conservation between species suggesting that this region has function. Not inconsistent with this proposition is the observation that mutant cnxF8 results from loss of the 34 C-terminal residues of CnxF. There is no obvious similarity of the CnxF C-terminal region with other proteins of known function. Two cnxF transcripts are found in low abundance and similar levels were observed in nitrate- or ammonium-grown cells.

The molybdenum cofactor is a ubiquitous molecule found in most organisms from bacteria to humans and is required for activity by a number of molybdoenzymes, including nitrate reductase and xanthine dehydrogenase (also known as purine hydroxylase I) (1). The first biochemical studies, by Nason and co-workers (2, 3) and Ketchum and Swarai (4) in the early 1970s, which indirectly demonstrated the existence of a molybdenum-containing component, made use of fungal cell-free extracts of the Neurospora crassa nit-1 mutant lacking the cofactor but containing apo-nitrate reductase. The presence and universality of the cofactor in bacterial cell-free extracts as well as acid-treated enzymes from diverse sources of biological material, such as cow milk or fowl liver, was suggested by their ability to reconstitute holo-nitrate reductase after mixing that source with nit-1 crude extracts.Later, Garrett and Cove (5) predicted the presence of the cofactor, using a similar approach, in the fungus Aspergillus nidulans.

Direct chemical studies by Rajagopalan (Refs. 1 and 6, and articles therein) suggested that the molybdenum cofactor of liver sulfite oxidase consists of a novel pterin called molybdopterin linked by its 6-alkyl side chain to a dithiolene group which coordinates molybdenum. Further biochemical and genetic work carried out in the prokaryote Escherichia coli has provided valuable information regarding the events that take place leading to the biosynthesis of a prokaryotic molybdenum cofactor variant, molybdopterin guanine dinucleotide (reviewed in Refs. 1 and 7). The proposed structure of the molybdenum cofactor and intermediates and their biosynthesis from a guanosine derivative are summarized in Fig. 1.

Even earlier than the biochemical work, genetic studies of molybdenum cofactor synthesis had been initiated by Cove and Pateman (8) and Pateman et al. (9). Using the fungus A. nidulans, they isolated mutants resistant to chlorate, an analogue of nitrate toxic to wild-type cells. One particular class of chlorate-resistant mutants was unable to utilize either nitrate or purines such as adenine, hypoxanthine, and xanthine as sole sources of nitrogen. This inability to grow on nitrate and hypoxanthine was found to be due to the concomitant diminution of nitrate reductase and xanthine dehydrogenase activities.
Aspergillus nidulans Strains, Plasmids, Media, and Transformation—The wild-type strain used was G051 (bi(a1)). Strains cnxF2, cnxF7, and cnxF8 were original mutants described by Cove and Pateman (8) and Pateman et al. (9). Further cnxF mutants were selected in this study on the basis of resistance to chloride (11) using chemically generated mutagenesis (8, 9). In this regard, N-methyl-N-nitro-N-nitrosourea was used to obtain mutant strains cnxF19, cnxF206, cnxF472, cnxF480, cnxF1242, cnxF1228, and cnxF1285 while 1,2,7,8-diepoxyoctane was used for cnxF142, cnxF1224, and cnxF1193. Chlorate was used at a concentration of 200 mM with proline as the sole source of nitrogen at 37 °C as described previously (8, 9). Assignment of mutations to cnx loci was carried out by the heterokaryotic complementation test, i.e. growth of pair-wise heterokaryons on nitrate as the sole nitrogen source against representative cnx mutants, cnxA9, cnxB11, cnxC3, cnxF7, cnxF4, and cnxH4 (8, 9). Standard Aspergillus growth media and handling techniques were as described by Clutterbuck (12). For transcript analysis, cultures were grown at 37 °C for 16 h in liquid minimal medium containing 10 mM sodium nitrate or 5 mM ammonium tartrate as the sole nitrogen source (15).

Molecular Methods—Standard procedures were used for propagation of cosmids and for subcloning and propagation of plasmids in E. coli strain DH5α. Conditions employed here for A. nidulans Southern and Northern blot analysis were as described previously (14). Nucleotide sequence of the A. nidulans wild-type cnxF gene was determined on both strands of genomic and cDNA clones using a Sequenase Version 2 DNA sequencing kit according to manufacturer instructions (Amersham plc, UK). This sequence can be found under GenBank accession number AF055287. For primer extension analysis, mRNA was prepared from mycelium grown in minimal medium containing 10 mM sodium nitrate as the sole nitrogen source at 25 °C for 18 h, using a Quickprep mRNA purification kit (Pharmacia plc, UK). mRNA (2 mg) was hybridized with 5' 32P end-labeled primer FP2 (5'-GTTCGCTTCGACTCGC, position +383) at 52 °C for 1 h and reverse-transcribed using a Primer Extension System as recommended by the manufacturer (Promega). The extension product was compared on a denaturing sequencing gel with a DNA sequence ladder prepared using the same end-labeled primer with pSTa508 as template. The A. nidulans cosmid library was purchased from the Fungal Genetics Stock Center (Kansas City, KS).

Aspergillus Cloning by Complementation of Mutant Phenotypes—An argB-based A. nidulans genomic bank cleaved by BamHI together with the autonomously replicating A. nidulans vector pHelp consisting of pUC18 and AMA1, an A. nidulans sequence with replication activity, were used to transform A. nidulans cells, basically the approach described by Gems and Clutterbuck (15). After transformation of the mutant cnxF7, nitrate-utilizing colonies were purified on selective medium (i.e. minimal medium containing nitrate as the sole source of nitrogen).

Hybrid plasmid molecules, containing pHelp and argB with cnxF7 complementing sequences, created by in vivo recombination and/or DNA ligation events (15) were isolated by conventional A. nidulans DNA extraction procedures and transferred or “rescued” by transforming E. coli DH5α cells and selecting for ampicillin resistance. Such plasmids were then analyzed by standard recombinant DNA procedures. Positive identification of clones was achieved by reintroducing the isolated hybrid plasmid into the A. nidulans cnxF7 mutant with the successful restoration of wild-type growth with nitrate.

Polymerase Chain Reaction Amplification and Mutant DNA Sequence Determination—Genomic DNA was prepared from mycelia grown in liquid culture for 16–18 h at 25 °C using a Nucleon BAC2C Kit (Scotlab Ltd, UK). DNA was cleaved with EcoRI, and around 100 ng was amplified using 2.5 units/ml Dynazyme (Flowgen) or 2.5 units/ml Taq Polymerase Chain Reaction Amplification and Mutant DNA Sequence Determination. Following removal of primers and unincorporated nucleotides by the Glassmax DNA Isolation Spin Cartridge System (Life Technologies), the amplified DNA

EXPERIMENTAL PROCEDURES

Cove and Pateman (8) astutely suggested that such mutants were defective in the synthesis of a cofactor common to both nitrate reductase and xanthine dehydrogenase and designated the mutants cnx (common component for nitrate reductase and xanthine dehydrogenase). Five cnx loci (namely cnxABC, cnxΕ, cnxF, cnxG, and cnxH) were identified (8, 9). With the exception of the complex cnxABC locus that is involved in the first section of the pathway (Fig. 1), namely the biosynthesis of the pterin precursor Z from a guanosine derivative (10), little or no information is available to suggest the function of cnx gene products or the nature of the pathway in eukaryotes.

We report here the isolation of the A. nidulans cnxF gene, analysis of the wild-type and mutant sequences, levels of precursor Z and molybdopterin intermediates in cnxF mutants, and the likely nature of CnxF involvement in molybdenum cofactor biosynthesis.

The A. nidulans cnxF Gene

In the early section of the pathway, a guanosine derivative (Guanosine-R) is converted to precursor Z by the CnxABC protein (10). In the intermediate section of the pathway, molybdopterin synthase is activated following the transfer of a reactive sulfur by the CnxF protein most likely regenerates the transferable sulfur of the molybdopterin. The CnxF protein most likely regenerates the transferable sulfur of the molybdopterin synthase (see “Discussion”). Finally, molybdopterin coordinates molybdenum to yield the molybdenum cofactor.

![Structure of molecules and the pathway for the synthesis of the molybdenum cofactor](image)

**FIG. 1. Structure of molecules and the pathway for the synthesis of the molybdenum cofactor (Ref. 1 and references therein).**

The assay above shows that the mutants were defective in the synthesis of a cofactor common to both nitrate reductase and xanthine dehydrogenase. Five cnx loci (namely cnxABC, cnxΕ, cnxF, cnxG, and cnxH) were identified (8, 9). With the exception of the complex cnxABC locus that is involved in the first section of the pathway, namely the biosynthesis of the pterin precursor Z from a guanosine derivative, little or no information is available to suggest the function of cnx gene products or the nature of the pathway in eukaryotes.

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1 The abbreviations used are: PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; kb, kilobase(s).
Purified compound Z was detected by excitation at 350 nm and emission at 450 nm. Form A dephosphorylated was detected by excitation at 370 nm and emission at 450 nm, respectively. Reversed phase HPLC analysis of compound Z and form A dephosphorylated per mg of protein from three independent experiments carried out for each strain.

Wild-type and cnxF mutant strains (Fig. 2B) were observed in the mutant strain (Fig. 2A). In contrast, molybdopterin was undetectable in the wild-type and the mutant strain (Fig. 2B). The complementing region of one of these (W17D04) was identified, by transformation of each of eight possible BamHI-generated fragments, as a 4-kb BamHI fragment that was subcloned into pUC13 and designated pSTA508. This recombinant vector complemented the cnxF mutation and was used as a template for DNA sequence determination.

**RESULTS**

**Determination of Precursor Z and Molybdopterin Levels in Wild-type and Mutant Strains**—The data presented in Fig. 2 show the levels of precursor Z and molybdopterin, as their oxidized derivatives compound Z and form A dephosphorylated, respectively, in wild-type and the mutant cnxF1242 (likely to be a null mutant as the mutation results in an intron boundary change, see below for details of the nucleotide lesion). The wild-type and mutant were grown with nitrate plus proline as the only nitrogen sources for 16 h at 30 °C and 250 rpm.

**Structure of the cnxF Gene**—A unique open reading frame encodes a protein of 560 amino acid residues (Fig. 3). Since no N-terminal data is available for the CnxF protein, we can only speculate as to the identity of the translational initiation codon. However, this ATG (nucleotide position +1) is the first in-frame ATG downstream from a major transcriptional start point that has been mapped at around 80 base pairs upstream.

**Comparison of the DNA sequence of genomic and an RT-PCR product, generated using primers FP3 and FP4, allowed the identification of two typical short fungal introns (86 and 62 base pairs) interrupting the putative CnxF protein coding region.**

**Analysis of the Predicted Protein**—Comparisons to the SwissProt protein data base using Blastp (20) showed that fungal CnxF shares considerable similarity with amino acid sequences belonging to an E. coli enzyme encoded by the moeb gene (21). This protein has also been shown to be required for the conversion of precursor Z to molybdopterin (22, 23). It appears to be involved in sulfur transfer and was recently termed molybdopterin synthase sulfurylase although the mechanisms and substrates are poorly defined.

The A. nidulans cnxF gene, at 560 residues, is much larger than the 249 residues of MoeB, the CnxF protein having a 150-residue extension at the N-terminus and an amino acid extension of 161 residues of MoeB, the CnxF protein, at 560 residues, is much larger than the 249 residues, see below) grown under the same conditions. These results suggest that the cnxF product is involved in the conversion of precursor Z to molybdopterin.

**Molecular Cloning of the A. nidulans cnxF Gene**—A 27-kb plasmid was isolated following co-transformation of the autonomously replicating A. nidulans plasmid pHELP with an A. nidulans argB-based genomic library as described under “Experimental Procedures.” This recombinant plasmid repaired the phenotype of the cnxF7 mutation, i.e. restoration of growth on nitrate as the sole nitrogen source. The size and the restric-
The A. nidulans cnxF Gene

| A. nidulans cnxF locus. Numbers on the left refer to nucleotides relative to the A of the start codon, numbered +1, and numbers on the right refer to amino acid residues. The introns are shown in lowercase. |
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Anabaena (25). Additionally, CnxF in common with MoeB (1) shares up to 36% identity with the yeast (Fig. 4B), plant, and human ubiquitin activating enzyme E1 (Uba1), a protein which also harbors the Gly-containing catalytic domain for nucleotide binding (26). The Gly-rich motifs of other, and functionally unrelated, nucleotide binding proteins often have associated with them several amino acid residues which are thought to be important for nucleotide binding (27, 28). Certain of these residues are also associated with the first Gly-rich sequence of CnxF, shown boxed in Fig. 4A and B. These include a stretch
of hydrophobic residues following the first Gly-rich motif encompassing a highly conserved Ala at position 186 in CnxF (conserved in MoeB, ThiF, HesA, and Uba1), a negatively charged Asp residue at position 197 (conserved in MoeB and ThiF), and a basic Arg residue at position 208 (conserved in MoeB, ThiF, HesA, and Uba1). The second Gly-X-Gly-X-Gly motif of CnxF (boxed in Fig. 4, A and B) is not conserved in MoeB, ThiF, or HesA but is present in Uba1 (Fig. 4B). However, this second motif does not appear to have those residues, conserved in equivalent positions, which are found in association with the first Gly-rich sequence.

As well as the Gly-X-Gly-X-Gly motif, there is an arrangement of conserved Cys residues resembling a metal-binding motif toward the C-terminal end of this central region of CnxF. These Cys-X-X-Cys pairs of Cys residues (boxed in Fig. 4, A and B) at positions 314 and 317 and positions 393 and 396 are also found in equivalent positions relative to the nucleotide binding motif in MoeB, ThiF, and HesA (Fig. 4A), but not Uba1, (Fig. 4B).

No significant sequence similarity was observed between the 150-residue N-terminal or the 161-residue C-terminal extension of CnxF and other E. coli proteins, including those required for the synthesis of the molybdenum cofactor. Similarly, comparison of the N- and C-terminal amino acid extensions with the SwissProt data base yields little illuminating information as to their potential function. Limited similarity of the 161 C-terminal residues was observed to a eukaryotic enzyme, rhodanese, a thiosulfate sulfurbisferase that is probably involved in the formation of iron-sulfur complexes and cyanide detoxification (29). However, none of the apparently conserved residues was associated with the known active site of rhodanese, and more stringent analysis using GCG Bestfit indicated that the similarity is unlikely to be significant.

There is high similarity of CnxF to hypothetical proteins recognized from genome sequencing. These include the 440 residue Yhr1 and the 401 residue YabA proteins from the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe, respectively, which possess maximum similarity (54 and 51% identical, respectively) to the central portion of CnxF, containing both possible Gly-X-Gly-X-Gly motifs and both Cys-X-X-Cys pairs in equivalent positions. Unlike MoeB, ThiF, HesA, and Uba1, similarity of the yeast proteins extends to the CnxF C-terminus with 33 and 34% identity to the S. cerevisiae (Fig. 4C) and S. pombe (not shown) proteins, respectively. At the N-terminus, CnxF is at least 100 residues longer than the yeast proteins and no striking similarity is seen except in a small stretch of 18 residues where 13 are identical in Yhr1 and 9 in YabA.

**Characterization of cnxF Mutants**—From several hundred cnxF mutants isolated on the basis of chlorate resistance at 37 °C and determined by lack of growth on nitrate or hypoxanthine as sole nitrogen source, ten in this study were found to be cnxF mutants after complementation in heterokaryons resulting in growth on nitrate as sole nitrogen source (see “Experimental Procedures”). Nine of these (cnxF119, cnxF200, cnxF472, cnxF480, cnxF1193, cnxF1224, cnxF1228, cnxF1242, and cnxF1285) were found to be temperature non-conditional, i.e. were unable to grow on either sole nitrogen source and were chlorate-resistant at both temperatures tested (25 and 37 °C). One mutant, cnxF142, failed to grow on nitrate or hypoxanthine at both temperatures, was fully chlorate-resistant at 37 °C, but showed almost wild-type sensitivity at 25 °C. In addition, five mutants (cnxF2, cnxF7, cnxF8, cnxF21, and cnxF24) originally isolated by Pateman and Cove (8, 9) were checked again phenotypically. The mutants cnxF2, cnxF7, and cnxF8 failed to grow on nitrate or hypoxanthine and were resistant to chlorate at both temperatures as expected from the reported work. The two reported temperature-sensitive mutants, cnxF21 and cnxF24, while growing significantly better on nitrate and showing sensitivity to chlorate at the permissive temperature, i.e. 25 °C as expected (30), completely failed to grow on hypoxanthine as the nitrogen source at the permissive temperature. Both mutants showed the expected phenotype at 37 °C, i.e. failure to grow on nitrate or hypoxanthine and resistance to chlorate. It was also noted that there were marked differences in growth and conidiation between cnxF21 and cnxF24 on complete medium.

**Sequence Analysis of Randomly in Vivo Generated cnxF Mutants**—Of the 15 mutations described above, 7 resulted from amino acid substitutions, the positions of which are shown in Fig. 4A. Four mutations were found to change highly conserved Gly residues and included the three with temperature-sensitive phenotypes—both cnxF21 and cnxF24 result in the same alteration of Gly-160 to Asp while in cnxF142, residue Gly-342 is converted to Ser. The fourth, a non-conditional mutation, namely cnxF1285, was found to alter Gly-178 to Asp, the last Gly residue of the N-proximal Gly-X-Gly-X-Gly motif. Three further interesting mutants that also substitute highly conserved amino acids are mutation cnxF200, which converts Arg-208 to Gln, mutation cnxF472, which forms Tyr from the Cys-263 residue, and finally, mutation cnxF119, where Glu-293 is changed to Lys.

Mutation cnxF8, despite being a single base pair deletion, is nevertheless potentially informative as the deletion occurs at nucleotide +1714 (Fig. 3), resulting in a frameshift at residue 522, only 38 amino acid residues preceding the CnxF C-terminus, and placing a stop codon in-frame after three residues (Fig. 4C). The protein in this mutant is therefore truncated with the loss of 34 amino acids at the C-terminus. This is the only mutation isolated thus far that is not in the central region of the CnxF protein.

The others are most likely null mutants. Seven of these strains were found to be nucleotide deletions (cnxF1224, loss of C at nucleotide +1108), intron boundary changes (cnxF1228, G to A at nucleotide +479, and cnxF1242, G to A at nucleotide +783), generation of a stop codon as a result of a base substitution (cnxF480, G to T at nucleotide +331), or gross DNA changes where not all the PCR products were obtainable (two original mutants, cnxF2 and cnxF7, and one mutant arising from this study, cnxF1193).

**cnxF Expression**—In Northern blot experiments, two weakly hybridizing transcripts of 1.9 and 2.55 kb present in poly(A)+ mRNA were observed after several days exposure to x-ray film using a probe derived from the cnxF coding region only (Fig. 5). Although the presence of more than one transcript is not uncommon in fungi as demonstrated by the control actA transcripts (31) in Fig. 5, Southern blot analysis confirms that there is a single copy of the cnxF gene in A. nidulans. The 2.55-kb transcript is present in higher abundance than the 1.9-kb one, and the relative abundance of both transcripts was similar in mRNA isolated from nitrate or ammonium grown cells. Approximately equal loadings of mRNA were present for each condition as judged by the hybridization intensity of actA, a constitutively expressed A. nidulans gene encoding actin (31). In contrast to cnxF, the actin transcript was visible after a few hours of autoradiographic exposure.

**DISCUSSION**

The Aspergillus nidulans cnxF gene, identified by Cove and Pateman (8, 9) more than 3 decades ago, is required for the conversion of precursor Z to molybdopterin (Fig. 1). This conclusion comes from the biochemical analysis of the null mutant strain cnxF1242 which accumulates precursor Z to levels 9-fold
The A. nidulans cnxF Gene

The A. nidulans cnxF protein, with 560 amino acid residues, is considerably larger than the prokaryotic MoeB (249 residues), with N- and C-terminal amino acid extensions of 150 and 161 residues, respectively. However, whether there are additional functions in these additional regions is uncertain. Data base similarity searching reveals little obviously significant similarity to other characterized proteins. Also, no potential function can be inferred from the position of amino acid substitution mutations analyzed since all (seven) residue changes are found in the CnxF region similar to MoeB (i.e. residues 153 to 398), with none observed in the 150-residue N-terminal or the 161-residue C-terminus extensions. This may suggest that the MoeB-homologous stretch is the most important or the only functional domain contained within the CnxF protein. At some variance with this suggestion, however, is the finding that the cnxF8 mutation in the C-terminal extension results in the lack of molybdopterin production. This particular mutation generates a frameshift at residue 522, placing a stop four codons distal. The mutant protein, therefore, missing 34 C-terminal amino acids may be generally unstable and/or subject to proteolysis with the resultant loss of sulfurylase activity. An alternative explanation would suggest that the last 38-residue stretch of CnxF is required specifically for function in eukaryotes. In support of this proposition is the observation that there is high similarity of the C-terminal 161 amino acids of CnxF to hypothetical yeast proteins identified by DNA sequencing.

The central portion of the CnxF protein (between residues 157 and 393) is clearly similar to further bacterial proteins, ThiF and HesA (Fig. 4A), and to yeast (Fig. 4C), plant, and human E1 ubiquitin activating protein (designated Uba1 in yeast). There is no immediately obvious physiological relationship between CnxF and ThiF, HesA, or Uba1 proteins. ThiF is thought to be involved in the synthesis of the thiazole ring of vitamin B1 in E. coli (25), while HesA is expressed following heterocyst induction in the cyanobacterium, Anabaena, and is required for efficient nitrogen fixation possibly by participating in electron transfer to nitrogenase (24). Uba1 is a ubiquitin activating enzyme that forms a thioester linkage between the C-terminal Gly of ubiquitin and a Cys within Uba1 during activation of ubiquitin as part of the process for ubiquitin-targeted degradation of proteins (32). In the ubiquitin system, this reaction is followed by transfer of the ubiquitin to another carrier protein, with the formation of a new thioester linkage. The suggestion has been made (1) that the mechanism, by which the proposed sulfurylase transfers sulfur to molybdopterin synthase, may resemble the process of ubiquitin activation by Uba1. By analogy, in the molybdopterin synthase sulfurylase reaction, molybdopterin synthase would form a thioester with CnxF. However, subsequent transfer of molybdopterin synthase to another protein would not occur, but instead a sulfide, possibly created by the metal center of CnxF, would be transferred to the carboxyl group of the synthase, creating a thioether bond. The reactive thioether of the molybdopterin synthase would then be the source of sulfur for the conversion of precursor Z to molybdopterin. The fact that ubiquitin, the small subunit of E. coli molybdopterin synthase, ThiC (a protein encoded by a cistron in the ThiF operon), and HesB (a protein from the HesA operon) all have C-terminal or C-terminal Gly of ubiquitin and a Cys within Uba1 during activating enzyme that forms a thioester linkage between the small subunit of the synthase and the carboxyl group of the ubiquitin and a Cys within Uba1 during activating enzyme that forms a thioester linkage between the small subunit of the synthase and the carboxyl group of the ubiquitin.

The CnxF primary sequence shows the presence of features typical of nucleotide binding sites for NAD, NADP, GTP, or ATP (residues 173 to 208). In this potential nucleotide binding region, the core motif Gly-X-Gly-X-Gly (residues 173 to 178) is very highly conserved. Replacement of the last conserved Gly with Asp (residue 178) in the cnxF285 mutant strain results in loss-of-function, supporting the proposal that this motif is essential for enzyme function. Evidence regarding the functional importance of this site in other enzyme systems comes from the recent site-directed mutagenesis of E. coli pyridine nucleotide transhydrogenase showing that the first Gly residue is also essential for function (28). Another intriguing parallel is that CnxF loss-of-function occurs when the highly conserved Arg-208 is replaced by Glu in A. nidulans mutant strain cnxF200. In the case of E. coli transhydrogenase, replacement of the Arg residue that locates at an identical distance 36 residues downstream from the first Gly residue to that in CnxF also leads to loss of activity. This basic residue of the transhydrogenase is thought to interact with the 2′-phosphate of NAD(P)H and is not found in a comparable position in proteins which possess NAD(H)-binding sites, but the crucial role of
played by this Arg residue is unclear. That the Arg residue is not found at these positions in NAD binding sites, it may be concluded that NADP, GTP, or ATP is the binding nucleotide. The functionality of the other Gly-X-Gly-X-Gly motif (CnxF residues 189–194) is unclear. However this motif may not be required for nucleotide binding since it is not found in association with other residues recognized in other nucleotide binding proteins and is not conserved in the prokaryotic proteins MoeB, ThiF, or HesA although there is a second Gly-X-Gly-X-Gly motif in yeast Uba1.

Two temperature-sensitive mutants (cnxF21 and cnxF24) result in an identical nucleotide change (G to A) to alter the Gly-160 residue to Asp, a result verified by resequencing further strain copies of these mutants. These are likely to be independently isolated mutations as reported originally since the two strains can be distinguished by their subtly different morphological phenotype. Their temperature-sensitive phenotype, i.e. growth on nitrate at 25 °C but not 37 °C would suggest that the enzyme has some activity at 25 °C but not 37 °C, and indicates that the Gly residue at position 160 is important to permit correct protein folding and hence catalysis. Also, it should be noted that both cnxF21 and cnxF24 mutants fail to grow on hypoxanthine at both temperatures. This totally mutant phenotype at both temperatures on hypoxanthine is in accord with other A. nidulans cnx temperature-conditional mutants such as cnxA140 (10), cnx20 (33), and several cnxH strains (30). It was suggested by these workers that xanthine dehydrogenase (purine hydroxylase I) has a more stringent requirement for the molybdopterin cofactor than nitrate reductase. The cnxF142 mutation is phenotypically distinct from cnxF21 and cnxF24 in that this mutant fails to grow on nitrate and hypoxanthine at both temperatures but is chlorate-resistant (mutant) at 37 °C while sensitive (wild-type) at 25 °C and hypoxanthine at both temperatures but is chlorate-resistant (mutant) at 37 °C while sensitive (wild-type) at 25 °C and hypoxanthine at both temperatures but is chlorate-resistant (mutant) at 37 °C while sensitive (wild-type) at 25 °C.

The regulation of molybdenum cofactor biosynthesis was first studied by Garrett and Cove in A. nidulans (5). Using an assay for formation of nitrate reductase activity in vitro similar to the nit-1 assay (in the Introduction), they observed levels of cofactor consistent with a degree of regulation depending on the nitrogen source and suggested that its formation is ammonium-repressed. Transcript analysis presented here shows that cnxF is expressed at the same level in cells grown with ammonium or nitrate as the sole nitrogen source. In contrast, the cnxABC transcript level is considerably higher in cells grown with nitrate than ammonium (10), which might suggest that, if there is transcriptional control of the molybdopterin biosynthetic genes, it is exerted on the cnxABC locus, at least, but not cnxF.

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