Eukaryotic Molybdopterin Synthase
BIOCHEMICAL AND MOLECULAR STUDIES OF ASPERGILLUS NIDULANS cnxG AND cnxH MUTANTS*

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Shiela E. Unkles‡§, Immanuel S. Heck¶, M. Virginia C. L. Appleyard§**, and James R. Kinghorn‡
From the ‡Department of Microbiology, Monash University, Clayton, Victoria 3168, Australia, the §Division of Environmental and Evolutionary Biology, School of Biology, University of St. Andrews, St. Andrews, Fife KY16 9TH, United Kingdom, and the ¶Institute for Plant Biochemistry, Correnstrasse 41, D-72076 Tubingen, Germany

We describe the primary structure of eukaryotic molybdopterin synthase small and large subunits and compare the sequences of the lower eukaryote, Aspergillus nidulans, and a higher eukaryote, Homo sapiens. Mutants in the A. nidulans cnxG (encoding small subunit) and cnxH (large subunit) genes have been analyzed at the biochemical and molecular level. Chlorate-sensitive mutants, all the result of amino acid substitutions, were shown to produce low levels of molybdopterin, and growth tests suggest that they have low levels of molybdoenzymes. In contrast, chlorate-resistant cnx strains have undetectable levels of molybdopterin, lack the ability to utilize nitrate or hypoxanthine as sole nitrogen sources, and are probably null mutations. Thus, on the basis of chlorate toxicity, it is possible to distinguish between amino acid substitutions that permit a low level of molybdopterin production and those mutations that completely abolish molybdopterin synthesis, most likely reflecting molybdopterin synthase activity per se. Residues have been identified that are essential for function including the C-terminal Gly of the small subunit (CnxG), which is thought to be crucial for the sulfur transfer process during the formation of molybdopterin. Two independent alterations at residue Gly-148 in the large subunit, CnxH, result in temperature sensitivity suggesting that this residue resides in a region important for correct folding of the fungal protein. Many years ago it was proposed, from data showing that temperature-sensitive cnxH mutants had thermolabile nitrate reductase, that CnxH is an integral part of the molybdenzyme nitrate reductase (MacDonald, D. W., and Cove, D. J. (1974) Eur. J. Biochem. 47, 107–110). Studies of temperature-sensitive cnxH mutants isolated in the course of this study do not support this hypothesis. Homologues of both molybdopterin synthase subunits are evident in diverse eukaryotic sources such as worm, rat, mouse, rice, and fruit fly as well as humans as discussed in this article. In contrast, molybdopterin synthase homologues are absent in the yeast Saccharomyces cerevisiae. Precursor Z and molybdopterin are undetectable in this organism nor do there appear to be homologues of molybdoenzymes.

Molybdoenzymes play essential roles in carbon, sulfur, and nitrogen cycles in most organisms. For instance, in higher eukaryotes including humans, sulfite oxidase is required for the degradation of sulfur amino acids converting sulfite to sulfate (1). In certain lower eukaryotes such as Aspergillus nidulans, another molybdoenzyme nitrate reductase is required for the important ecological process of nitrate assimilation to ammonium (2). Additionally, a few molybdoenzymes exist in both eukaryotic groups, including xanthine dehydrogenase, which is important in the breakdown process of purines to uric acid (3, 4).

For catalytic activity, these enzymes require the molybdenum cofactor, a prosthetic group that consists of a novel pterin called molybdopterin linked by its 6-alkyl side chain to a di-thiolene group, which coordinates molybdenum. Its chemical structure and likely biosynthetic pathway were proposed by Rajagopalan (5) and are shown in Fig. 1. The presence of molybdenum cofactor has been demonstrated indirectly in a variety of biological material, such as cow milk and rabbit or fowl liver, by its ability to assemble active holo-nitrate reductase in cell-free extracts of a nit-1 mutant from the related fungus Neurospora crassa (6). The nit-1 mutant lacks the cofactor but contains inactive apo-nitrate reductase. Similarly, the presence of the molybdenum cofactor has also been demonstrated indirectly in the fungus, A. nidulans (7).

Pleiotropic loss of human molybdoenzymes, including sulfite oxidase and xanthine dehydrogenase, results in a severe clinical disease for which no known therapy exists (8). In contrast to this extreme phenotype, impaired molybdoenzymes in the lower eukaryotes such as A. nidulans result in differences in simple growth characteristics, *vis à vis* the inability to grow on nitrate or hypoxanthine as the sole nitrogen source. Such phenotypes are easily observable and permit convenient genetic analyses (9, 10). These features make the study of the molybdenum cofactor in A. nidulans attractive and indeed allowed Cove and Pateman (9) and Pateman et al. (10) to isolate molybdenum cofactor-deficient mutants some 3 decades ago. Their genetic analyses of mutants, isolated on the basis of screening for the inability to grow on nitrate as the sole source of nitrogen, indicated the presence of five unlinked loci. The mutants were shown to lack nitrate reductase and xanthine dehydrogenase, and this suggested that these loci were involved in the synthesis of a cofactor common to both nitrate reductase and xanthine dehydrogenase, accordingly designated as *cnx* (common component for nitrate reductase and xanthine dehydrogenase) with the gene allocations, *cnxABC*, *cnxE*, *cnxF*, *cnxG*, and *cnxH*.

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†To whom correspondence should be addressed. Tel.: 44-3-9905-4323; Fax: 44-3-9905-4811; E-mail: shiela.unkles@med.monash.edu.au.

‡Present address: Dept. of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland, United Kingdom.

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In addition to screening for lack of growth on nitrate, improved methods were found to allow selection of cnx mutants. One procedure involved direct selection for nitrate non-utilization by the putrescine starvation procedure (11, 12). Here a putrescine auxotrophic mutant strain (pu2A) grown on minimal medium containing nitrate as the nitrogen source and a severely limiting concentration of putrescine produced small compact colonies. From these colonies, rapidly growing, sporey sectors of growth were observed which, on isolation, were found to result from a secondary mutation affecting nitrate utilization. Although the underlying mechanism and physiology of the putrescine starvation method are unclear, fortuitously a proportion of these sectors was found to be cnx mutants. In this way, cnx mutants could be easily and directly selected on the basis of nitrate non-utilization. A second selection technique involved the isolation of mutants resistant to the toxic substance, chlorate (13, 14). Although the mechanisms of chlorate toxicity are unclear, this approach afforded the rapid and convenient isolation of large numbers of cnx mutants. Among the cnx mutants isolated on the basis of nitrate non-utilization (either by screening or by putrescine starvation selection), some were found to be chlorate-resistant, but intriguingly others were found to be sensitive to chlorate, both classes of mutants occurring in all five cnx loci (11, 14).

The study of the A. nidulans cnx mutants yielded limited biochemical information regarding the function of their gene products (2). Mutations in the cnxH gene were found that resulted in a temperature-sensitive phenotype on nitrate, but not hypoxanthine, as sole nitrogen source. Unexpectedly, these mutants were reported (21) to be thermolabile nitrate reductase, and this led MacDonald and Cove (15) to propose that the cnxH gene produces a protein that is an integral part of the molybdooenzyme, nitrate reductase.

In this article, we describe biochemical and molecular characteristics of cnxG and cnxH genes and their products as well as the characterization of chlorate-resistant, chlorate-sensitive, and temperature-sensitive mutants.

EXPERIMENTAL PROCEDURES

A. nidulans Strains, Plasmids, and Media—The wild-type strains, with regard to nitrogen metabolism, were the biotin auxotroph, biA1, the yellow methionine auxotroph, yA2 methH2, or the putrescine auxotroph, pu2A. Strains cnxG2, cnxG4, cnxH1, and cnxH3, and cnxH4 are the original mutants described by Pateman and colleagues (9, 10). Also used in this study are cnxH35 and cnxH36 classified by MacDonald and Cove (15) as cnxH (cnxE35 and cnxE36). All other cnxG and cnxH mutants were selected during this study at 37 °C following chemical mutagenesis with N-methyl-N-nitro-N-nitrosoguanidine and cnxH20 following 4-nitroquinoline-1-oxide treatment. Mutation cnxG141 arose spontaneously. Mutant strains cnxG100, cnxG141, cnxG166, cnxH86, cnxH89, cnxH255, and cnxH804 were generated following mutagenesis treatment with N-methyl-N-nitro-N-nitrosoguanidine and cnxG20 following 4-nitroquinoline-1-oxide treatment. Mutation cnxG141 arose spontaneously. Mutant strains cnxH35 and cnxH36 were isolated by HPLC using the method described by Johnstone and Rajagopalan (24, 25) with modifications detailed in Unkles et al. (21).

Determination of Nitrate Reductase Thermolability—The procedure of MacDonald and Cove was followed (15). Strains were grown at 25 °C for 21 h in minimal medium with 10 mM nitrate as sole nitrogen source. Freshly harvested mycelium was ground in a mortar precooled to 4 °C with acid-washed sand and 10 volumes of 0.1 M sodium orthophosphate buffer, pH 7.0, containing 10% glycerol at 4 °C. The crude homogenate was centrifuged at 80,000 × g for 20 min at 4 °C, and a 4-ml sample of the supernatant was incubated at 35 °C. Aliquots (0.2 ml) were taken at timed intervals up to 80 min and transferred to glass test tubes held at 0 °C in an ice bath. Nitrate reductase was assayed at 25 °C (26).

RESULTS

Isolation of cnxG and cnxH Mutants—Mutations cnxG10, cnxG24, cnxG100, cnxG166, cnxH43, cnxH86, cnxH89, cnxH255, and cnxH804 were generated following mutagenesis treatment with N-methyl-N-nitro-N-nitrosoguanidine and cnxG20 following 4-nitroquinoline-1-oxide treatment. Mutation cnxG141 arose spontaneously. Mutant strains cnxG10, cnxG141, cnxG166, cnxH86, and cnxH89 were selected on the basis of chlorate resistance, whereas cnxG10, cnxG20, cnxG24, and cnxH43 were isolated as spidery nitrate non-utilizing sectors during putrescine starvation of strain pu2A. Of these latter nitrate non-utilizing mutants isolated by the putrescine starvation technique, cnxG20 and cnxG24 are chlorate-sensitive whereas cnxG10 and cnxH43 are chlorate-resistant.

As we were unable to obtain the original temperature-sensitive cnxH mutants reported by MacDonald and Cove (15) to have thermolabile nitrate reductase, we screened the chlorate-resistant strains selected in this study at 37 °C for a temperature-sensitive phenotype. Strains were therefore tested for sensitivity to chlorate and for the ability to utilize nitrate as the sole nitrogen source at 25 °C. Two temperature-sensitive mutants, cnxH255 (isolated by selection for chlorate resistance) and cnxH804 (putrescine starvation technique), were detected during this screening and are discussed below. No temperature-sensitive cnxG mutants were identified (using either mutant isolation route), a result similar to that observed previously (15).

In addition to new mutant strains above, several mutants originally isolated by screening for nitrate non-utilization (9, 10) were studied, including the chlorate-sensitive strains cnxG2 and cnxH1, as well as chlorate-resistant strains cnxG4, cnxH3, and cnxH4. Mutant strains provided as cnxE35 and cnxE36, which were selected by the putrescine starvation procedure and reported by MacDonald and Cove (15) to be tem-

1 The abbreviations used are: HPLC, high performance liquid chromatography; RACE, rapid amplification of cDNA ends; kb, kilobase pair(s).
per Temperature sensitive on nitrate, were found to be cnxH mutants on the basis of complementation patterns in heterokaryons and consequently redesignated cnxH35 and cnxH36.

**Determination of Precursor Z and Molybdopterin Levels in Chlorate-resistant and Chlorate-sensitive cnxG and cnxH Mutant Strains**—The levels of intermediates of the molybdenum cofactor biosynthetic pathway (Fig. 1A), namely precursor Z and molybdopterin, were measured as their oxidized derivative, compound Z, and dephosphorylated molybdopterin form A, respectively, in the wild-type, chlorate-resistant (cnxG4 and cnxH3), and chlorate-sensitive mutants (cnxG2 and cnxH1). The level of precursor Z was observed to be markedly increased in all mutants compared with the wild-type (Fig. 1B).

Molybdopterin is undetectable in the chlorate-resistant mutants, cnxG4 and cnxH3, whereas up to 2% and 10% of the wild-type level is found in the chlorate-sensitive strains cnxG2 and cnxH1, respectively (Fig. 1B).

**Growth of cnxG2 and cnxH1 Chlorate-sensitive Mutants on nitrate and Hypoxanthine**—To determine if low levels of molybdopterin observed in chlorate-sensitive cnxG2 and cnxH1 mutants are sufficient for production of low levels of nitrate reductase and xanthine dehydrogenase activities in vivo and hence to permit some growth on nitrate or hypoxanthine as sole sources of nitrogen, these mutants were grown for an abnormally long incubation period (7 days at 37 °C). Mutant cnxH1 does indeed show limited ability to grow on both nitrate and hypoxanthine with sparse but conidiating colonies (Fig. 2), most likely reflecting low levels in vivo of active nitrate reductase and xanthine dehydrogenase. Although growth of the cnxH1 mutant is poor on either nitrogen source, it is clearly better on nitrate than hypoxanthine. This ability of the cnxH1 strain to utilize nitrate or hypoxanthine, albeit on prolonged incubation, is in contrast to the chlorate-resistant strain cnxH3 in which molybdopterin is undetectable and which is completely incapable of growth on either nitrogen source (Fig. 2). The cnxG2 mutant also shows low but significant growth after prolonged incubation periods both on nitrate and hypoxanthine compared with the chlorate-resistant cnxG4 mutant, which completely lacks the ability to grow on either nitrogen source. Similar to the cnxH1 strain, mutant cnxG2 can utilize nitrate better than hypoxanthine.

**Studies of the Temperature-sensitive Mutants**—Fig. 3A shows the chlorate toxicity (column 1) and growth responses on nitrate (column 2) or hypoxanthine (column 3) of strains incubated at 25 °C or 37 °C for the standard incubation period of 3 days. At both temperatures, the wild-type is sensitive to chlorate but grows vigorously on nitrate or hypoxanthine, whereas the negative control, mutant cnxH3, is chlorate-resistant and fails to grow on either nitrate or hypoxanthine. The mutant cnxH255 is chlorate-resistant at 37 °C but sensitive to chlorate at 25 °C. Further, it shows considerable growth at 25 °C on nitrate as a nitrogen source but not at 37 °C. In contrast, no growth of the cnxH255 strain is seen on hypoxanthine at both temperatures, i.e. 25 °C and 37 °C, after the standard incubation period of 3 days. However, on prolonged incubation at 25 °C (but not at 37 °C), cnxH255 shows poor but observable growth on hypoxanthine. It should be noted that the temperature sensitivity in mutant cnxH255 is restricted to chlorate toxicity and nitrate utilization because it grows as wild-type on other nitrogen sources such as ammonium at both temperatures.

Precursor Z and molybdopterin levels were measured in mutant cnxH255 as well as the wild-type strain grown at 25 °C (the permissive temperature) or at 37 °C (the nonpermissive temperature) (Fig. 3B). At both 25 °C and 37 °C, there is accumulation of precursor Z in the mutant strain relative to the wild-type. For molybdopterin, however, although at 37 °C it is undetectable in the mutant strain, at 25 °C mutant cnxH255
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Cloning and Structure of the cnxG and cnxH Genes—Both genes were isolated by self-cloning techniques assisted by the autonomous plasmid, pHELP (23), as used for the isolation of the A. nidulans cnxF gene (22). Clones obtained were used as probes to isolate genomic copies from an independently generated A. nidulans genomic library to circumvent potential problems of sequence rearrangement during the original isolation procedure. Unique ORFs encode proteins of 91 and 195 amino acid residues and molecular sizes of 9.6 and 21.6 kDa, respectively, for CnxG and CnxH (Fig. 4). Because no N-terminal amino acid sequence data are available for these proteins, we can only speculate as to the identity of the translational initiation codons. However, in both cnxG and cnxH, ATGs at nucleotide position +1 are the first in-frame downstream from a transcriptional start point that has been mapped by 5' RACE at nucleotide positions –86 (cnxG) and –23 (cnxH). No other in-frame ATG codons exist in the cnxG ORF or until nucleotide 517 in cnxH. Canonical core transcriptional TATAAA or CCAAT motifs are not present in the 5'-nontranslated region of either gene, but this is not entirely unusual for fungal genes (27). No introns appear to be present in either gene in contrast to cnxABC (21), cnxF (22), and probably most fungal genes (27).

Analysis of the Predicted CnxG and CnxH Proteins and the Location of Mutations—Comparisons with the Swiss-Prot data base using Blastp (28) showed that fungal CnxG and CnxH share considerable similarity with amino acid sequences belonging to the small and large subunits, respectively, of the E. coli molybdopterin synthase (encoded by the moaD and moaE genes) (Fig. 5), which is involved in the conversion of precursor Z to molybdopterin (5, 29–31).

The A. nidulans CnxG protein has 23% identity with E. coli MoaD (encoding the small subunit of molybdopterin synthase), this identity being concentrated near the C terminus (9 of 13 residues) (Fig. 5A). To allow comparison of CnxG with a eukaryotic homologue, a corresponding cDNA clone was identified from the human EST data base, and the complete sequence was obtained (32). The predicted human protein has 35% identity with CnxG. Seven mutant forms of A. nidulans cnxG were recovered, each with a different amino acid substitution. Of the seven strains containing mutant forms of cnxG, four strains are chlorate-resistant (cnxG4, cnxG100, cnxG114, and cnxG166) and three are sensitive (cnxG2, cnxG20, and cnxG24). Three mutations, cnxG24 (Ala-10 to Asp), cnxG100 (Glu-21 to Lys), and cnxG4 (the C-terminal Gly-91 to Arg), resulted in changes to residues whose position is conserved in bacterial, fungal, and human proteins. Three mutations, cnxG2, cnxG20, and cnxG141, caused substitution of residues that are conserved in the fungus and human but not the prokaryote. In mutant cnxG2 the aliphatic Ile-84 is replaced by the amide Asn. The nonpolar, hydrophobic residue, Phe, is found in the position equivalent to Ile-84 in the MoaD protein. In the cnxG20 mutant Ala-12 is replaced by Val. Mutation cnxG141 results in the conversion of Tyr-8 to Cys, this Tyr residue being represented in the MoaD protein by another aromatic amino acid, Phe. In the final amino acid substitution mutant, cnxG166, the putative translational initiator is converted to Ile.

Finally, mutation cnxG10 results in the conversion of the codon for residue Gln-77 to a stop codon effectively deleting the region of high similarity to the bacterial and human proteins at the C terminus (Fig. 5A). Six further cnxG mutations were observed that resulted in frameshifts, most likely destroying the CnxG protein. All seven mutants are chlorate-resistant.

produces a small but measurable level of molybdopterin (approximately 6% of the wild type value).

Previous studies by MacDonald and Cove (15) showed that temperature-sensitive mutants in cnxH had a nitrate reductase with a much shorter half-life (e.g. as low as 3.8 min) compared with the wild-type value (18.8 min). In contrast, three independent experiments in this study carried out using the same protocol showed that the stability of nitrate reductase in the wild-type (average half-life 19.25 min) is similar to the temperature-sensitive mutant cnxH255 (average half-life 17.7 min). A further temperature-sensitive mutant, cnxH604, was isolated by the putrescine starvation method. This mutant is phenotypically identical to mutant cnxH255 with regard to chlorate toxicity as well as nitrate and hypoxanthine utilization at 25 °C and 37 °C on standard and prolonged incubations. Strain cnxH604 was shown to possess nitrate reductase with wild-type stability.

A second class of mutants with respect to temperature-sensitive phenotype is represented by strains cnxH35 and cnxH36. Unlike mutants cnxH255 and cnxH604, strains cnxH35 and cnxH36 are sensitive to chlorate at both 25 °C and 37 °C. Also, whereas mutants cnxH255 and cnxH604 appear unable to utilize nitrate or hypoxanthine as the sole nitrogen source at 37 °C after the standard 3-day incubation, mutants cnxH35 and cnxH36 show appreciable growth on both nitrogen sources at this temperature (although marginally better on nitrate than hypoxanthine). At 25 °C, growth of the two mutants is almost as good as a wild-type strain on nitrate although poorer on hypoxanthine after 3 days of incubation.

Fig. 3. Growth phenotypes, precursor Z, and molybdopterin levels in the temperature-sensitive strain cnxH255. A, chlorate toxicity responses of wild-type and mutants cnxH255 and cnxH3 were determined on minimal medium containing 150 mM chlorate and 5 mM uric acid as the nitrogen source at 25 °C or 37 °C (column 1). Strains were grown also on minimal medium containing 10 mM nitrate (column 2) or 5 mM hypoxanthine (column 3) as the sole nitrogen sources at 25 °C or 37 °C. B, levels of precursor Z and molybdopterin were estimated (see legend to Fig. 1) in wild-type and cnxH255 cells grown in medium containing 10 mM proline plus 10 mM nitrate at 25 °C or 37 °C.
The *A. nidulans* CnxH protein shows 29% sequence identity with *E. coli* MoaE, which encodes the large subunit of molybdopterin synthase, whereas a human homologue identified as above has 31% identity (Fig. 5B). The DNA sequence of the *cnxH* gene in four chlorate-resistant strains (cnxH3, cnxH43, cnxH86, and cnxH89) and one chlorate-sensitive strain (cnxH1) was determined. Two mutations, cnxH89 (Gly-74 to Asp) and cnxH43 (Glu-166 to Lys), resulted in changes in amino acid residues whose position is conserved in *E. coli*, *A. nidulans*, and human proteins. The chlorate-sensitive *A. nidulans* strain, cnxH1, contains an alteration of the translational stop codon such that it is translated to give the amino acid residue Cys, now residue 196, followed by 31 novel amino acids before the next observed stop codon (Fig. 5B).

Two temperature-sensitive strains with respect to response to chlorate toxicity and utilization of nitrate (cnxH255 and cnxH604) were examined at the nucleotide level. In strain cnxH255 there is an addition of three nucleotides with the result that Gly is added after residue 148, whereas in mutant cnxH604 this Gly-148 (Ala in the equivalent position in humans and *E. coli*) is replaced with Asp. The second class of mutants cnxH35 and cnxH36, which have temperature-sensitive responses to growth on nitrate but not to chlorate toxicity, have an identical alteration, i.e. Ala-108 to Thr. The residue in the equivalent position in the human protein is not conserved.

Finally, the putative translation initiation Met-1 is altered to Ile in mutant cnxH86, mutation cnxH3 leads to the conversion of residue Trp-163 to a stop, and five other mutations analyzed,2 including cnxH4, generated stops or single-base insertions, again resulting presumably in the synthesis of aberrant proteins. All these mutant strains are chlorate-resistant.

**FIG. 4.** DNA and deduced amino acid sequence of *A. nidulans* *cnxG* and *cnxH*. Numbers on the left refer to nucleotides relative to the A of the start codon, numbered +1, and numbers on the right to amino acid residues. The vertical arrows indicate the transcriptional start points determined by 5' RACE.

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**DISCUSSION**

The data presented here show that the *cnxG* and *cnxH* gene products of the eukaryote, *A. nidulans*, are involved in the conversion of precursor Z to molybdopterin, the intermediate section of the molybdenum cofactor biosynthetic pathway. First, *cnxG* and *cnxH* mutant strains, most likely loss-of-function, have negligible molybdopterin compared with wild-type levels while having vastly increased levels of precursor Z. Second, amino acid sequence comparisons with prokaryotes indicate that the CnxG and CnxH proteins are the eukaryotic
homologues of the small and large subunits, respectively, of molybdopterin synthase, which is required to synthesize molybdopterin from precursor Z (8, 24, 25). Between the eukaryotic CnxG and E. coli MoaD (29), the small subunit, the overall similarity is low (23% identity) and concentrated mainly in the 13 C-terminal amino acid residues with 9 of 13 identical. Several amino acid substitutions in mutant strains (see below) locate either in this C-terminal conserved region or in the first 21 residues at the N terminus, in contrast a region of limited similarity. Although only a small number of mutants have been analyzed at the nucleotide level, such localization of mutations could possibly indicate functional domains of the protein. The human homologue, MOCO1-A (32), is more similar to the A. nidulans CnxG protein with 39% identity, and there are residues in the central section, as well as the N and C termini, conserved in both eukaryotic proteins that might additionally be involved in function. In contrast to the molybdopterin synthase small subunit proteins, similarity of the large subunit proteins (around 29% identity between the three species) is distributed more or less evenly over their primary structure, the bacterial protein, MoaE (29), being somewhat smaller than its eukaryotic counterparts, CnxH and MOCO1-B (32).

We have taken advantage of the ease of cnx mutation isolation in A. nidulans to identify amino acid residues important for function of molybdopterin synthase small and large subunits. Six amino acid residues were found to be essential in CnxG, the small subunit. Of these residues, three are conserved in the bacterial, fungal, and human proteins. Ala-10 and Glu-21 are within the region of low similarity near the N termini of the proteins. The third, the C-terminal residue of the small subunit, Gly-91, has been implicated in the catalytic mechanism of the conversion of precursor Z to molybdopterin. In the mechanism proposed from studies in E. coli, molybdopterin synthase adds dithiolene sulfur to the pterin side chain of precursor Z to form molybdopterin (5). The available evidence suggests that molybdopterin formation requires molybdopterin synthase to be in a sulfur-charged state. This activation of the synthase is thought to occur when molybdopterin synthase sulfurylase, encoded by the cnxF gene in A. nidulans (22) and moeB in E. coli (30, 31, 34), adds sulfur atoms to the molybdopterin synthase small and large subunits, respectively, of the synthase small subunit proteins, similarity of the large subunit proteins (around 29% identity between the three species) is distributed more or less evenly over their primary structure, the bacterial protein, MoaE (29), being somewhat smaller than its eukaryotic counterparts, CnxH and MOCO1-B (32).

We have taken advantage of the ease of cnx mutation isolation in A. nidulans to identify amino acid residues important for function of molybdopterin synthase small and large subunits. Six amino acid residues were found to be essential in CnxG, the small subunit. Of these residues, three are conserved in the bacterial, fungal, and human proteins. Ala-10 and Glu-21 are within the region of low similarity near the N termini of the proteins. The third, the C-terminal residue of the small subunit, Gly-91, has been implicated in the catalytic mechanism of the conversion of precursor Z to molybdopterin. In the mechanism proposed from studies in E. coli, molybdopterin synthase adds dithiolene sulfur to the pterin side chain of precursor Z to form molybdopterin (5). The available evidence suggests that molybdopterin formation requires molybdopterin synthase to be in a sulfur-charged state. This activation of the synthase is thought to occur when molybdopterin synthase sulfurylase, encoded by the cnxF gene in A. nidulans (22) and moeB in E. coli (30, 31, 34), adds sulfur atoms to the molybdopterin synthase small subunit, i.e. CnxG in A. nidulans. The precise mechanisms are unclear but the proposed sulfur transfer might resemble the activation of ubiquitin by ubiquitin-activating enzyme E1 (35). Central to this mechanism is the presence of a C-terminal Gly in CnxG, which forms a thioester bond with an internal Cys residue of CnxF and subsequently acts as the acceptor for reactive sulfur via a thioester bond from an as yet unidentified sulfur donor (Fig. 7). The significance of the short region of identity between the three organisms, immediately upstream of this C-terminal Gly residue, is unknown but could be associated with this sulfur transfer process. It has been suggested (5) that Gly-Gly may be required at the C terminus but, whereas MOCO1-A and MoaD both have C-terminal Gly-Gly residues, Ser is the penultimate residue in the A. nidulans CnxG protein. Of the remaining three substitution mutations, which occur in residues conserved in both eukaryotes but not in the prokaryotic protein, Ile-85 is located in this region of high similarity toward the C terminus. Near the N terminus, replacement of Tyr-8 with a non-aromatic residue (Cys) in the short sequence Tyr-Phe-Ala, conserved between fungus and human and Phe-Phe-Ala in E. coli, sug-
gests that an aromatic residue at this position is necessary for protein function. Finally Ala-12, present in fungal and human proteins, is not conserved in the prokaryotic protein.

In organisms such as bacteria and higher plants, generation of mutants defective in molybdenum cofactor biosynthesis is usually achieved by selection for chlorate resistance. In *A. nidulans*, mutants can additionally be selected on the basis of nitrate non-utilization using the putrescine starvation technique, and a proportion of these mutants are sensitive to chlorate. Therefore, in *A. nidulans* two classes of cnx mutants with regard to chlorate toxicity can be obtained. Chlorate-resistant mutants *cnxG4* and *cnxH3* fail to produce molybdopterin and completely lack the ability to grow on nitrate even after prolonged incubation. In contrast, the chlorate-sensitive mutants *cnxG2* and *cnxH1* elaborate some molybdopterin and show slow but significant growth on nitrate after long incubation periods (7 days) probably reflecting low in vivo levels of nitrate reductase activity. Such results suggest that in order to become chlorate-resistant, a mutant requires the complete abolition of nitrate reductase activity, whereas the retention of some activity renders a mutant strain sensitive to chlorate. In this way, we can predict which *cnx* mutants are leaky, producing low levels of molybdopterin, and which are loss-of-function, completely lacking molybdopterin, simply by testing for sensitivity or resistance to chlorate toxicity. Therefore, we would expect that low but detectable levels of molybdopterin are present in chlorate-sensitive strains, *cnxG24* and *cnxG20*. On this basis, replacement of CnxG residues, Ala-10 or Ala-12, in these mutants leads to reduced molybdopterin synthase activity but not loss-of-function. By extension, in chlorate-resistant strains, *cnxG141*, *cnxG100*, *cnxH89*, and *cnxH43*, substitution of residues Tyr-7 or Glu-21 of CnxG, and Gly-74 or Glu-166 of CnxH, respectively, results in absence of molybdopterin due to inactivity of the molybdopterin synthase.

Temperature-sensitive mutants *cnxH255* and *cnxH604* affect the same location in the CnxH protein, *cnxH255* having an addition of a Gly residue after Gly-148 and *cnxH604* being an alteration in Gly-148 itself. This region of the protein does not appear to show similarity to the human or bacterial proteins. A likely interpretation is that the conformation of the CnxH protein in these mutant strains is altered substantially at the nonpermissive temperature leading to complete loss-of-function of the molybdopterin synthase. Because no molybdopterin is produced, no active nitrate reductase is formed, and hence the strains are chlorate-resistant. At the permissive temperature, the conformation of the CnxH polypeptide is such that a small but significant level of molybdopterin synthase activity is possible, molybdenum cofactor is synthesized permitting active nitrate reductase, and the strains are chlorate-sensitive. Strains *cnxH35* and *cnxH36* have a less extreme temperature-sensitive phenotype than *cnxH255* and *cnxH604*. Replacement of Ala-108 therefore also affects reduced molybdopterin synthase efficiency due to alteration of protein conformation, although the structural perturbation is less severe than changes affecting Gly-148. In contrast to mutants *cnxH255* and *cnxH604*, at the nonpermissive temperature of 37 °C, strains *cnxH35* and *cnxH36* are chlorate-sensitive and thus probably produce a low level of molybdopterin. Finally, it is noteworthy that no temperature-sensitive mutations have been isolated in the *cnxG* gene despite fairly extensive searches previously (15) and in this study. The significance of this is unclear, but the lack of temperature-sensitive mutations may reflect the fact that *cnxG* is a small gene.

A further point emerges from the study of the chlorate-sensitive mutants with regard to the interaction of the molybdopterin synthase subunits and the *cnxF* gene product, molybdopterin synthase sulfurylase. Previous analysis of pairwise double mutants between chlorate-sensitive strains, *cnxH1*, *cnxG2*, and *cnxF11*, showed that a *cnxH1cnxF11* double mutant remains chlorate-sensitive (therefore producing molybdopterin), whereas *cnxG2cnxH1* and *cnxF2cnxF11* double mutants become resistant (unable to produce molybdopterin) (14). Study of combinations of mutations leading to chlorate sensitivity could therefore yield information on the tolerance of conformational changes during protein-protein interactions.

The demonstration that temperature-sensitive *cnxH* mutants possessed thermolabile nitrate reductase led to the hypothesis advanced by MacDonald and Cove (15) that the CnxH protein is an integral part of the nitrate reductase molecule, a homodimer of around 180 kDa (36, 37) consisting of two identical 91-kDa polypeptide subunits (38). Because the validity of this proposal has never been resolved and the original *cnxH* mutants with thermolabile nitrate reductase properties are lost or not available to us, two new temperature-sensitive strains, *cnxH255* and *cnxH604*, were isolated in this study. These mutants were found to have nitrate reductase with wild-type thermolability. The *cnxH* gene encodes the molybdopterin synthase large subunit, and there is no evidence of nitrate reductase containing polypeptides other than the two subunits in any organism in which the enzyme has been purified including *A. nidulans* (38). These observations, coupled with the wild-type thermal stability of nitrate reductase in our mutants, makes the proposition that CnxH is part of the nitrate reductase molecule appear less attractive.

Chlorate-sensitive mutants, such as *cnxG2* and *cnxH1*, grow better on nitrate than hypoxanthine after extended growth periods. This is also true for the temperature-sensitive mutant *cnxH255* at the permissive temperature. Such mutants have been shown to produce low but measurable levels of molybdopterin. The reasons for the growth differences on nitrate and hypoxanthine are unclear, but it has been suggested that xanthine dehydrogenase has a more stringent requirement for the integrity of molybdenum cofactor than nitrate reductase (15) or that nitrate reductase has a greater affinity for the cofactor than xanthine dehydrogenase (39).

The study of the *A. nidulans* molybdenum cofactor biosynthetic genes may aid the identification of essential residues in other eukaryotic homologues. These are clearly observed in EST data bases including worm, rice, fruit fly, mouse, and human.2 Until now, human molybdenum cofactor deficiency has been recognized as a rare disease that leads to fatality at a very young age (1, 3), but recently, late-onset symptoms have been described in two individuals who appear to have less severe forms of molybdenum cofactor deficiency (40). By extrapolation from the studies of *A. nidulans* chlorate-sensitive *cnx* mutants, these milder symptoms in humans could be the result of alteration of certain residues leading to low molybdo-terin production with concomitant decrease in the elaboration of molybdoenzymes. The position of the *A. nidulans* CnxG residues which result in a leaky phenotype is conserved in the human protein. Reduced levels of molybdopterin due to residue changes may be less easily diagnosed in humans than severe deficiency and escape detection. Therefore, milder forms of human molybdenum cofactor deficiency could be more common in the population than originally thought.

It is perhaps surprising that both *cnxG* and *cnxH* genes are absent from the yeast *Saccharomyces cerevisiae*,2 an organism of immense technological interest as well as being a model eukaryote. Their absence however supports our inability to demonstrate the presence of precursor Z and molybdopterin in
The A. nidulans and Cove (15). The results are presented in Fig. 8. Nitrate reductase values in the mutant result from variation in this initial increase in most organisms from bacteria to man, it seems likely that at some point during evolution S. cerevisiae has lost one of the cofactor biosynthetic genes, making redundant the genes encoding the remainder of the pathway and those specifying molybdoenzymes.

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Note Added in Proof—The half-life of nitrate reductase in temperature-sensitive mutants cnxH604 and cnxH604 was reported to be approximately wild-type. However, the nitrate reductase assay was used that normally used for N. crassa (26). To circumvent the unlikely possibility that this was the reason our results differed from MacDonald and Cove (15), we repeated the analysis of cnxH604 and wild-type with the A. nidulans nitrate reductase assay procedure used by MacDonald and Cove (15). The results are presented in Fig. 8. Nitrate reductase activity in the wild-type was maximal after 2.5 min. In contrast, the maximum activity of cnxH604 was not reached until 10 min, after an initial increase in activity of up to 27%. The higher standard deviation values in the mutant result from variation in this initial increase (which was not observed in two of the three experiments). Even although formation of active nitrate reductase may not have a significant effect on the apparent rate of inactivation in the later stages of the experiment, there is a clear and significant superimposition of nitrate reductase formation and inactivation at the start of the incubation period. Consistent with the low specific activity of nitrate reductase from the mutant, these findings might be explained by a limitation of nitrate reductase formation in vivo, resulting in accumulation of precursor form(s) that can form nitrate reductase activity in vitro. Notwithstanding this initial increase in nitrate reductase activity in the mutant, we can see no evidence that the half-life of nitrate reductase is significantly lower in our temperature-sensitive cnxH mutants than in the wild-type.

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