Mutational Analysis of the Gephyrin-Related Molybdenum Cofactor Biosynthetic Gene *cnxE* From the Lower Eukaryote *Aspergillus nidulans*

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ABSTRACT

We report the identification of a number of mutations that result in amino acid replacements (and their phenotypic characterization) in either the MogA-like domain or domains 2 and 3 of the MoeA-like region of the *Aspergillus nidulans cnxE* gene. These domains are functionally required since mutations that result in amino acid substitutions in any one domain lead to the loss or to a substantial reduction in all three identified molybdoenzyme activities (*i.e.*, nitrate reductase, xanthine dehydrogenase, and nicotinate hydroxylase). Certain *cnxE* mutants that show partial growth with nitrate as the nitrogen source in contrast do not grow on hypoxanthine or nicotinate. Complementation between mutants carrying lesions in the MogA-like domain or the MoeA-like region, respectively, most likely occurs at the protein level. A homology model of CnxE based on the dimeric structure of *E. coli* MoeA is presented and the position of inactivating mutations (due to amino acid replacements) in the MoeA-like functional region of the CnxE protein is mapped to this model. Finally, the activity of nicotinate hydroxylase, unlike that of nitrate reductase and xanthine dehydrogenase, is not restored in *cnxE* mutants grown in the presence of excess molybdate.

THE molybdenum cofactor is an identical structural and functional component of most molybdoenzymes, catalysts that carry out key metabolic reactions necessary for sustaining the sulfur, nitrogen, and carbon cycles in organisms ranging from bacteria to human (Rajagopalan 1996 and references therein). The first evidence for the biosynthetic pathway of this ubiquitous and ancient molecule came from genetic studies of nitrate assimilation in the lower eukaryote *Aspergillus nidulans*; these studies, by Pateman and Cove, of a number of *cnx* mutants resulted in the identification of five gene loci (Cove and Pateman 1963; Cove *et al.* 1964; Pateman *et al.* 1964). Mutant *cnxE* strains were found to be unique among *cnx* mutants in that they could be repaired for growth on minimal medium with nitrate or purines (such as hypoxanthine) as the sole sources of nitrogen by addition of relatively high concentrations (33 mm) of sodium molybdate (Cove *et al.* 1964; Arst *et al.* 1970). This phenotypic repair has been shown to be due to the partial restoration of nitrate reductase and xanthine dehydrogenase activities, respectively (Arst *et al.* 1970). Partial rectification of enzyme activity led to the proposal that the CnxE protein acted directly on molybdate when present at low concentrations to incorporate it into the cofactor. As the final step in the biosynthetic pathway, its catalytic activity became largely unnecessary in cells grown in the presence of excess molybdate concentrations, probably as a result of mass action.

Pioneering work on the chemical structure of the molybdenum cofactor and the gene/enzyme relationships implicated in the biosynthesis of the molybdenum cofactor, including *mogA* and *moeA* cistrons in *Escherichia coli* (discussed below), was provided by Rajagopalan (1996) and colleagues. On the basis of the illuminating work of Rajagopalan, our *A. nidulans* studies have suggested the likely involvement of the *cnx* loci (including *cnxE*) in molybdenum cofactor biosynthesis (Unkles *et al.* 1997, 1999; Appleyard *et al.* 1998).

Kamdar *et al.* (1994) isolated and sequenced the eukaryotic gene Cinnamon from *Drosophila melanogaster*. Their interesting comparison studies showed that the N-terminal and C-terminal sections of the fruit fly protein are similar in amino acid sequence to MogA and MoeA, respectively, which are single proteins implicated in the later stage of the molybdenum cofactor biosynthetic pathway in *E. coli* (Rajagopalan 1996). Cinnamon is also highly similar in amino acid sequence to the rat protein Gephyrin (Kamdar *et al.* 1994), which appears to have two functions: one anchoring neurotransmitter receptors to the cytoskeletal structures and the other involving the biosynthesis of the molybdenum cofactor (Feng *et al.* 1998). Recent studies have shown that *A. nidulans* CnxE is the ortholog of these eukaryotic fused proteins (Millar *et al.* 2001). Curiously, in the

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plant ortholog, Cnx1, the fused MogA-like and MoeA-like domains are in the opposite orientation to the fungal, mammalian, and fruit fly proteins (Stallmeyer *et al.* 1995, 1999).

The precise biochemical role of *E. coli* MogA and MoeA (or the counterpart MogA- and MoeA-like fused proteins in eukaryotes) remains unclear. MogA and its eukaryotic counterpart domain have been implicated in molybdopterin binding while MoeA has also been shown to bind molybdopterin, albeit with a lower affinity (Schwarz *et al.* 1997; Stallmeyer *et al.* 1999; Kuper *et al.* 2000). However, no binding of molybdenum (in the form of molybdate) by either domain has been observed. Whatever the exact mechanisms of the later stages of the pathway, it is clear from the Aspergillus *cnxE* molybdate repairability growth test studies that CnxE (and *inter alia*, homologs) is needed only to synthesize the molybdenum cofactor when the concentration of molybdate available to the organism is low. Indeed, recent studies by Leimkühler and Rajagopalan (2001) have demonstrated directly, using an *in vitro* system, that MogA and MoeA reactions are largely dispensable in the presence of higher concentrations of molybdate.

Recent research has solved the crystal structure of several proteins involved in molybdenum cofactor biosynthesis, including MogA (Liu *et al.* 2000; Sola *et al.* 2001) and MoeA (Schrag *et al.* 2001; Xiang *et al.* 2001). Although no definitive biochemical role is obtained from these reports, several important observations have been made. First, while MogA is composed of a single globular domain, MoeA consists of four discrete domains. In this article, therefore, we use the terms MogA-like domain and MoeA-like region (containing the four domains). Second, sequence and structural similarities between MogA and the third domain of MoeA suggest that they bind similar ligands and therefore have similar functions (Schrag *et al.* 2001). It has been suggested that the MogA-like domain and MoeA-like region in the eukaryotic protein may form a composite binding region (Xiang *et al.* 2001). High-resolution crystal structures provide the position of the highly conserved ThrThrGlyGlyThrGly motif, which is thought to be involved in molybdopterin binding.

Here, we report the results of a mutation experimental approach to study this eukaryotic complex fusion protein. Although mutants have been isolated previously in several eukaryotes (Cove and Pateman 1963; Cove *et al.* 1964; Pateman *et al.* 1964; Kuper *et al.* 2000; Reiss *et al.* 2001), only one eukaryotic amino acid replacement mutant is thus far (*i.e.*, the molybdate-repairable *chl-6* mutant of *Arabidopsis thaliana*; Schwarz *et al.* 2000) identified at the nucleotide level with the residue change related to the phenotypic consequences of the resultant mutation within the natural eukaryotic host. We describe in this article studies of the phenotypic consequences of a series of *cnxE* mutations, identified at the amino acid level and within the natural host, *A. nidulans*, and the complementation patterns of these mutations within heterokaryons.

MATERIALS AND METHODS

***A. nidulans* strains and mutant isolation:** Standard wild-type (with regard to nitrogen metabolism) strains used for the isolation of mutants on the basis of chlorate resistance (see below) were (a) G1070, yellow *yA2* with no other known markers or (b) G001, a biotin auxotroph, *biA1*. The *puA2* putrescine auxotrophic strain (G071) was used to isolate mutants on the basis of selection for nitrate nonutilization. Standard Aspergillus growth media and handling techniques were as described before (Clutterbuck 1974). The generation of mutations was carried out using the chemical mutagen 4-nitroquinoline-1 oxide (Bal *et al.* 1977). After mutagenesis, mutant selection was carried out on the basis of (i) resistance to 150 mm chlorate toxicity with 10 mm proline as the sole nitrogen source or (ii) nitrate nonutilization using the putrescine starvation method (Cove 1976a,b and references therein). The subsequent characterization and identification of *cnxE* mutants were by growth tests according to those described by Cove (1976a,b). All *cnxE* mutants containing the putrescine auxotrophic marker (*puA2*) were outcrossed to provide *cnxE* putrescine auxotropic strains for characterization. For growth testing of molybdate repair with nicotinate or hypoxanthine, *cnxE* mutants were crossed to mutant *crnA-niiA-niaD*Δ*506* (abbreviated in the article to *niaD*Δ*506*), a deletion extending through the nitrate assimilation gene cluster and therefore lacking nitrate reductase (as well as nitrite reductase and nitrate transport) activity (Tomsett and Cove 1979). These *cnxE niaD*Δ*506* double-mutant strains were used to circumvent molybdate toxicity effects that occur when these two nitrogen sources are used in molybdate repair growth tests (Arst *et al.* 1970).

Isolation of a *cnxE* mutant in the *mogA*-like domain by *in vitro* mutagenesis: pMON5, containing the entire *cnxE* gene in pUC19, was digested with *Eag*I and *Spe*I. The 6-kb fragment was isolated, blunt-ended with Klenow, and ligated to give pΔ36-130. This removed a 375-bp fragment encoding amino acid residues 36–130, but left the reading frame unchanged. The construct pΔ36-130, linearized with *Xba*I, was transformed into *cnxE13* and transformants selected by growth on nitrate as the sole nitrogen source. DNA was isolated from 17 transformants and subjected to PCR amplification using primers situated on either side of the deletion (5′-GTGCCTGAGGTG TCAAT and 5′-GATGATGATCATGTTGTGAC). All the transformants contained a 781-bp fragment expected for the wild-type *cnxE*. However, the amplification products of transformant T1 contained, in addition, a fragment of 406 bp expected from pΔ36-130, suggesting that T1 was a heterokaryon. Since the conidia of *A. nidulans* are uninucleate, single colonies were isolated from a conidial suspension of T1, thus permitting isolation of homokaryotic strains. When tested for the ability to utilize nitrate as the sole nitrogen source, some of these colonies were unable to grow. PCR amplification of DNA from these colonies using the above primers showed that those that grew on nitrate contained the 781-bp fragment alone, while those that did not grow contained only the 406-bp fragment. A 406-bp fragment from colony C3 was sequenced and found to contain the expected deletion of nucleotides encoding residues 36–130, but leaving the CnxE start codon in frame with the rest of the protein. Finally, the entire *cnxE* coding region in C3 was sequenced following the finding that the only mutation present was the 375-bp deletion. This strain was designated *cnxE*Δ*3*.

Mutant DNA sequence determination: The DNA sequence

of mutant *cnxE* genes was determined by automated sequencing following PCR amplification of genomic DNA as described before (Unkles *et al.* 1999). The entire coding region was amplified in five overlapping sections using primers E1A and E1B (5′-GTGCCTGAGGTGTCAAT and 5′-AGTGACTTGT GTCGGGT, nucleotide positions −119–481 relative to the A of the start codon as 1; Millar *et al.* 2001), E2A and E2B (5′-GTTGAAGGTCACGCCTT and 5′-CAGCATATCCATCA ACG,positions 371–987), E3A and E3B (5′-CGAGGTTCCGG TGAATA and 5′-CCCGTAGGCTTTGTTCC, positions 881–1575), E4A and E4B (5′-CTGAAATCATGGGGGAT and 5′-CTTTGGGAGACGCTGTA, positions 1494–2031), and E5A and E5B (5′-CCCTTGTAGCAGTAAC and 5′-GAATAAGTCT TTGGAGG, positions 1942–2336).

Molecular modeling of CnxE: The homology model was constructed from the coordinates of *E. coli* MoeA (PDB code 1FC5; Schrag *et al.* 2001) using the homology module of *INSIGHT* (Molecular Simulations, San Diego) based on a multiple sequence alignment of the MoeA homologous domains of CnxE, Cnx1, Cinnamon, and Gephyrin with *E. coli* MoeA. After assignment of coordinates of equivalent residues, the model was energy minimized in *CNS* (Brünger *et al.* 1998).

HPLC analysis of molybdenum cofactor precursor levels in cell-free extracts: Shake flask cultures were grown for 12 hr at 30° and 250 rpm in liquid minimal medium (Cove 1966) containing 10 mm proline plus 10 mm nitrate as the sole nitrogen sources. Preparation of samples for HPLC was carried out using the following modification of the method reported by Johnson and Rajagopalan (1987). Mycelium (0.8 g), suspended in 3.5 ml of distilled water, was homogenized by sonication and centrifuged for 20 min at 20,000 × *g*. Cell-free extracts corresponding to 1 mg of protein were mixed with 125 μl of iodine solution (1% I_2, 2% KI, 0.25 m HCl) and water to a final volume of 1.125 ml. After ~16 hr at room temperature, 138 μl 1% ascorbic acid and 0.5 ml 0.25 m Tris base were added and samples were cleared using a table-top centrifuge. To the supernatant, 13 μl 1 m $MgCl_2$ and 1 unit alkaline phosphatase dissolved in 85 μl water were added and incubated for 4 hr at 37° to carry out dephosphorylation. The samples were bound to 0.5 ml of QAE Sephadex (acetate form). After washing the columns with water, form A dephospho was eluted with 5 ml of 10 mm acetic acid and compound Z was eluted with 13 ml of 10 mm HCl. The eluates were stored at −70° for HPLC analysis. The samples were used without pH titration or a concentration step. HPLC buffer for form A dephospho analysis was 7% methanol, 50 mm ammonium acetate, and 5% methanol, 50 mm triethylammonium acetate, pH 7.0 for compound Z. Reverse phase (C18) HPLC of form A dephospho samples (1 ml) and compound Z (0.5 ml) samples was carried out with fluorescence detection set at 350/450 nm and 360/440 nm, respectively. Internal standards of form A dephospho and compound Z added during iodine oxidation showed an extraction efficiency of ~95%, which significantly decreased when extracts corresponding to several milligrams of protein were applied to the QAE Sephadex columns. Form A dephospho is derived from all sources of molybdopterin, including the cofactor itself, molybdopterin bound to nonmolybdoenzymes, and free molybdopterin.

Nitrate reductase, xanthine dehydrogenase, and nicotinate hydroxylase activities in mutants and their repair by molybdenum: Mycelial cells were grown in orbital shake flask culture at 25° according to previous methods (Cove 1966; Arst *et al.* 1970). Cultures grown on minimal medium with 5 mm urea as the sole source of nitrogen, with and without sodium molybdate (33 mm), were induced by the addition of 10 mm nitrate (for nitrate reductase activity) or 0.6 mm uric acid (for xanthine dehydrogenase) for 5 hr. For nicotinate hydroxylase assays, *cnxE* strains were first crossed to a mutant in the structural gene (*hxA1*) encoding xanthine dehydrogenase (Scazzocchio 1994) to generate *cnxE hxA1* double mutants. The *hxA1* mutation abolishes possible interfering xanthine dehydrogenase activity (Sealy-Lewis *et al.* 1978). Cells of the double mutants were grown as before, except that for induction, the mycelium was washed in deionized water and suspended in fresh minimal medium containing 10 mm nicotinic acid as the sole nitrogen source and incubated for a further 5 hr. After induction, mycelia were harvested and stored at −70° until assay.

Cell-free extracts from 0.5 g of cells were prepared by homogenization using sonication with 3 ml of 100 mm phosphate buffer, pH 7.5 (for nitrate reductase) or 100 mm pyrophosphate buffer pH 9.4 (xanthine dehydrogenase and nicotinate hydroxylase) and centrifuged 20 min at 20,000 × *g*.

Nitrate reductase activity at 25° (nanomoles of nitrite produced per minute per milligram) was determined according to Heck and Ninnemann (1995). Xanthine dehydrogenase and nicotinate hydroxylase activities were determined using described procedures (Sealy-Lewis *et al.* 1978; Scazzocchio 1994). Specific activity (nmol/min/mg) was calculated as the reduction of cytochrome c (from horse heart) using a millimolar extinction coefficient of 29.5. Xanthine dehydrogenase and nicotinate hydroxylase activities were determined once in each of three independent mycelial cultures, while nitrate reductase was determined in triplicate in each of three independent cultures.

Protein content was estimated by the Bradford method (Bio-Rad) with BSA as the standard.

***E. coli* strains, plasmids, and media:** Standard growth media and handling techniques for *E. coli* were employed. Standard procedures were used for propagation of plasmids and for subcloning and propagation of plasmids in *E. coli* strain DH5α.

RESULTS

Generation and sequence analyses of *in vivo cnxE* mutants: A total of ~2000 mutants was isolated on the basis of resistance to chlorate toxicity or the nonnitrate utilization/putrescine starvation method using random chemical mutagenesis. From growth tests, 205 were observed to be *cnx* mutants, of which 25 were found to be *cnxE* mutants on the basis of complementation tests in heterokaryons (Cove and Pateman 1963; Cove *et al.* 1964; Pateman *et al.* 1964). Chlorate toxicity screening yielded 20 *cnxE* mutants while 5 mutants came from the nonnitrate utilization approach. The latter crop of mutants were all found to be chlorate resistant. In addition, 6 mutants (*cnxE3*, *-12*, *-13*, *-14*, *-16*, *-22*) originally isolated by Cove and Pateman were included in this study. Strain *cnxE14* did not conform to the phenotype previously described, *i.e.*, sensitivity to chlorate toxicity (Arst *et al.* 1970). Figure 1A summarizes the changes in the CnxE protein in representative *cnxE* mutants analyzed at the nucleotide level.

One amino acid replacement, in mutant *cnxE22*, was found in the MogA-like domain of the CnxE protein (Figure 1), resulting in Gly79 being replaced with Asp. Nine mutations were found to result in amino acid substitutions within the MoeA-like region (Figures 1 and 2): *cnxE12* (Gly343 to Asp) and *cnxE849* (Ala372 to Ser) within domain 2 and *cnxE107*, *cnxE601*, and *cnx140* (all

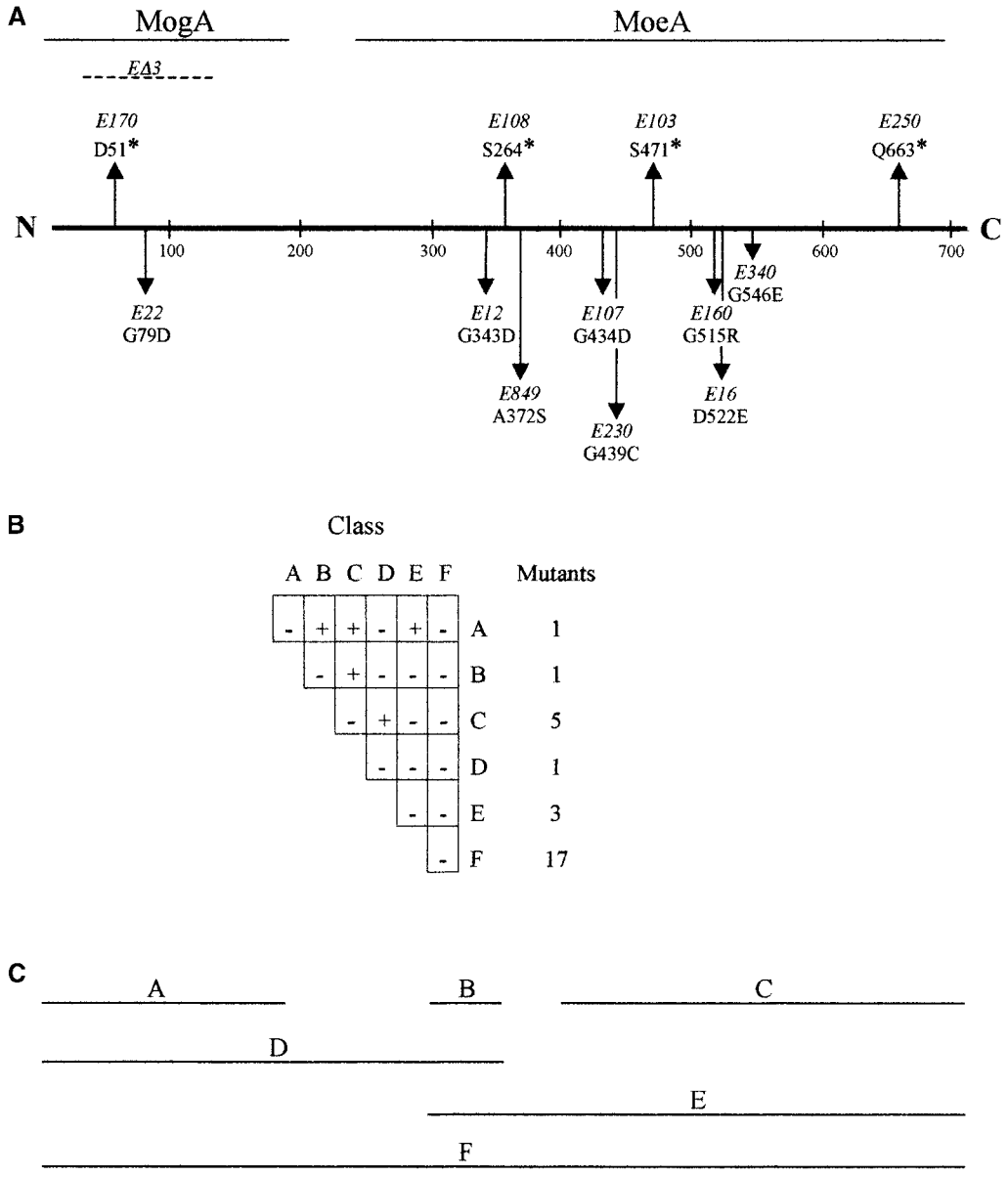

FIGURE 1.—The position of *cnxE* mutant changes within the MogA-like domain and MoeA-like region in the CnxE protein and genetic complementation data. (A) The thick line represents the 709 amino acid residues of the CnxE protein (MILLAR *et al.* 2001). The thin lines above show the extent of the MogA-like domain and the MoeA-like region while the dashed line indicates the position of the deletion in strain *cnxEΔ3*. Arrows show the position of mutations with the amino acid change given in single letter code. Stops are indicated by an asterisk. (B) Results of pairwise mutant complementation tests in heterokaryons. +, vigorous growth of heterokaryons; −, no detectable growth. Hypoxanthine (5 mM) was chosen as the nitrogen source since individual *cnxE* mutants are completely unable to grow on this nitrogen source. Mutants (28) were divided into six complementation classes, A–F, as indicated in the rightmost column. (C) Establishment of a complementation map derived from testing pairwise heterokaryon combinations (FINCHAM 1966, 1994).

Gly434 to Asp), *cnxE230* (Gly439 to Cys), *cnxE160* (Gly515 to Arg), *cnxE16* (Asp522 to Glu), and *cnxE340* (Gly546 to Glu), all within domain 3. Four single-base-pair substitution mutations, which resulted in stop codons, were used in complementation studies: *cnxE170* at residue 51, *cnxE108* at 364, *cnxE103* at 471, and *cnxE250* at 663 (Figure 1). An additional 13 mutations resulted in stop codons: *cnxE104* at residue 4, *cnxE360* at 62, *cnxE106* at 149, *cnxE116* at 183, *cnxE101* at 185, *cnxE109* at 187, *cnxE170* at 256, *cnxE14* at 429, *cnxE3* at 502, *cnxE150* at 520, *cnxE13* at 542, *cnxE4* at 552, and *cnxE190* at 598. Finally, only one substantial deletion was obtained with this mutagen: *cnxE505* with a 10-bp deletion starting at residue 606. Surprisingly, three mutants appeared to be unchanged at the DNA level and are being examined further.

Isolation of a *cnxE* mutant in the MogA-like domain by *in vitro* mutagenesis: Since only one *in vivo* mutation (*i.e.*, *cnxE22*) has been found to reside in the MogA-like domain coding region (Figure 1) and since *cnxE22* is not a complete loss-of-function mutation (see below), an in-frame deletion mutant was generated (see MATERIALS AND METHODS). This mutation, designated *cnxEΔ3*, results in deletion of a section of the MogA-like domain from amino acid residues 36–130 to create a mutant devoid of MogA-like function.

Genetic complementation relationships: Twenty-eight mutants were analyzed to identify complementation patterns**.** Mutants were co-inoculated in all pairwise combinations to form heterokaryons on hypoxanthine as the sole nitrogen source. From the results of complementation that restored growth approximating wild type on hypoxanthine (Figure 1), six complementation classes, A–F, were derived (Figure 1C). Mutants *cnxE22*

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CnxE       PRAHTTPSERRSNDPAAGATRRYRESPYPMLSVDEALRQVSAHTPEPEVIEVPVNIDLVG  273
MoeA       ----------------------MEFTTGLMSLDTALNEMLSRVTPLTAQETLPLVQCFG   37
Cnx1       --------------MEGQGCCGGGGGKTEMIPTEEALRIVFGVSK-RLPPVIVSLYEALG   45
Cinnamon   -----AQKSHICPHKTGTGTDSDRNSPYPMLPVQEVLSIIFNTVQ---------KTANLN  219
Gephyrin   -------------------ARRHRMSPFPLTSMDKAFITVLEMTP-VLGTEIINYRDGMG  352

CnxE       YVIAEDVYAAEAVPAYLASIVDGYAVIAPESPDDGHSTKGIFPVASITHANEEGALAPLE  333
MoeA       RILASDVVSPLDVPGFDNSAMDGYAVRLADIASG-----QPLPVAGKSFAGQP-YHGEWP   91
Cnx1       KVLAEDIRAPDPLPPYPASVKDGYAVVASDGP-------GEYPVITESRAGNDGLGVTVT   98
Cinnamon   KILLE-MNAPVNIPPFRASIKDGYAMKSTGFS-------GTKRVLGCIAAGDSPNSLPLA  271
Gephyrin   RVLAQDVYAKDNLPPFPASVKDGYAVRAADGP-------GDRFIIGESQAGEQ-PTQTVM  404
                      *    *  ****                      *

                   D                          S
                   |E12                      |E849
CnxE       PGTIARITTGAPLPPNANAVVMVEDTLLASSTPDGKEEATVEILTGEIKPNENVRQPGSD  393
MoeA       AGTCIRIMTGAPVPEGCEAVVMQEQTEQMDN--------GVRFTA-EVRSGQNIRRRGED  142
Cnx1       PGTVAYVTTGGPIPVGADAVVQVEDTKVIG--DVSTEAKRVKILI-QTKKGTDIRRVGCD  155
Cinnamon   EDECYKINTGAPLPLEADCVVQVEDTKLLQLDKNGQES-LVDILV-EPQAGLDVRPVGYD  329
Gephyrin   PGQVMRVTTGAPIPCGADAVVQVEDTELIRESDDGTEELEVRILV-QARPGQDIRPIGHD  463
                  ** * *     **  * *              *             *  * *

                                              D    C
                                         E107|     |E230
CnxE       VALGSRILQRGDLITPVGGEIGLLAATGTRTVKVFKKPVVGVLSTGDELVEHDDPRSLQG  453
MoeA       ISAGAVVFPAGTRLTTA--ELPVIASLGIAEVPVIRKVRVALFSTGDELQLPGQ--PLGD  198
Cnx1       IEKDATVLTTGERIGAS--EIGLLATAGVTMVKVYPMPIVAILSTGDELVEPTA-GTLGR  212
Cinnamon   LSTNDRIFPALDPSPVV--VKSLLASVGNRLILS--KPKVAIVSTGSELCSPRN--QLTP  383
Gephyrin   IKRGECVLAKGTHMGPS--EIGLLATVGVTEVEVNKFPVVAVMSTGNELLNPED--DLLP  519
                             *  *           *   *** **        *

CnxE       GQIRDSNRPSILSCLKSWGIPAVDLGIARDTPAGELEQSLRDALRGVGKSNTSVDVIITT  513
MoeA       GQIYDTNRLAVHLMLEQLGCEVINLGIIRDDDPH-ALRAAFIEADSQ-------ADVVISS  250
Cnx1       GQIRDSNRAMLVAAVMQQQCKVVDLGIVRDDRK-ELEKVLDEAVSSG------VDIILTS  265
Cinnamon   GKIFDSNTTMLTELLVYFGFNCMHTCVLSDTFQ-RTKESLLELFEV-------VDFVICS  435
Gephyrin   GKIRDSNRSTLLATIQEHGYPTINLGIVGDNPD-DLLNALNEGISR-------ADVIITS  571
           * * * *                      *                      *

            R      E                      E
            |E160  |E16                   |E340
CnxE       GGVSMGELDLLKPTIERSLGGTIHFGRVSMKPGKPTTFATVPFKPTSSAAGQQERSSRLI  573
MoeA       GGVSVGEADYTKTILEE--LGEIAFWKLAIKPGKPFAFGKLSNS--------------WF  294
Cnx1       GGVSMGDRDFVKPLLEE--KGKVYFSKVLMKPGKPLTFAEIRAKPTESML----GKTVLA  319
Cinnamon   GGVSMGDKDFVKSVLED-LQFRIHCGRVNIKPGKPMTFASRKDK--------------YF  480
Gephyrin   GGVSMGEKDYLKQVLDIDLHAQIHFGRVFMKPGLPTTFATLDID----------GVRKII  621
           **** *  *  *                  *** *  *

CnxE       FSLPGNPASALVTLNLFVLPSLHKLIGLGQKQAALGIAPALGLPLVAVTLSHAFPLDPKR  633
MoeA       CGLPGNPVSATLTFYQLVQPLLAKLSGNTAS-------GLP--ARQRVRTASRLKKTPGR  345
Cnx1       FGLPGNPVSCLVCFNIFVVPTIRQLAGWTS--------PHP--LRVRLRLQEPIKSDPIR  369
Cinnamon   FGLPGNPVSAFVTFHLFALPAIRFAAGWDR--------CKCSLSVLNVKLLNDFSLDS-R  531
Gephyrin   FALPGNPVSAVVTCNLFVVPALRKMQGILD--------PRP--TIIKARLSCDVKLDP-R  670
             ***** *          *      *                              *

CnxE       TEYHRAIVTASPKD----GRLYATSTGAEGVGQRSSRVGSLASANSLLVLQPGKGSIAQG  689
MoeA       LDFQRGVLQRNADG-----ELEVTTTG----HQGSHIFSSFSLGNCFIVLERDRGNVEVG  396
Cnx1       PEFHRAIIKWKDNDGSGTPGFVAESTG----HQMSSRLLSMRSANALLELPATGNVLSAG  425
Cinnamon   PEFVRASVISKSGE-----LYASVNGN-----QISSRLQSIVGADVLINLPARTSDRPLA  581
Gephyrin   PEYHRCILTWHHQE----PLPWAQSTG----NQMSSRLMSMRSANGLLMLPPKTEQYVEL  722
              *                          * *    *         *

CnxE       SLVEALMMGPIVREGAAVAL  709
MoeA       EWVEVEPFNALFGGL-----  411
Cnx1       SSVSAIIVSDISAFSID---  442
Cinnamon   KAGEIFPASVLRFDFISKYE  601
Gephyrin   HKGEVVDVMVIGRL------  736
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FIGURE 2.—Altered residues in the MoeA-like region of the *A. nidulans* CnxE protein. Mutant designations are shown beside the vertical lines indicating the alteration. Domains 2 and 3 of the *E. coli* MoeA protein (second line) are indicated in boldface and italic type, respectively. The amino acid sequences of eukaryotic orthologs, *A. thaliana* Cnx1, *D. melanogaster* Cinnamon, and *Rattus norvegicus* Gephyrin, are shown for comparison. Numbers to the right refer to residues in the respective proteins. Conserved residues are indicated below by an asterisk.

and *cnxEΔ3* belong to complementation classes A and D, respectively (encompassing the MogA-like domain; Figure 1A) whereas *cnxE16*, *cnxE160*, *cnxE107*, *cnxE230*, and *cnxE340* form complementation class C (domain 3 of the MoeA-like region of CnxE; Figure 2). Mutant *cnxE12*, representing complementation class B, is located within domain 2 of the MoeA-like region (Figure 2). The largest complementation class, F, consists, not unexpectedly, of most of the chain termination mutants, including *cnxE170* and *cnxE108* (shown in Figure 1A), which did not show complementation with any other classes. Surprisingly perhaps, *cnxE849*, an amino acid substitution residing within domain 2 of the MoeA-like region, is included in class F. Finally, class E, as exempli-

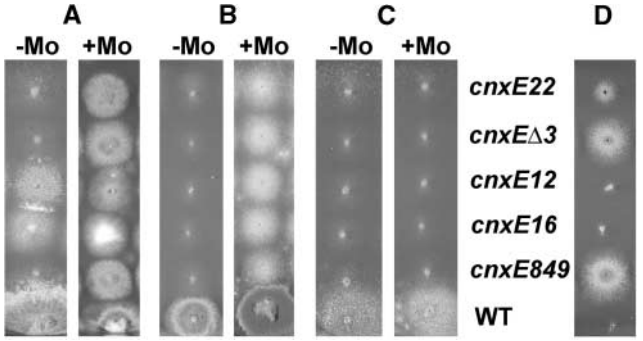

FIGURE 3.—Growth and chlorate toxicity tests of *cnxE* mutants. Growth is shown in the presence (+Mo) or absence (−Mo) of 33 mM sodium molybdate on minimal medium containing the nitrogen sources as follows: (A) 10 mM nitrate: the mutants had no other markers associated with nitrogen metabolism other than the *cnxE* allele indicated. Mutants *cnxE12* and *cnxE16* show intermediate levels of growth between the wild type and a loss-of-function mutant such as *cnxEΔ3* (the narrow line of growth between *cnxE12* and *cnxE16* is due to heterokaryon formation and genetic complementation of the two alleles). (B) 5 mM hypoxanthine or (C) 10 mM nicotinate as the sole source of utilizable nitrogen: for growth tests on these nitrogen sources, all *cnxE* mutants were in a *niaDΔ506* genetic background (see MATERIALS AND METHODS). The *niaDΔ506* mutant grows as wild type on these nitrogen sources (TOMSETT and COVE 1979; J. R. KINGHORN, unpublished results). Protection against molybdate toxicity was afforded by this strain combination (*i.e.*, in *cnxE niaDΔ506* double mutants) together with 10 mM nitrate being added to hypoxanthine or nicotinate minimal medium. For further details of nitrate protection against molybdate toxicity, see ARST *et al.* (1970). (D) Resistance to toxicity of 150 mM chlorate with 10 mM proline as the sole source of nitrogen.

fied by the chain termination mutants *cnxE250* and *cnxE103*, differs from class F in that the two class E mutants complement class A mutants (Figure 1, B and C).

Phenotype of mutants: All *cnxE* mutants failed to grow on nitrate as the sole source of nitrogen (reflecting the absence of nitrate reductase activity) with the exception of mutants *cnxE12* and *cnxE16*, which showed significant growth on nitrate, and are described below as "leaky" (Figure 3A, −Mo). No growth of mutants, including *cnxE12* and *cnxE16*, was observed with hypoxanthine, indicating the loss of xanthine dehydrogenase activity (Figure 3B, −Mo). Testing on growth regimes that reflect the level of nicotinate hydroxylase activity with (i) nicotinate as the sole nitrogen (Figure 3C, −Mo) or (ii) hypoxanthine, allopurinol, nicotinamide containing minimal media (J. R. KINGHORN, unpublished results) revealed no growth on either medium by any of the *cnx* mutants, including *cnxE12* and *cnxE16*.

All *cnxE* single mutants grew substantially better on nitrate when molybdate was present in the medium over a range of concentrations (1, 10, 20, and 33 mM; Figure 3A, +Mo). Restoration of growth was observed, but to a lesser extent relative to nitrate, on hypoxanthine with molybdate supplementation up to 10 mM. Above this concentration, wild-type growth is reduced due to molybdate toxicity as previously observed (ARST *et al.* 1970). To overcome this problem of toxicity exhibited at higher molybdenum concentrations, mutants in domain *cnxE22*, *-Δ3*, *-12*, *-16*, or *-849* were each combined with the deletion allele *niaDΔ506* (lacking nitrate reductase activity; see MATERIALS AND METHODS) to yield *cnxE niaDΔ506* double mutants, which no longer are subject to molybdate toxicity due to protection afforded by nitrate added to the medium but not available for use as a nitrogen source by the *niaDΔ506* mutant (ARST *et al.* 1970). *cnxE niaDΔ506* double mutants do not show growth on hypoxanthine/nitrate (in molybdate unsupplemented minimal media) due to the lack of xanthine dehydrogenase activity (J. R. KINGHORN, unpublished results). However, substantial restoration of growth of all five *cnxE niaDΔ506* double mutants examined was observed on hypoxanthine/nitrate supplemented with excess (20 or 33 mM) molybdate concentrations (Figure 3B, +Mo). In contrast, no repair of the ability to grow on nicotinate with molybdate supplementation was exhibited by any of the single *cnxE* mutants (J. R. KINGHORN, unpublished results). This lack of phenotypic repair was confirmed when it was observed that *cnxE22*, *-Δ3*, *-12*, *-16* , and *-849* double mutants (again each *cnxE* mutation being in combination with *niaDΔ506*) failed to grow on nicotinate/nitrate minimal medium containing molybdate concentrations of up to 33 mM (Figure 3C, +Mo).

Finally, all *cnxE* mutants isolated in this and previous studies (COVE and PATEMAN 1963; PATEMAN *et al.* 1964; ARST *et al.* 1970), such as *cnxEΔ3* and *cnxE849* (Figure 3D), are highly resistance to chlorate toxicity, with the exception of mutants *cnxE12* and *cnxE16*, which are sensitive to chlorate toxicity with the three sole nitrogen sources tested (*i.e.*, arginine, proline, or uric acid) and *cnxE22*, which showed a low/intermediate level of resistance.

Nitrate reductase, xanthine dehydrogenase, and nicotinate hydroxylase activities in mutants and their repair by molybdenum: *cnxE* strains, mutant in the MogA-like domain (*cnxE22, cnxEΔ3*), domain 2 (*cnxE12*), or domain 3 (*cnxE16*, *-849*, *-230*) of the MoeA-like region, were examined for the restoration of enzyme activities in cells supplemented with 33 mM sodium molybdate (Table 1). All mutant strains examined showed a similar increase in nitrate reductase or xanthine dehydrogenase on supplementation. In contrast, no increase in nicotinate hydroxylase activity was observed in any mutant cells grown in the presence of high molybdate concentrations. It is noteworthy that the values obtained for the wild type in cells grown in the presence of molybdate are lower for nitrate reductase and nicotinate hydroxylase. This is probably connected with the fact that high concentrations of molybdate reduce the growth rate (see above), although it is not clear to us why this is not also the case for xanthine dehydrogenase.

TABLE 1

Molybdate repair of nitrate reductase, xanthine dehydrogenase, and nicotinate hydroxylase activities in the MogA-like domain or the MoeA-like region *cnxE* mutants

	Nitrate reductase		Xanthine dehydrogenase		Nicotinate hydroxylase	
Strain	−Mo	+Mo	−Mo	+Mo	−Mo	+Mo
Wild type[a]	166.5 ± 12.1	127.3 ± 12.1	18.48 ± 1.60	21.16 ± 5.51	43.06 ± 10.57	17.46 ± 2.31
cnxE22	1.3 ± 0.1	20.4 ± 2.1	<0.03	1.26 ± 0.25	<0.03	<0.2
cnxEΔ3	<0.5	16.8 ± 3.0	<0.03	0.84 ± 0.08	<0.03	<0.2
cnxE12	3.7 ± 0.2	17.6 ± 1.4	0.13 ± 0.02	1.80 ± 0.05	<0.03	<0.2
cnxE849	<0.5	16.6 ± 0.3	<0.03	1.62 ± 0.32	<0.03	<0.2
cnxE16	4.2 ± 0.1	21.7 ± 0.3	<0.03	1.46 ± 0.18	ND	ND
cnxE230	<0.5	15.0 ± 0.3	<0.03	1.26 ± 0.22	ND	ND

Growth of strains, determination of enzyme activities, and units are described in MATERIALS AND METHODS. For nicotinate hydroxylase assays, double mutants between all *cnxE* and the *hxA1* mutant strain (lacking xanthine dehydrogenase activity) were used to circumvent the possible uncertainty of residual xanthine dehydrogenase being mistakenly interpretated as nicotinate hydroxylase activity. For −Mo conditions, cells were incubated in the absence of molybdate supplementation (*i.e.*, with 4.55 μM trace levels added routinely to minimal medium for growth). For +Mo conditions, cells were incubated in the presence of 33 mM sodium molybdate. ND, not determined.

[a] The "wild type" used for nicotinate hydroxylase assays was the *hxA1* strain that completely lacks xanthine dehydrogenase activity.

HPLC analysis of molybdenum cofactor precursor Z and molybdopterin levels in cell-free Aspergillus extracts: All *cnxE* mutants assayed appeared to synthesize similar levels of molybdopterin as the wild type in cells growing on nitrate/proline as the sole nitrogen sources (Table 2). In contrast, precursor Z concentrations were found to be significantly higher than wild-type concentrations in the *cnxE* mutants examined.

TABLE 2

Levels of precursor Z and molybdopterin in wild type and *cnxE* mutants

Strain	Precursor Z[a]	Molybdopterin[a]
Wild type	4.38 ± 0.26	3.17 ± 0.90
cnxE22	9.78 ± 0.41	4.69 ± 1.33
cnxEΔ3	7.74 ± 0.69	5.34 ± 0.49
cnxE12	13.64 ± 2.83	4.27 ± 0.36
cnxE849	9.48 ± 1.12	5.37 ± 1.66
cnxE107	9.70 ± 0.99	4.48 ± 0.08
cnxE230	10.48 ± 0.63	4.02 ± 0.32
cnxE16	7.16 ± 1.40	5.09 ± 1.03
cnxE160	9.60 ± 2.77	3.92 ± 0.56
cnxE340	11.96 ± 0.58	3.66 ± 0.61

Growth of strains, determination by HPLC of precursor Z, and molybdopterin levels are described in MATERIALS AND METHODS.

[a] Intermediates of molybdenum cofactor biosynthesis (and the cofactor itself) are sensitive to oxygen and to measure levels of the intermediates, precursor Z and molybdopterin (including molybdopterin from the cofactor) were chemically converted to their stable, inactive oxidation products, compound Z and dephosphorylated molybdopterin form A, respectively.

DISCUSSION

The MogA-like domain and the MoeA-like region of CnxE are both required for activity: A number of randomly generated mutations within the *cnxE* gene result in amino acid replacements in the MogA-like domain as well as in domains 2 and 3 (identified from the crystal structure; see below) of the MoeA-like region. No discernible phenotypic differences could be observed between mutants in either the MogA-like domain or in domains 2 or 3 of the MoeA-like region of CnxE. First, loss-of-function mutants in either MogA-like domain or domains 2 or 3 of the MoeA-like region lead to the complete abolition of all three *A. nidulans* molybdoenzyme activities (*i.e.*, nitrate reductase, xanthine dehydrogenase, and nicotinate hydroxylase), resulting in the inability to use nitrate, hypoxanthine, or nicotinate, respectively, as sole sources of nitrogen. Second, nitrate reductase and hypoxanthine dehydrogenase activities of all mutants are repaired to similar levels by molybdate supplementation (as judged by growth tests or direct assay), but not nicotinate hydroxylase (see below). Third, pathway intermediate levels in all the mutants are higher (precursor Z) than or similar to those of wild type (molybdopterin). The MogA-like domain and domain 3 of the MoeA-like region are structurally similar (SCHRAG *et al.* 2001; XIANG *et al.* 2001) and so they may have similar roles in molybdenum cofactor synthesis and processing. Nevertheless, the characteristics of the mutants indicate that the fused MogA-like domain and MoeA-like region of the eukaryotic protein are both required for CnxE function.

Genetic complementation occurs between mutants in different domains of CnxE: Complementation tests

were carried out between pairwise combinations of mutants. The results support the independent functional nature of the MogA-like domain and MoeA-like region of CnxE since mutants in the MogA-like domain, *i.e.*, *cnxE22* and *cnxEΔ3*, clearly complement mutants (*cnxE16*, *-160*, *-230*, *-340*) within MoeA-like domain 3, which is similar in sequence and structure to the MogA domain. Also, complementation provides evidence that the CnxE protein is multimeric in structure, in agreement with the physical data obtained from crystallography studies of the trimeric MogA-like N-terminal domain gephyrin (Sola *et al.* 2001). Moreover, mutant *cnxEΔ3* (in which most of the MogA-like domain is deleted) will complement *cnxE16* (or *cnxE160*, *-230*, *-340*), suggesting that the MoeA-like domain can function when expressed with the MogA-like domain *in trans*. This would indicate that the complete, although mutant, CnxE16 (or CnxE160, CnxE230, CnxE340) polypeptide can aggregate with the truncated CnxEΔ3 peptide to yield a functional hybrid CnxE protein, consisting of both MogA- and MoeA-like partial activities.

A structural model for CnxE and the position of mutations therein: The availability of crystal structures for orthologs MogA (Liu *et al.* 2000; Sola *et al.* 2001) and MoeA (Schrag *et al.* 2001; Xiang *et al.* 2001) has aided our interpretation of the effects of mutations in CnxE. Only one amino acid substitution mutation affecting the MogA-like domain of CnxE was observed. Gly79 is the last Gly in the conserved GlyGlyThrGly motif contained within a loop that forms the base of a cavity proposed to be the site of molybdopterin binding (Sola *et al.* 2001). Replacement of this residue with Asp in mutant *cnxE22* most likely disrupts the loop directly affecting the active site conformation. A homology model of CnxE was built from the dimeric structure of *E. coli* MoeA (PDB code 1FC5; Schrag *et al.* 2001). Seven amino acid substitution mutations in the MoeA-like domain (Figure 2) were mapped on the CnxE model (Figure 4). Most of these mutations are replacements of residues containing sidechains for Gly and most are located along the border of the putative active site cleft formed between the two monomers of the dimer, providing supportive genetic evidence for the importance of this cleft in the function of the protein. Additionally, all the amino acid substitution mutations except *cnxE849* are located in regions of high sequence conservation among similar proteins from different species.

Four mutations lie within the third domain of the MoeA-like region of the protein as predicted from the model. The mutation in strain *cnxE160*, resulting in Gly515 being replaced with Arg, involves the Gly residue located in the putative active site. Introduction of the long Arg sidechain at residue 515 disrupts the geometry of the ThrThrGlyGly loop and acidic cluster that are conserved in MoeA and MogA homologs. The backbone conformation observed for Gly515 would be highly strained upon introduction of an Arg residue and some local conformational changes would be likely. The Arg sidechain also disrupts the electrostatic potential in the region of the conserved acidic cluster. In mutant *cnxE230*, a Cys replacement of Gly439 introduces two extra atoms into the area of the putative ligand-binding site. This residue is adjacent to Gly515 of the conserved ThrThrGlyGly signature motif and the presence of a sidechain probably interferes sterically with ligand binding. The change of Asp to Glu at residue position 522 in mutant strain *cnxE16* extends a fourth negative charge into the acidic cluster and may introduce both electrostatic and steric influences on ligand binding. However, in contrast to the other domain 3 mutants (*i.e.*, *cnxE107*, *-160*, and *-230*), mutant *cnxE16* possesses sufficient nitrate reductase activity to allow limited growth on nitrate (*i.e.*, leaky), indicating that this conservative replacement permits low levels of the molybdenum cofactor to be synthesized. The *cnxE340* mutation results in Gly546 being replaced by Glu and this change

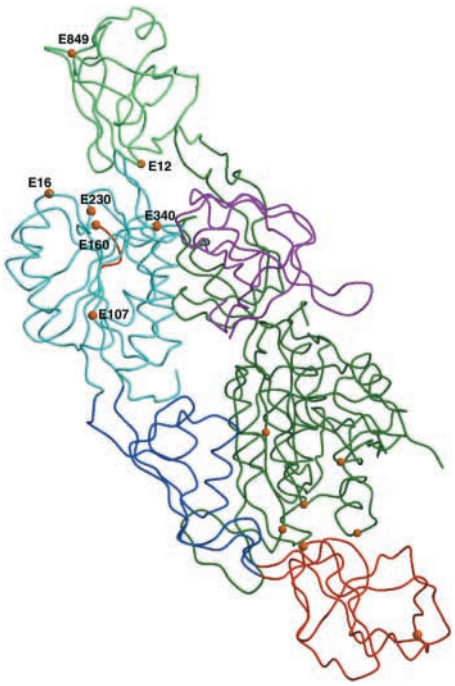

Figure 4.—Structural model of the dimer of the CnxE MoeA-like region. Domain 3 of monomer A is shown in cyan, domain 4 in magenta, domain 1 in blue, and domain 2 in red. Domain 2 of monomer B is light green and the remainder of monomer B is in dark green. The orange spheres mark the locations of the mutations. The TTGG signature sequence is marked in red within monomer A domain 3. Insertions in the *A. nidulans* amino acid sequence relative to that of *E. coli* were not modeled and so certain loops that should be present in *A. nidulans* are not shown. One such insertion involves residue 372 (mutation *E849*). Since this residue was not in the model, the adjacent residue 373 was marked, indicating the approximate location of the mutation. This model was made using MOLSCRIPT (Kraulis 1991) and Raster3D (Merritt and Murphy 1994; Merritt and Bacon 1997).

introduces a negative charge into a hydrophobic cluster. The backbone conformation of this residue is readily adopted by glycine, but is strained for other residues and will undoubtedly be altered as a result of the mutation. These local conformational changes will alter the shape of the cleft and could influence ligand binding.

The *cnxE12* mutation results in change of Gly343, a highly conserved residue located in the putative ligand-binding cleft, to Asp. Unlike the four previously mentioned mutations in domain 3, Gly343 is located in domain 2 and on the opposite side of the putative active site cleft. The replacement of Gly343 by Asp introduces a negative charge that neutralizes the positive charge of Arg482, suggesting both electrostatic and steric influences on ligand binding. However, these effects probably do not cause complete inactivation of the protein, as strain *cnxE12* is leaky (similar to mutant *cnxE16*), showing limited growth with nitrate due to low levels of nitrate reductase activity. Not unexpectedly perhaps, the counterpart mutant of Arabidopsis (*chl-6*) also possesses considerable levels of nitrate reductase (Schwarz *et al.* 2000). The model, moreover, may explain why *cnxE12* is the only mutant within the MoeA-like region that can complement other missence mutants within the same region; *i.e.*, it may explain why it appears in a complementation group by itself. This could be because the *cnxE12* mutation lies in a different domain from the other mutations and so aggregation of monomers with mutations in different domains could produce an active protein by a mechanism of conformational correction as proposed by Fincham (1966, 1994). This mechanism proposes that the packing constraints imposed on monomers within a hybrid oligomer can induce the correct conformation of a faulty polypeptide. Alternatively, domains 2 and 3 may have different and distinct functions that could complement *in trans* within the oligomeric protein. Isolation and analysis of more mutants within these domains, in combination with structural studies of mutant proteins, would be necessary to resolve these possibilities.

Of the final two mutants modeled, Gly434, which is located in a β-strand in the core of domain 3, is replaced by Asp in *cnxE107*, introducing a charged sidechain into a hydrophobic environment. This mutation is likely to alter the folding of the protein, at least locally, and may make the protein unstable. The Ala 372-to-Ser change in *cnxE849* is located in domain 2, far from the putative active site cleft. There is no clear indication from the modeling as to why this mutation inactivates the protein. However, the fact that this mutant is unable to complement any other *cnxE* mutant (although complementation with other *cnx* mutants is normal) suggests that it may affect the overall oligomerization of the protein.

Nicotinate hydroxylase is not repairable by molybdate supplementation: An interesting feature of the enzyme activity restoration studies is that, unlike nitrate reductase and xanthine dehydrogenase, nicotinate hydroxylase activity is not repaired by molybdate supplementation of the growth medium. The reason for this lack of enzyme restoration is unclear to us. One possibility may be that nicotinate hydroxylase requires for activity a dinucleotide derivative of the molybdenum cofactor such as is commonly found in prokaryotes (Rajagopalan 1996). A second possibility is that nicotinate hydroxylase may have a lower affinity for the molybdenum cofactor than xanthine dehydrogenase or nitrate reductase, as suggested before (Macdonald and Cove 1974; Arst 1997). Another possible explanation for the lack of nicotinate hydroxylase repairability requires molybdopterin to be chelated with molybdenum before the resulting molybdenum cofactor insertion into molybdoenzymes. Failure to repair nicotinate hydroxylase by molybdate supplementation could be due simply to lack of accessibility or hindrance in this enzyme in the absence of CnxE.

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