MOLECULAR CLONING OF THE nemA GENE ENCODING N-ETHYLMALEIMIDE REDUCTASE FROM Escherichia coli

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Using the gene mapping membrane technique, we identified a gene (nema) that encodes N-ethylmaleimide reductase in Escherichia coli. The open reading frame encodes a polypeptide of 365 amino acids with a molecular mass of 39,514 Da. The deduced amino acid sequence showed a high degree of homology (87% identical) with the pentaerythritol tetranitrate reductase of Enterobacter cloacae and the morphinone reductase of Pseudomonas putida (52% identical).

KEY WORDS Escherichia coli; N-ethylmaleimide reductase; unsaturated fatty acid; Old Yellow Enzyme

N-Ethylmaleimide (NEM) is a specific thiol reagent and a highly cytotoxic compound.\(^1\)
We have previously identified a NADPH-dependent NEM reducing activity in crude extracts of Escherichia coli strain W2252 cultured in the presence of polyunsaturated fatty acids such as linoleic acid\(^2\) and designated the enzyme as NEM reductase. Thereafter, we also found NEM reductase activity in the yeast Yarrowia (Candida) lipolytica and purified it to homogeneity.\(^3\) The yeast enzyme had a molecular mass of 47 kDa by SDS-PAGE and reduced the α, β-unsaturated five-membered ring compounds. To elucidate the physiological function of NEM reductase, we cloned and sequenced the E. coli NEM reductase gene.

![Fig. 1. Analysis of the Expression of the nemA Gene by SDS-PAGE](image)

Lane 1, crude extract from E. coli strain XL1-blue containing the pNEM5 (15 μg of protein). Lane 2, NEM reductase purified from strain DH5 (1.2 μg of protein). Samples were separated by SDS-PAGE in a 12% gel and stained with Coomassie brilliant blue R250.

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Fig. 2. Alignment of Amino Acid Sequences of the NEM Reductase of *Escherichia coli* (NEMA), the Pentaerythritol Tetranitrate Reductase of *Enterobacter cloacae* (ONR), and the Morphinone Reductase of *Pseudomonas putida* (MORB)

Gaps were inserted to achieve maximum homology using the Clustal W program. Numbers are residue numbers. Identical amino acid residues are shown by black boxes.

NEM reductase was purified from *E. coli* strain DH5. Purified NEM reductase was homogeneous by SDS-PAGE, having a molecular mass of 40 kDa and specific activity of 38 μmol/min/mg under standard assay conditions (Fig. 1, Lane 2). NEM reductase activity was determined by measuring the rate of decrease in absorbance associated with NADPH at 340 nm. Purified protein was cleaved with lysylendopeptidase (Wako). Two peptides termed L6 and L7 were separated and sequenced. The N-terminal sequence of peptide L6 was AELNPQRAESFY(GGGAEXYT) and that of peptide L7 was RGIAYLHMSEP(WAGXXP), where residues shown in parentheses indicate uncertain assignments, and X indicates any amino acid. The following oligonucleotides corresponding to the above peptide sequences were synthesized:

L6F, 5'-AATCTAGA(A/G)(C/T)TNAA(C/T)CCNCA(A/G)(A/C)GNGCNGA-3';
L6R, 5'-AATCTAGAA(N/A)(G/C)(C/T)TCNGCNC(G/T)(C/T)TGNGG(A/G)(G/T)-T-3';
L7F, 5'-AATGCAGGNAT(A/C/T)GCNTA(C/T)TNCA(C/T)ATG-3'; and
L7R, 5'-AATGCGAGG(C/T)TCN(A/T)(G/C)CAT(A/G)TGN(A/G)(A/G)-3'.

Using two primer sets L6R and L7F, a 280-bp DNA fragment was amplified by PCR from the genomic DNA prepared from *E. coli* strain W3110. Using this DNA fragment as a probe and the *E. coli* Gene Mapping Membrane (Takara), the gene was located at 37.1 min on the *E. coli*
chromosome. DNA from Kohara λ phage 317,5) which contains the 37-min region of E. coli chromosome, was isolated and a 2.8-kbp PsiI-BamHI DNA fragment was excised and cloned into pGEM-3Zf(+), generating plasmid pNEM5. The pNEM5 transformant contained NEM reductase activity of 2.9 μmol/min/mg, while the parent strain XL1-blue contained 0.002 μmol/min/mg. Large amounts of a protein co-migrated with purified NEM reductase on SDS-PAGE (Fig. 1). Thus, the entire structural gene for the E. coli NEM reductase5) was included in the pNEM5 plasmid and designated nemA. The DNA sequence of the portions of the pNEM5 were determined using an ABI PRISM dye terminator cycle sequencing kit (Applied Biosystems) and the walking primer method. The nemA gene is 1,098 bp in length and encodes a protein of 365 amino acids (molecular mass of 39,514 Da) containing all the peptide sequences determined previously (Fig. 2).

Comparison of the deduced sequence using the BLAST homology search program7) with the DDBJ/EMBL/GenBank databases revealed a high degree of homology (87% identical) with the pentaerythritol tetrinate reductase of Enterobacter cloacae (accession number U68759), the morphinone reductase of Pseudomonas putida3) (U37350) (Fig. 2), and several flavoprotein oxidases/dehydrogenases belonging to the class I flavin dehydrogenase/oxidase family9) such as Old Yellow Enzymes of Saccharomyces sp.10-12) Old Yellow Enzyme reduces the olefinic bond moiety of ω, ω-unsaturated carbonyl compounds as well as NEM reductase.13) Thus, it will be of interest to compare biochemical properties between NEM reductase and Old Yellow Enzyme. The relationship and characterization of these enzymes will be reported elsewhere.

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REFERENCES AND NOTES
6) The sequences reported in this paper have been submitted to the DDBJ/EMBL/GenBank databases under accession number D86931.

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