

by LC/ESI/MS, we accounted for 85% of the amino acid sequence. Furthermore, taking into account additional sequence information obtained by CID analysis and Edman sequencing, 96% of the 746 amino acid sequence was determined by tryptic mapping.

Acknowledgments

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[8] Cloning of Mammalian Topa Quinone-Containing Enzymes

By DAVID MU and JUDITH P. KLINMAN

Introduction

Topa quinone-dependent enzymes appear to be ubiquitous *in vivo*, with enzymes having been purified from a variety of sources, including mammals, plants, yeast, fungi, and bacteria (cf. McIntire and Hartman¹ for a complete summary). To date, all known topa-dependent enzymes are copper-containing and capable of catalyzing the reaction of oxidative deamination. Since the earliest study reported,² the majority of investigations have focused on understanding the enzymatic and biochemical properties of these copper amine oxidases.^{1,3} Molecular biological studies of topa quinone enzymes were not initiated until the late 1980s and early 1990s, leading to the cloning of only a few nonmammalian enzymes, including yeast amine oxidase from *Hansenula polymorpha*,⁴ lentil seedling amine oxidase,⁵ and bacterial amine oxidase from *Arthrobacter* P1.⁶ Although an extensive back-

¹ W. S. McIntire and C. Hartman, in "Principles and Applications of Quinoproteins" (V. L. Davidson, ed.), p. 97. Dekker, New York, 1993.

² E. A. Zeller, *Proc. R. Soc. London, Ser. B.* **161**, 153 (1938).

³ J. P. Klinman and D. Mu, *Annu. Rev. Biochem.* **63**, 299 (1994).

⁴ P. G. Bruinenberg, M. Evers, H. R. Waterham, J. Kuipers, A. C. Amberg, and G. Ab, *Biochim. Biophys. Acta* **1008**, 157 (1989).

⁵ A. Rossi, R. Petruzzelli, and A. Finazzi-Agró, *FEBS Lett.* **301**, 253 (1992).

⁶ X. Zhang, J. H. Fuller, and W. S. McIntire, *J. Bacteriol.* **175**, 5617 (1993).

ground of mechanistic and spectroscopic data exists for copper amine oxidases from porcine and bovine plasma (cf. McIntire and Hartman¹ and Klinman and Mu³ and references therein), efforts at cloning one of these mammalian proteins had not been successful until very recently. This chapter reports the strategy and methods for the cloning of the first mammalian topa quinone-containing protein. The availability of appropriate cDNA probes is expected to prove extremely valuable in elucidating the as yet unresolved physiologic function of the various mammalian copper amine oxidases located both intra- and extra-cellularly.

Strategy

The initial obstacle in the cloning of a plasma amine oxidase was the uncertainty of the enzyme's site of biosynthesis. Even though aorta smooth muscle^{7,8} and small intestine⁹ have been independently implicated to be the source of serum topa enzymes, a commercially prepared bovine liver cDNA library was chosen for trial experiments in light of the fact that most serum proteins are secreted from liver.¹⁰ Initial screening of this cDNA library used oligonucleotide probes deduced from the bovine serum amine oxidase (BSAO) active-site peptide sequence.^{11,12} Although these studies yielded several positive recombinants with small cDNA inserts (<1 kb), the translated amino acid sequences were not found to correspond to any known bovine serum amine oxidase peptide sequences.

A turning point came during a routine protein sequence data bank search using known topa quinone-containing peptide sequences. This search revealed a previously cloned protein, amiloride-binding protein from human kidney,¹³ with extensive homology to pig kidney diamine oxidase as well as bovine serum amine oxidase. Subsequent demonstration of amiloride binding to several copper amine oxidases led to the redesignation of

⁷ R. M. Hysmith and P. J. Boor, *Biochem. Cell. Biol.* **66**, 821 (1987).

⁸ R. Lewinsohn, *J. Pharm. Pharmacol.* **33**, 569 (1981).

⁹ L. D'Agostino, S. Pignata, B. Daniele, R. Ventriglia, G. Ferrari, C. Ferrari, S. Spaguolo, P. Lucchelli, and G. Mazzacca, *Biochim. Biophys. Acta* **993**, 228 (1989).

¹⁰ C. A. Alper, in "Hematology" (W. J. Williams, E. Beutler, A. J. Erslev, and M. A. Lichtman, eds.), p. 1616. McGraw-Hill, New York, 1990.

¹¹ D. Mu, S. M. Janes, A. J. Smith, D. E. Brown, D. M. Dooley, and J. P. Klinman, *J. Biol. Chem.* **267**, 7979 (1992).

¹² S. M. Janes, M. M. Palcic, C. H. Scaman, A. J. Smith, D. E. Brown, D. M. Dooley, M. Mure, and J. P. Klinman, *Biochemistry* **31**, 12147 (1992).

¹³ P. Barbry, M. Champe, O. Chassande, S. Munemitsu, G. Champigny, E. Lingueglia, P. Maes, C. Frelin, A. Tartar, A. Ullrich, and M. Lazdunski, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7347 (1990).

amiloride-binding protein as human kidney diamine oxidase.¹⁴ This fortuitous finding provided a chance to employ a more sensitive, polymerase chain reaction¹⁵ (PCR)-based method to approach bovine serum amine oxidase cloning. As discussed herein, this has yielded a full-length cDNA from a bovine liver cDNA library, providing an answer to the long-standing question regarding the source of the circulating serum topa quinone amine oxidases. Subsequent sequence alignments among eukaryotic topa-containing enzymes have also led to an estimation of the conserved copper-binding ligands.¹⁴

Cloning of a Partial BSAO cDNA by PCR

The ability of PCR to generate micrograms of a specific DNA fragment has been used to design an efficient method to yield cDNA probes for molecular cloning, termed mixed oligonucleotide primed amplification of cDNA (MOPAC).¹⁶ By synthesizing perfectly matched cDNA probes, one can perform hybridization under very stringent conditions, thereby eliminating spurious hybridization signals. A limitation of this method compared to the conventional protocols is that some sequence information flanking the desired fragment is required. At the time the experiments described herein were performed, several bovine serum amine oxidase peptide sequences were available (Table I).¹⁷ However, their relative positions in the enzyme were unknown. The finding that a recently cloned and sequenced human kidney amiloride-binding protein is, in actuality, a topa quinone-dependent amine oxidase¹⁴ was triggered by the observation that peptides I and II of bovine serum amine oxidase (Table I) are highly homologous to two separate regions in human kidney amiloride-binding protein. Assuming a similar relative position for these peptides in bovine serum amine oxidase, the MOPAC technique could be used to amplify the authentic cDNA of ca. 0.7 kb flanked by the two peptides (Fig. 1).

Although a tolerance of up to a 20% base pair mismatch between the primer and the template has been shown for the MOPAC reaction,¹⁸

¹⁴ D. Mu, K. F. Medzihradzky, G. W. Adams, P. Mayer, W. M. Hines, A. L. Burlingame, A. J. Smith, D. Cai, and J. P. Klinman, *J. Biol. Chem.* **269**, 9926 (1994).

¹⁵ K. Mullis, F. Faloona, S. Scharf, R. Saiki, G. Horn, and H. Erlich, *Cold Spring Harbor Symp. Quant. Biol.* **51**, 263 (1986).

¹⁶ C. C. Lee, X. Wu, R. A. Gibbs, R. G. Cook, D. M. Muzny, and C. T. Caskey, *Science* **239**, 1288 (1988).

¹⁷ D. Mu, Ph.D. Thesis, University of California at Berkeley (1993).

¹⁸ C. C. Lee and C. T. Caskey, in "PCR Protocols: A Guide to Methods and Applications" (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds.), p. 46. Academic Press, San Diego, CA, 1990.

TABLE I
COMPARISON OF BOVINE SERUM AMINE OXIDASE (BSAO) PEPTIDE SEQUENCES TO HUMAN KIDNEY
AMILORIDE-BINDING PROTEIN^a

BSAO ^b (active site-derived peptide I)	SVSTMLNYDYVW ^c DMVFYPNGAIEVK
Human kidney amiloride-binding protein ^d	⁴⁵³ TTSTVYNYDYIWDFIFYPNGVMEAK ⁴⁷⁷
BSAO (peptide II) ^e	DLVAWVTAGFL <u>HIPHAED</u> IPNTVTVGNGVGFLLRPYNF
Human kidney amiloride-binding protein ^d	⁶⁶² DLVAWVTVGFLHIPHSEDIPNTATPGNSVGFLLRPYNF ⁶⁹⁹

^a Human kidney amiloride-binding protein is now designated human kidney diamine oxidase.¹⁴

^b Data from Janes *et al.*¹² Instead of topa quinone at position 8, we include its precursor, tyrosine.¹¹ Underlined region represents the sequence used for the synthesis of the degenerate sense MOPAC primer.

^c This previously unidentified residue was shown to be tryptophan (cf. Mu¹⁷ and [7], this volume).

^d Barbry *et al.*

^e Mu *et al.*¹⁴ Underlined region represents the sequence used for the synthesis of the degenerate antisense MOPAC primer.

selection of amino acids with minimal degeneracy is desired. Figures 2A and 2B show the number of codons for each amino acid residue in peptides I and II. For the purpose of reducing degeneracy, the residue 8–15 of peptide I and the residue 12–19 of peptide II were selected for primer synthesis. A mixed sense primer [5'-TA(C/T)GA(C/T)TA(C/T)GT(C/G)TGGGA(C/T)ATGGT-3'] was synthesized to the amino acid residue 8–15 (Y-D-Y-V-W-D-M-V) of the active-site peptide I and a mixed anti-sense primer [5'-AT(G/A)TC(T/C)TCGGC(G/A)TG(G/T)GG(T/G/A)AT(G/A)TG-3'] was synthesized to the amino acid residue 12–19 (H-I-P-H-A-E-D-I) of the peptide II close to the C terminus. The degeneracies for the sense and antisense primers are 32- and 96-fold, respectively. This is within the range of the recommended primer degeneracy by Compton.¹⁹ Since most serum proteins are known to originate from liver,¹⁰ a commercially prepared bovine (female) liver cDNA library (in λ gt10 vector, Clontech, Palo Alto, CA) was chosen for amplification by MOPAC. A typical MOPAC experiment consists of the following: 50% glycerol, 20 μ l; 100 mM Tris, pH 8.3/500 mM KCl, 10 μ l; sense primer, 120–150 pmol (5 μ l); antisense primer, 120–150 pmol (5 μ l); 2 μ l of bovine liver cDNA library (10⁶ plaque-forming units, frozen and thawed twice) mixed with 52 μ l water; 4 mM of dNTP, 5 μ l; 1–3 units of *Taq* polymerase (Perkin

¹⁹ T. Compton, in "PCR Protocols: A Guide to Methods and Applications" (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds.), p. 39. Academic Press, San Diego, CA, 1990.

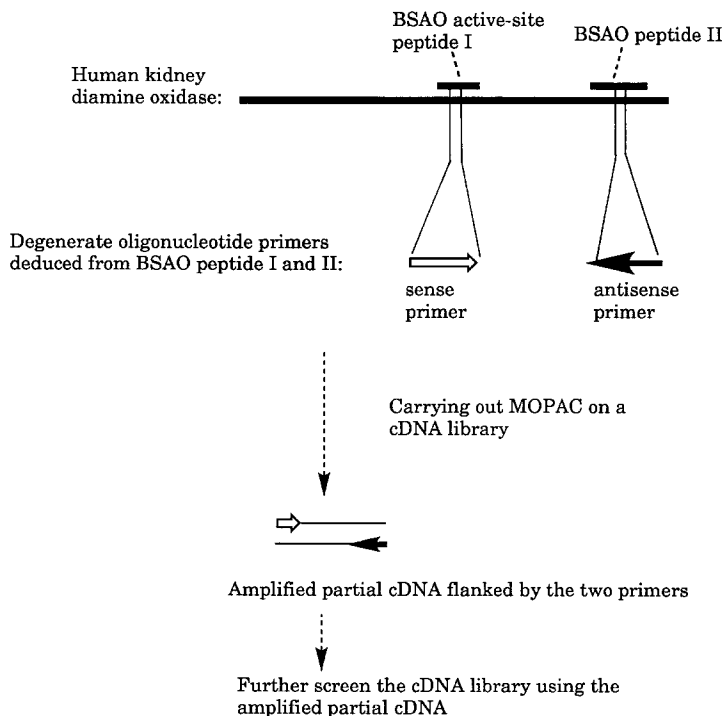


FIG. 1. Scheme for the MOPAC experiments which are based on the fact that two bovine serum amine oxidase (BSAO) peptides are highly homologous to two segments in human kidney diamine oxidase.

Elmer/Cetus, Norwalk, CT), 1 μ l. Reactions are overlaid with 25–30 μ l of mineral oil and amplified using a Perkin Elmer DNA thermal cycler by following this program: step (i) 94° for 5 min; step (ii) 94° for 30 sec, 44–51° (variable annealing temperatures) for 30 sec, 72° for 1 min; step (iii) 72° for 1 min. Amplification step (ii) is repeated 30 times. Following amplification, the reactions are analyzed by gel electrophoresis of 5 μ l of the reaction mix on 2% agarose gels. The key to success in applying degenerate primers in PCR is to find conditions for optimal complementation between the target template and the primers. Initially, reactions are performed at a low, nonstringent annealing temperature of 44°, leading to the expected 0.7-kb product DNA (Fig. 3, lane 1). The gel in Fig. 3 shows that unknown DNA, at higher molecular weight, is amplified as well. To check the authenticity of the 0.7-kb DNA, experiments are repeated under more stringent conditions of elevated annealing temperatures (46–51°). As shown in Fig. 3 (lanes 2–5), band intensities at higher molecular weight decrease to zero through

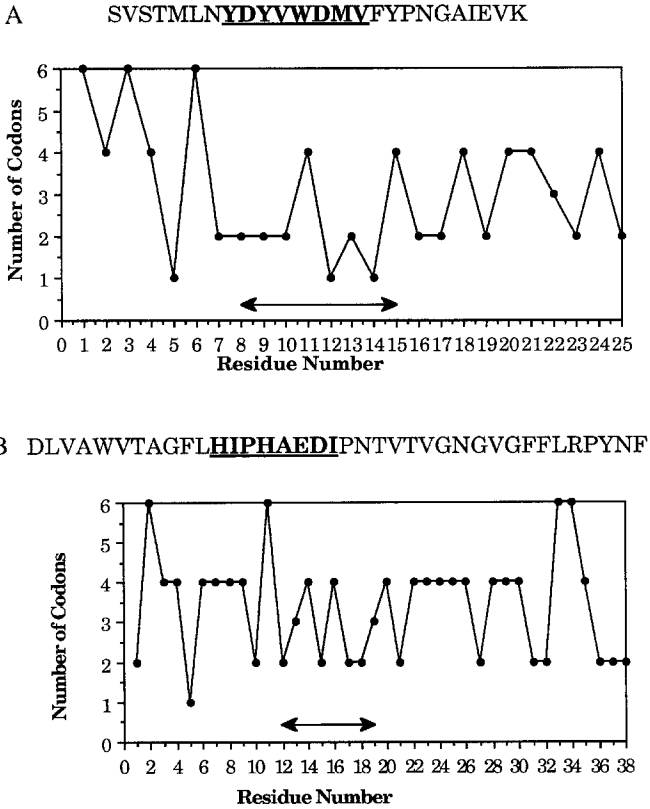


FIG. 2. Number of codons for each amino acid residue in the bovine serum amine oxidase peptide I (A) and peptide II (B). The corresponding peptide sequence is shown above the graph. The arrow covers the region selected for the synthesis of degenerate MOPAC primers.

the experiments, indicating that the 0.7-kb dominant band is likely to be the authentic cDNA fragment flanked by the two degenerated primers. Since the primers for MOPAC are incorporated at the ends of the 0.7-kb MOPAC DNA, the same primers can be used to obtain the nucleotide sequence near the ends of gel-purified MOPAC product DNA. Comparison of the translated amino acid sequence to the original peptide sequence confirmed the authenticity of the 0.7-kb DNA fragment (Fig. 4).

Isolation of Clones Containing Complete Coding Sequence

To isolate the remainder of the coding sequence for the bovine serum amine oxidase cDNA, the 0.7-kb MOPAC product is used to screen the

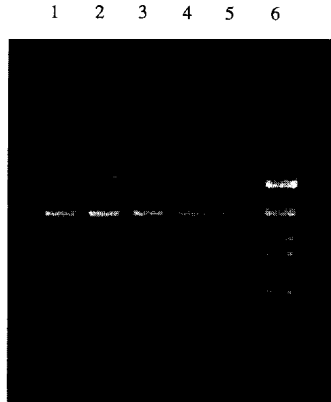


FIG. 3. Analysis of the MOPAC reaction products on bovine liver cDNA library by agarose (2%) gel electrophoresis. Lanes 1–5 are the MOPAC reactions performed at different annealing temperatures: lane 1, 44°; lane 2, 46°; lane 3, 48°; lane 4, 50°; and lane 5, 51°. Molecular weight markers were run in lane 6 with bands (from top) of 1000-, 700-, 500-, 400-, 300-, 200-, 100-, and 50-bp linear DNA (from Research Genetics). It appears that an annealing temperature of 49° represents the optimal condition. (Reproduced with permission from Mu *et al.*¹⁴)

same bovine liver cDNA library. Preparation of the probes for screening the library is initiated by gel purifying one optimal MOPAC reaction using a GeneClean kit (Bio 101, San Diego, CA) and dissolving it in 40 μ l of 1 mM Tris–HCl, pH 8.0, containing 0.1 mM EDTA. Twenty-five nanograms of this purified DNA is then labeled with [α -³²P]dATP using a random priming kit (“prime-a-gene,” Promega, Madison, WI) to a specific activity of ca. 6×10^8 counts/min/ μ g.

Approximately 1.0×10^6 recombinants from the bovine liver cDNA library are plated and duplicated plaque lifts made using standard proce-

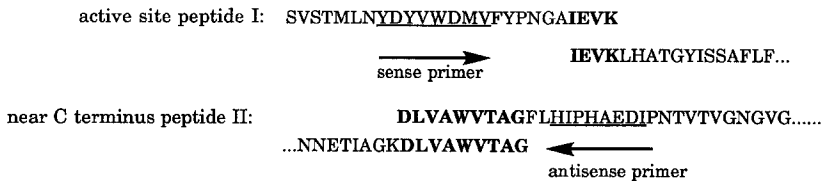


FIG. 4. The translated amino acid sequence near the ends of the initially amplified DNA of 0.7 kb generated by MOPAC. The complete cDNA-deduced primary structure of bovine serum amine oxidase is published in Mu *et al.*¹⁴

dures.²⁰ Filters are prehybridized for 2–3 hr at 42° in 50% formamide, 0.15 M NaCl in 15 mM sodium citrate ($6 \times \text{SSC}$), 0.1% sodium dodecyl sulfate (SDS), $5 \times$ Denhardt's solution,²¹ 150 $\mu\text{g/ml}$ denatured salmon sperm DNA, 50 mM Tris-HCl, pH 7.5. Then 8×10^5 counts/min/ml of ³²P-labeled probe are added and the hybridization is continued for 24–36 hr. Following hybridization the filters are washed twice for 20 min each at room temperature in $2 \times \text{SSC}/0.1\%$ SDS, washed three times each for 1 hr at 68° in $0.1 \times \text{SSC}/0.1\%$ SDS, air-dried, and autoradiographed at –70° with an intensifying screen for 3–7 days.

The initial round of library screening for BSAO cDNA resulted in the isolation of 25 recombinants. To analyze the size of the cDNA inserts and to eliminate false positive signals, each of the 25 clones was subject to two verifying PCR reactions:

- i. For cDNA insert size: amplification using primers complementary to the *EcoRI* linker which flanks all library cDNA inserts
- ii. To eliminate spurious hybridization signals: amplification using the two primers for MOPAC.

The former PCR reaction was performed using the cDNA insert screening amplimers (Clontech) according to the manufacturer's protocol; conditions for the latter PCR reaction and analysis of reaction products are identical to those described earlier. Thirteen of the initial 25 clones failed to yield the expected 0.7-kb amplified DNA. One of the 12 verified positive clones was found to be full-length as judged by the cDNA insert size (2.7 kb). Subsequently, the 1 phage recombinant containing this 2.7-kb cDNA was isolated using lambda-sorb (Promega) as recommended by the manufacturer, digested with *EcoRI*, and the insert subcloned into *EcoRI*-cut pGEM3Z (Promega) by following the standard exonuclease III-directed deletion method.²⁰ The DNA sequence of the insert (both strands) was then determined using a sequenase 2.0 dideoxy-sequencing kit (US Biochemical, Cleveland, OH). The translated amino acid sequence was matched exactly with a total of 11 peptide sequences²² isolated from native bovine serum amine oxidase, confirming the authenticity as the cDNA of bovine serum amine oxidase.¹⁴

In summary, the use of degenerate PCR primers provides a powerful approach for the identification of mammalian topa quinone-containing enzymes. The fact that the two MOPAC primers, derived from bovine serum

²⁰ T. Maniatis, E. F. Fritsch, and J. Sambrook, "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Lab., Cold Spring Harbor, NY, 1982.

²¹ D. T. Denhardt, *Biochem. Biophys. Res. Commun.* **23**, 641 (1966).

²² G. W. Adams, P. Mayer, K. F. Medzirhadszky, W. H. Hines, and A. L. Burlingame, in preparation.

amine oxidase peptides I and II, are conserved between bovine serum amine oxidase and human kidney diamine oxidase suggests that this set of primers may be used as generic cloning primers for isolating additional mammalian topa quinone enzymes.

[9] Isolation of Active Site Peptides of Lysyl Oxidase

By HERBERT M. KAGAN and PING CAI

A variety of copper-dependent amine oxidases have been isolated from mammalian, plant, and yeast sources. These enzymes play critical roles in the metabolism of a variety of organic amines, including neurotropic monoamines as well as diamines such as putrescine and cadaverine.¹ In addition to the common requirement for a tightly bound Cu(II) cofactor at their active sites, this group of enzymes is catalytically inactivated by reagents known to form covalent adducts with active site carbonyl functions, including phenylhydrazine, semicarbazide, and others.^{1,2} Indeed, several of these enzymes are known to contain trihydroxyphenylalanine (topa) quinone.^{3,4} This quinone residue, which appears to derive post-translationally from a tyrosyl residue,³ is the site of reaction with carbonyl reagents.^{3,4} It is likely that this carbonyl cofactor serves as a transient electron sink operating during the oxidative deamination of the amine substrate.⁵

Lysyl oxidase shares certain features in common with this group of catalysts. Thus, this connective tissue amine oxidase contains a tightly bound Cu(II) cofactor at its active site^{6,7} and is inactivated by various carbonyl

¹ U. Bachrach, in "Structure and Functions of Amine Oxidases" (B. Mondovi, ed.), p. 5. CRC Press, Boca Raton, FL, 1985.

² W. G. Bardley, in "Structure and Functions of Amine Oxidases" (B. Mondovi, ed.), p. 135. CRC Press, Boca Raton, FL, 1985.

³ D. Mu, S. M. Janes, A. J. Smith, D. E. Brown, D. M. Dooley, and J. P. Klinman, *J. Biol. Chem.* **267**, 7979 (1992).

⁴ D. E. Brown, M. A. McGuirl, D. M. Dooley, S. M. Janes, D. Mu, and J. P. Klinman, *J. Biol. Chem.* **266**, 4049 (1991).

⁵ D. M. Dooley, M. A. McGuirl, D. E. Brown, P. N. Turowski, W. S. McIntire, and P. F. Knowles, *Nature (London)* **349**, 262 (1991).

⁶ H. M. Kagan, in "Biology of Extracellular Matrix" (R. P. Mecham, ed.), Vol. 1, p. 321. Academic Press, Orlando, FL, 1986.

⁷ S. N. Gacheru, P. C. Trackman, M. A. Shah, C. Y. O'Gara, P. Spacciapoli, F. T. Greenaway, and H. M. Kagan, *J. Biol. Chem.* **265**, 9022 (1990).