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Expression of Lysyl Oxidase From cDNA Constructs in Mammalian Cells: The Propeptide Region Is Not Essential to the Folding and Secretion of the Functional Enzyme

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Abstract Rat aortic lysyl oxidase cDNA was expressed under a metallothionein promoter in Chinese hamster ovary cells using a dihydrofolate reductase selection marker. One methotrexate-resistant cell line, LOD-06, generated by transfecting with full-length cDNA, yielded lysyl oxidase proteins consistent with the 50 kDa proenzyme and a 29 kDa mature catalyst. A second cell line, LOD32-2, was generated by transfection with a truncated cDNA lacking sequences which code for the bulk of the propeptide region. Both cell lines secreted apparently identical, 29 kDa forms of mature lysyl oxidase each of which catalyzed the deamination of human recombinant tropoelastin and alkylamines, consistent with the known specificity of lysyl oxidase. The secreted enzyme forms were inhibited by chemical inhibitors of lysyl oxidase activity, including β-aminopropionitrile, phenylhydrazine, ethylenediamine, α,α′-dipyridyl, and diethyldithiocarbamate. Sensitivity to these agents is consistent with the presence of copper and carbonyl cofactors in the expressed enzymes, characteristic of lysyl oxidase purified from connective tissues. These results indicate the lack of essentiality of the deleted proprotein sequence for the proper folding, generation of catalytic function, and secretion of lysyl oxidase.

Key words: crosslinkages, elastin, collagen, amine oxidase, lysyl oxidase

Lysyl oxidase (protein-lysine 6-oxidase, EC 1.4.3.13) is a copper-dependent amine oxidase which oxidatively deaminates the ε-amino group of specific peptidyl lysine and hydroxylysine residues of collagen and of lysine in elastin. The resultant aldehyde residues can spontaneously condense with neighboring peptidyl aldehydes or with unreacted ε-amino groups to form the inter- and intramolecular crosslinkages which stabilize the fibrous forms of these structural proteins [Kagan, 1986]. The participation of this enzyme is critical, therefore, to the development of structurally sound connective tissues of the respiratory, cardiovascular, skeletal, and other systems of the body. The expression of lysyl oxidase has been found to be markedly elevated in several instances and models of fibrotic disease [Kagan, 1986]. Moreover, the expression of the enzyme is markedly suppressed in fibroblasts derived from human Menkes disease [Gacheru et al., 1993], although it appears likely that the molecular defect in this disease involves a gene coding for a copper transporting ATPase [Vulpe et al., 1993]. In addition to the involvement of lysyl oxidase in these processes, recent studies have indicated the apparent identity of a mouse fibroblast gene product, *rrg*, which suppresses *Ha-ras*-induced transformation of mouse fibroblasts, with lysyl oxidase [Kenyon et al., 1991]. Thus, this enzyme plays pivotal roles in biology which may encompass a broader scope than previously suspected.

Studies of the pathway of biosynthesis of lysyl oxidase in rat aorta smooth muscle cells have revealed that this catalyst is synthesized as a 46 kDa preproenzyme. Following signal peptide

Abbreviations: BAPN, β-aminopropionitrile; CHO, chinese hamster ovary; DDTC, sodium diethyldithiocarbamate; DHFR, dihydrofolate reductase; DP, α,α′-dipyridyl; EDA, ethylenediamine; LO, lysyl oxidase; PH, phenylhydrazine; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

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cleavage and N-glycosylation, the resultant 50 kDa proenzyme form is secreted and then proteolytically cleaved to a 32 kDa species in the extracellular space [Trackman et al., 1992]. The molecular mass of this cleaved product is consistent with several reports identifying 28–32 kDa proteins as the catalytically active form of this enzyme isolable from various connective tissues [Kagan, 1986]. The cloning and sequencing of lysyl oxidase cDNA, first reported for the rat aorta gene product [Trackman et al., 1990, 1991], has also been accomplished with cDNA from human [Mariani et al., 1992; Hamalainen et al., 1993], mouse [Contente et al., 1993], and chick libraries [Wu et al., 1992]. These studies reveal a considerable degree of sequence conservation among these species and each of the protein sequences predicted from these cDNA sequences are consistent with the biosynthetic pathway found in smooth muscle cells. It is of some interest that the sequence corresponding to the propeptide region is considerably less conserved among these various forms of prolysyl oxidase than is the C-terminal two-thirds of the sequence of the proenzyme which corresponds to the mature, functional catalyst. In the present report, a mammalian cell culture expression system for the production of catalytically functional lysyl oxidase from cDNA constructs is described. This new tool for the study of structure-function relationships in lysyl oxidase and its proenzyme has been used here to assess the importance of the propeptide region to the production of the functional enzyme. In view of the limiting quantities of lysyl oxidase obtainable by purification from tissue sources, conventional analyses of structure-function relationships of this protein by protein chemistry remain as formidable tasks. Thus, the development of an expression system permitting site-directed mutagenesis of this catalyst should prove of considerable value in assessing the roles of specific residues previously implicated in enzyme function. Potential targets include an active site histidine residue [Gacheru et al., 1988], a covalently incorporated carbonyl cofactor [Williamson et al., 1986], and copper-binding sites [Gacheru et al., 1990], as well as residue sites of posttranslational modification of the proenzyme.

MATERIALS AND METHODS

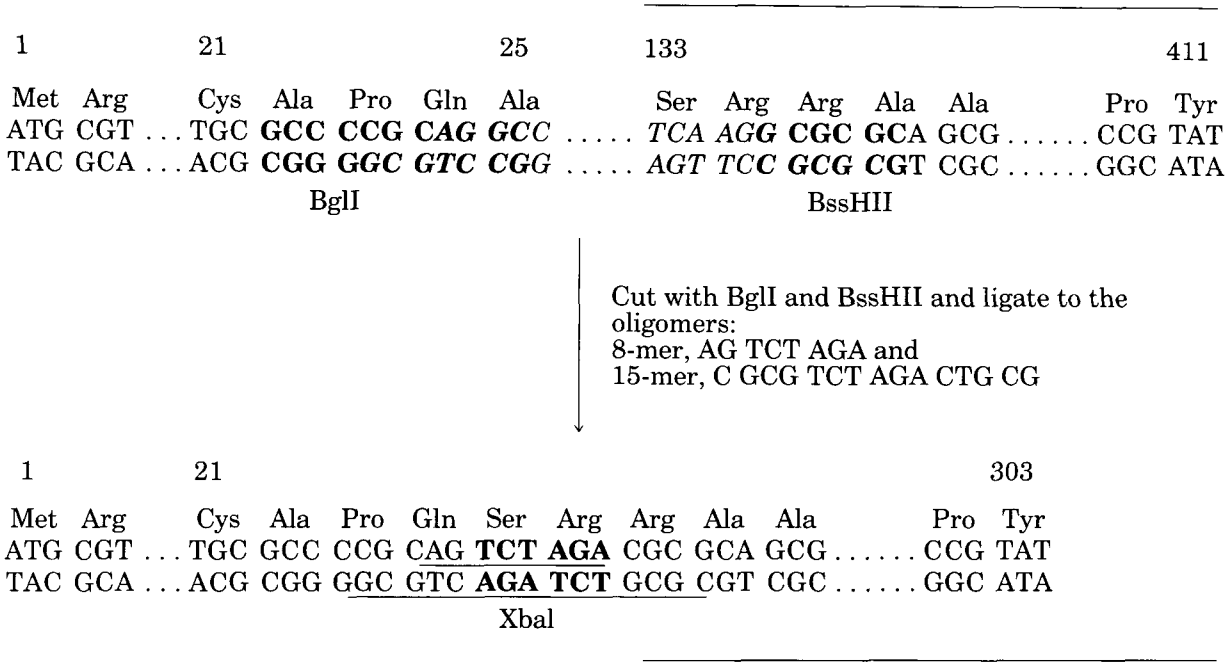
Restriction and DNA modifying enzymes were obtained from New England Biolabs (Beverly, MA). DNAs were transformed into competent DH-5α *E. coli* (Gibco Life Technologies, Grand Island, NY). Tissue culture media and reagents were purchased from Sigma (St. Louis, MO) and Gibco Life Technologies. DNA fragments from agarose gels were purified by the Gene Clean method (Bio 101, Inc., La Jolla, CA). Mutant dhfr-deficient CHO cells were obtained from the American Type Culture Collection (ATCC CRL 9096).

Construction of Expression Vectors

pMLD. Mouse metallothionein promoter, contained in a 2 kb sequence, was engineered as an EcoRI-XhoI fragment [Reddy et al., 1987] and an SV40 polyadenylation signal sequence (240 bp) was engineered as a XhoI-BamHI fragment [Reddy et al., 1987; Adams et al., 1991]. These two fragments were cloned into pML, a derivative of pBR322 [Lusky and Botchan, 1981], between EcoRI and BamHI sites. The resulting plasmid, pMTXS, was linearized with BamHI and ligated to a BamHI fragment (1.9 kb) containing an SV40 early promoter, mouse dihydrofolate reductase (DHFR) cDNA, and an SV40 poly A signal [Subramani et al., 1981], yielding plasmid pMXD. pMXD was cut with XhoI and ligated to the SalI fragment of rat lysyl oxidase [Trackman et al., 1990, 1991]. The ligated DNAs were treated with XhoI and transformed into competent DH5α *E. coli* cells. Colonies resistant to ampicillin were analyzed by restricting the mini-prep DNAs with EcoRI and HindIII, and the plasmid, pMLD, with the correct orientation of LO cDNA was selected. Large scale plasmid DNA was prepared from stationary cultures and purified on cesium chloride gradients.

pMLD32. The full-length rat lysyl oxidase cDNA, as previously reported [Trackman et al., 1990, 1991], has a BglI site after the signal peptide sequence and a BssHII site at a putative protease recognition sequence. Deletion of the cDNA coding sequence between these two sites will produce, after signal peptide removal, a truncated LO peptide approximately corresponding to the mature 32 kDa enzyme. To facilitate the cloning of this truncated LO, the cDNA was first cloned into pUC19 plasmid at the SalI site in the opposite orientation of lacZ transcription. The pUCLO DNA was cut with a mixture of BamHI and BssHII and the two fragments (3,795 bp and 500 bp) were isolated on agarose gels. The small fragment was cut with BglI and ligated to the large fragment combined with a mixture of 8-mer and 15-mer oligonucleotides

with ends complementary to the BglI and BssHII cohesive ends, while, at the same time, creating an XbaI site, as shown:



After ligation, the reaction mixture was treated with BssHII and transformed into competent DH5α. The resulting colonies were screened with SalI and XbaI, and, as expected, they all contained truncated 1.2 kb LO cDNA SalI inserts. The truncated, 1.2 kb LO cDNA fragment was cloned into pMXD as described for the full-length LO cDNA. The vector carrying the truncated LO cDNA was designated as pMLD32. The construction of the plasmids containing the full-length or truncated versions of lysyl oxidase cDNA is summarized in Figure 1.

Transfection

DHFR-deficient CHO cells were grown in F12 medium supplemented with 10% FBS and plated in 100 mm dishes at 0.5×10^6 cells per dish. pMLD and pMLD32 DNAs were coprecipitated with calcium phosphate and the precipitates were uniformly mixed over the cells for transfection, as described [Subramani et al., 1981; Kaufman et al., 1986; Mory et al., 1986]. Two days after transfection, the cells were harvested by treatment of the cell layers with trypsin, diluted 1:5 and spread into 100 mm dishes, and then fed with alfa-modified MEM culture medium containing 10% dialyzed FBS and 0.02 μM methotrexate [Kaufman et al., 1986; Mory et al., 1986]. The medium in the dishes was changed two times a week for 3 weeks. Colonies appearing during the third week were isolated with cloning rings and each clone was then isolated and cultured in six-well plates in methotrexate-supplemented selection medium until the cells reached confluency. Cells were then transferred for continued expansion in T-25 or T-75 flasks in the same medium. Cells were harvested from these flasks at confluency. Ten clones derived from cells transfected with full-length lysyl oxidase cDNA were isolated and identified as LOD-01 through LOD-010. In similar fashion, ten additional clones, LOD32-1 through LOD32-10, were isolated from cells transfected with truncated lysyl oxidase cDNA.

Enzyme Assay

Media from the cell lines isolated and cultured as described above as well as conditioned medium of cultures of CHO cells transfected only with a chimeric DHFR gene were assayed for LO activity with a human recombinant tropoelastin substrate using a discontinuous tritium release assay procedure [Bedell-Hogan et al., 1993].

Western Blots

CHO cells transfected with DHFR gene as well as LOD cell lines were grown to confluence

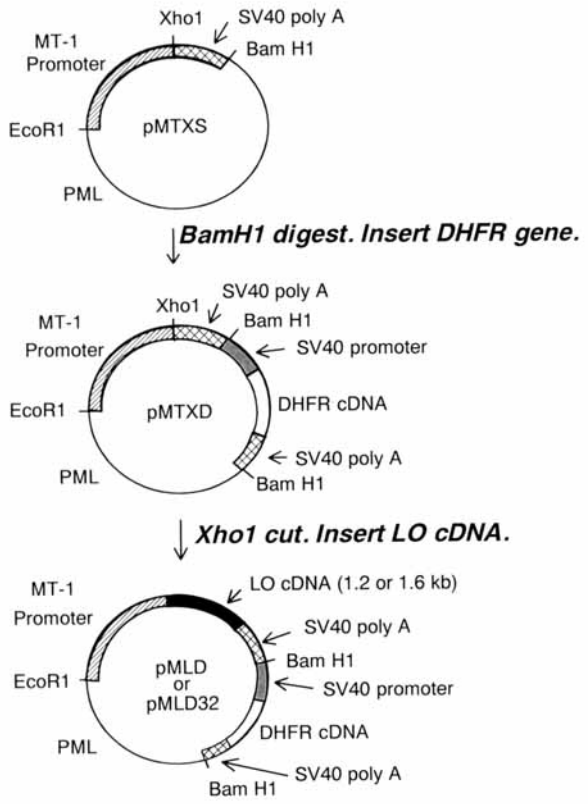


Fig. 1. Construction of expression vectors, pMLD and pMLD32. Mouse metallothionein (MT-1) promoter and SV40 poly-A are separated by the cloning site, XhoI. Full-length or truncated lysyl oxidase cDNAs (1.6 kb or 1.2 kb) are cloned as SalI fragments into XhoI site. The complementary SalI and XhoI ends, after ligation, result in the loss of both the SalI and XhoI sites in the final vectors, pMLD and pMLD32. The chimeric DHFR gene within the BamHI fragment consists of SV40 early promoter, mouse DHFR cDNA, and SV40 poly-A fragments.

in alfa-modified MEM and then fed with serum-free medium (CHO-S-SFM) for 24 h. Aliquots (20 μl) of the conditioned medium samples were boiled in SDS-PAGE loading buffer and proteins were resolved by electrophoresis on 10% SDS-polyacrylamide gels [Laemmli, 1970]. The proteins were transferred to Hybond-ECL nitrocellulose membrane by electroblotting as suggested by the manufacturer (Amersham). The membrane was first treated with 5% blocking reagent in tris-buffered saline-Tween (TBS-T) for 2 h at room temperature and incubated with rabbit anti-lysyl oxidase primary antibody diluted at 1:800 in 0.5% blocking reagent in TBS-T for 1 h. The membrane was washed for 1 h in five changes of TBS-T and incubated for 1 h with HRP-labeled goat anti-rabbit secondary antibody diluted to 1:1,000. The membrane was again washed in five changes of TBS-T and treated with enhanced chemiluminescence reagent (Amersham) containing a mixture of luminol and hydrogen peroxide. The chemiluminescent signals were detected by exposing the membrane to Dupont (Boston, MA) Reflection NEF-496 X-ray films.

Northern Blotting for Estimation of RNA

Total RNA was extracted by the acid guanidinium thiocynate-phenol-chloroform extraction procedure [Chomczynski and Sacchi, 1987] from the cell layers of cultures of LOD-06 and LOD32-2, as well as from CHO cells transfected with mouse DHFR cDNA construct, alone. Ten micrograms of each of the RNA preparations were electrophoresed on formaldehyde-agarose gels, transferred to nitrocellulose membrane, and hybridized with ^{32}P-labeled LO cDNA probe, using hybridization and washing conditions as described [Sambrook et al., 1989].

RESULTS

Western Blotting

Initial screening for expressed forms of lysyl oxidase was performed by Western blotting of proteins secreted into the serum-free medium of selected clones derived from cells transfected with the full-length or truncated lysyl oxidase constructs described. Four positive, full-length clones (LOD-01, -03, -06, and -07) and six positive, truncated clones (LOD32-1 through -6) were identified in this fashion. In each case, immunoreactive bands corresponding to a molecular weight of 29 kDa were seen. Clones LOD-06 and LOD32-2 were selected for further analyses. As shown (Fig. 2), 29 kDa bands appear in the Western blots of the conditioned medium of LOD-06 (lane 2) and LOD32-2 (lane 3). In addition, a band is seen at approximately 50 kDa derived from the cell lysate of LOD-06 (lane 5), whereas a band occurs at 35 kDa derived from the lysate of LOD32-2 (lane 6). A nonspecific, high molecular weight band is seen in the nontransfected, control cell medium sample (lane 1) and control cell lysate sample (lane 4), as well as in each of the other lanes. Bands were not seen corresponding to proteins with the molecular weights expected for mature or precursor forms of lysyl oxidase in the lanes corresponding to the control cells lacking the lysyl oxidase construct (lanes 1 and 4). Purified bovine aorta lysyl oxidase migrates as a 32 kDa protein (lane 7). The bands at 50 kDa seen here are consistent with the expected size of N-glycosylated prolysyl oxidase [Trackman et al., 1992] and those at 29 kDa seen in the medium

are consistent with the size of processed forms of this enzyme which have been reported to vary between 28 and 32 kDa when active enzyme is isolated from different tissues and species [Kagan, 1986]. The calculated molecular weight of the protein derived from the truncated cDNA is 32,533 Da after signal peptide cleavage between residues 21 and 22 [Trackman et al., 1990, 1991]. The observed molecular weight of 35 kDa suggests that this product may have undergone posttranslational modification, a probable form of which is N-glycosylation at the -Asn-Arg-Thr- consensus sequence which would persist in this product at residues equivalent to residues 138–140 in the full proenzyme sequence [Trackman et al., 1990, 1991] (see Fig. 5, Discussion). This possibility was borne out by the observation that treatment of the 35 kDa band with peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase converted this to a protein migrating as a 32 kDa species on SDS-PAGE. Similar analysis of the full-length product confirmed that this species was also N-glycosylated (data not shown).

Catalytic Properties

The optimal expression of an enzyme is expected to yield a catalytically functional protein. This proved to be true in the present case. Aliquots of the conditioned medium of clones LOD-06 and LOD32-2 both catalyzed the oxidative deamination of lysine in a recombinant human tropoelastin substrate. In contrast, negligible activity was found in the conditioned medium of CHO cells transfected only with a DHFR gene but not with lysyl oxidase cDNA (Table I). Comparing the amount of activity of each clone against the tropoelastin substrate with that obtained with known quantities of purified bovine aorta lysyl oxidase permits the estimation that clones LOD-06 and LOD32-2 secrete approximately 2 mg of active enzyme per liter of medium in 24 h. Although the specific activities of the two clones were not established, the densities of the bands at 29 kDa of each seen in Western blots of conditioned medium were similar, consistent with the conclusion that similar amounts of lysyl oxidase protein were used in the assays to yield the data shown in Table I. The specific activities of the functional forms derived from the full-length and truncated constructs thus appear to be similar. Since lysyl oxidase can also oxidize primary alkyl monoamines and diamines, the relative activities against representative alkylamines of the LOD-06, LOD32-2, and bovine aorta lysyl oxidase were compared. It was found that each of the recombinant cell lines expressing lysyl oxidase utilized both n-hexylamine and 1,5-diaminopentane as substrates, assaying with a peroxidase-coupled fluorescent method [Trackman et al., 1981]. The ratios of the initial, BAPN-inhibitable rates of oxidation of 1,5-diaminopentane to that for n-hexylamine were 2.1, 2.2, and 2.4 for the enzyme activities of LOD-06, LOD32-2, and purified bovine aorta lysyl oxidase, respectively, indicating that the substrate specificities among these three enzyme sources are at least highly similar. Notably, CHO cells transfected only with a DHFR gene did not exhibit BAPN-inhibitable

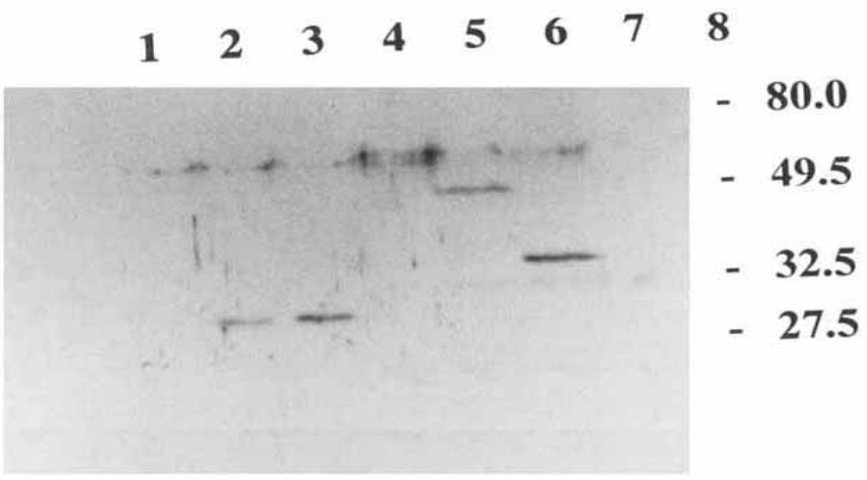


Fig. 2. Western blot analysis of lysyl oxidase expressed in CHO cells. *Lane 1:* medium from control CHO cells transfected with chimeric DHFR gene alone; *lanes 2 and 3:* media from LOD-06 and LOD32-2 cell lines, respectively; *lane 4:* cell lysate from CHO cells transfected with DHFR gene alone; *lanes 5 and 6:* cell lysates from LOD-06 and LOD32-2 cell lines, respectively; *lane 7:* lysyl oxidase purified from bovine aorta; and *lane 8:* molecular weight markers.

TABLE I. Activity of Recombinant Forms of Lysyl Oxidase against Tropoelastin*

Enzyme source	[^{2}H]H_2O, cpm/2 h
LOD-06 (760 μl)	969 ± 64
LOD32-2 (760 μl)	987 ± 73
Bovine aorta lysyl oxidase (2 μg)	987 ± 1
CHO Cells (760 μl)	33 ± 29

*Data are presented as the means ± standard deviations of assays in triplicate.

rates of oxidation with either of these substrates (not shown).

Inhibition Profiles

The enzyme activity in the conditioned media of clones LOD-06 and LOD32-2 was assayed in the presence and absence of agents known to inhibit purified lysyl oxidase. As shown (Table II), each expressed enzyme is inhibited to similar degrees by phenylhydrazine, consistent with the presence of a functional carbonyl cofactor [Williamson et al., 1986]; by ethylenediamine, previously implicated as forming a covalent pyrazine adduct with purified lysyl oxidase [Gacheru et al., 1989]; by α,α′-dipyridyl and diethyldithiocarbamate, consistent with a functional copper cofactor [Gacheru et al., 1990]; and by β-aminopropionitrile, a mechanism-based, irreversible inhibitor of lysyl oxidase [Tang et al., 1983]. Preincubation of aliquots of the conditioned medium of LOD-06 and LOD32-2 in the presence or absence of 5 mM BAPN or 10 mM ethylenediamine at 37°C for 1 h followed by exhaustive dialysis against 50 mM sodium borate, pH 8, at 4°C resulted in the complete loss of enzyme activity in the samples incubated with either of these inhibitors but not in samples of these conditioned media which had been incubated in the absence of inhibitors and dialyzed correspondingly (data not shown). Thus, each of these agents inhibit both of the enzyme preparations irreversibly. The sensitivities to BAPN of the lysyl oxidase species produced by the two positive clones were compared and found to be essentially the same with IC_{50} values of 1 to 2 μM seen in each case (Fig. 3). This range of values is in excellent agreement with the IC_{50} values of 2.6 and 1.4 μM reported for the 32 kDa lysyl oxidase enzymes purified from bovine lung and aorta, respectively [Cronlund and Kagan, 1986].

Messenger RNA Expression

Total RNA was prepared from CHO cells transfected with the full-length or truncated forms of lysyl oxidase cDNA. As shown, cells not transfected with lysyl oxidase cDNA constructs do not yield a visibly hybridizing band on Northern blots probed with ^{32}P-labelled, full-length lysyl oxidase cDNA (Fig. 4). In contrast, total RNA isolated from LOD-06 displays a band at ca. 2 kb, while that from clone LOD32-2 displays a band at ca. 1.7 kb. The estimated lengths of these transcripts are consistent with those expected of the lysyl oxidase mRNA species derived from the two constructs used to generate these two clones.

DISCUSSION

The present report describes the successful in vitro expression of catalytically functional lysyl oxidase in CHO cells transfected with cDNA coding either for the full-length, 46 kDa proenzyme or for a truncated form of the proprotein. The active enzyme appearing in the medium in both cases exhibits catalytic properties typical of the 32 kDa catalyst purified from bovine connective tissues. Thus, the catalysts derived from the protein products of the full-length or truncated inserts each are irreversibly inhibited by BAPN and exhibit the expected sensitivity to this inhibitor of lysyl oxidase. Other amine oxidases have been shown to be much less sensitive or insensitive to this aminonitrile [Tang et al., 1989], further supporting the identity of the enzymes expressed here as lysyl oxidase. Each of the expressed enzymes is also inhibited with apparently similar sensitivities by specific copper chelating agents, consistent with the presence of a copper cofactor. Moreover, each is inhibited irreversibly by phenylhydrazine and ethylenediamine, agents which can form a covalent adduct with the carbonyl cofactor in lysyl oxidase, again consistent with the known properties of this catalyst. Most importantly, the expressed enzymes oxidatively deaminate tropoelastin, a natural substrate of lysyl oxidase, as well as simple alkyl mono- and diamine substrates previously shown to be oxidized by lysyl oxidase purified from connective tissues [Trackman et al., 1981]. The identity of the expressed proteins was further verified by reaction with anti-lysyl oxidase raised against the purified aortic enzyme [Trackman et al., 1992].

TABLE II. Inhibition of Lysyl Oxidase Activity Against Tropoelastin*

Inhibitor	LOD-06		LOD32-2	
	[^{3}H]H_2O release, cpm/2 h	Percent control	[^{3}H]H_2O release, cpm/2 h	Percent control
Control	551 ± 26	100 ± 5	441 ± 30	100 ± 6.8
PH (1 mM)	71 ± 0.4	12.9 ± 0.1	71 ± 0.4	12.9 ± 0.1
EDA (1 mM)	24.3 ± 0.2	4.4 ± 0	26 ± 2.9	4.7 ± 0.5
DP (5 mM)	105 ± 8.6	19 ± 1.6	100 ± 7.5	18.2 ± 1.4
DDTC (1 mM)	183 ± 16.3	33.3 ± 3	125 ± 4.7	22.6 ± 0.8
BAPN (0.5 mM)	95 ± 5	17.3 ± 0.8	95 ± 4.5	17.3 ± 0.8

*Data are presented as the means ± standard deviations of assays in triplicate.

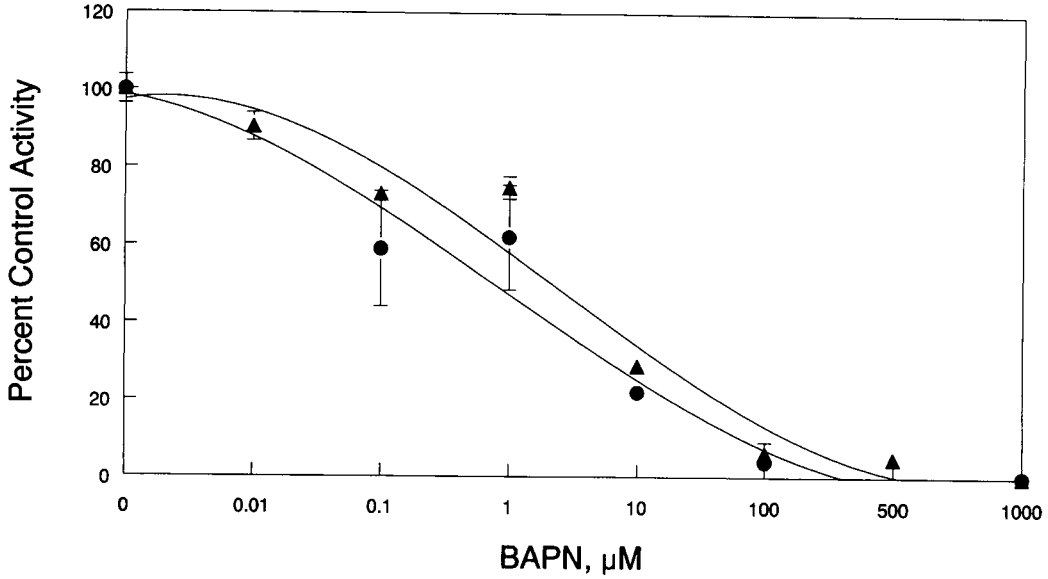


Fig. 3. Inhibition of recombinant lysyl oxidase activity against tropoelastin by BAPN. ●, enzyme activity in medium of LOD-06; ▲, enzyme activity in medium of LOD32-2. The heights of the vertical bars represent the standard deviations obtained from assays in triplicate.

The truncated version of the enzyme expressed in these studies was constructed to incorporate the signal peptide sequence contained within residues 1–21 of the preproprotein. This signal sequence was continuous with residues 134–411 in the product of LOD32-2, the latter region containing the functional, mature enzyme portion of the proenzyme, as represented in Figure 5. Hence, the cDNA portion coding for the bulk of the propeptide sequence (residues 25–132) was omitted from the truncated construct. Notably, functional enzymes with apparently the same molecular weights and at least very similar specific activities and catalytic properties appeared in the medium of both clones. As has been demonstrated in arterial smooth muscle cell cultures, preprolysyl oxidase loses its signal peptide, becomes N-glycosylated, and then is secreted as a 50 kDa species which is proteolytically processed to the active, 32 kDa species in the medium by a metalloenzyme which is also secreted by these cells [Trackman et al., 1992]. The size of the protein derived from the truncated construct was intended to exclude the structural information represented by propeptide residues 25–132 while including the dibasic Arg-Arg sequence (residues 134 and 135) as a possible proenzyme processing site. Dibasic sites as -Lys-Arg- and -Arg-Arg- have been shown to be selectively cleaved upon the activation of precursor forms of neuropeptides by specific endoproteases [Benjannet et al., 1991]. Cleavage at this Arg-Arg site in prolysyl oxidase predicts the release of a 32 kDa enzyme derived from the C-terminal sequence of the proenzyme [Trackman et al., 1990, 1991]. The 29 kDa molecular masses of the lysyl oxidase species produced in the CHO cell medium suggests that cleavage

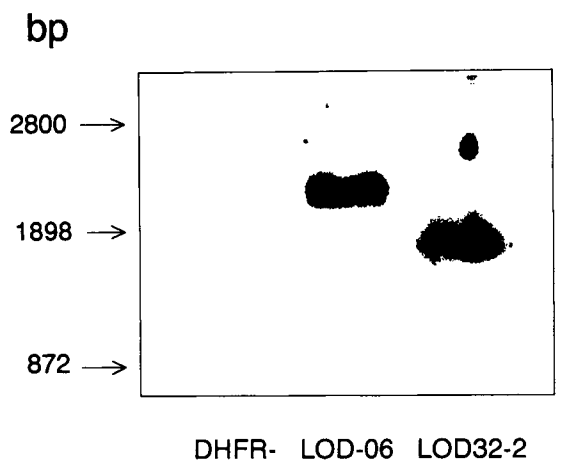


Fig. 4. Northern blot analysis of RNAs from lysyl oxidase producing cell lines. *First lane:* RNA from CHO cells transfected with chimeric DHFR gene alone; *second lane:* RNA from LOD-06 cell line; and *third lane:* RNA from LOD32-2 cell line. Migration positions of the molecular weight standards (bp) are shown.

occurred at a site which is more C-terminal than the dibasic site. Consistent with this possibility, other studies in progress in this laboratory support the conclusion that the cleavage site of the expressed proprotein occurs 26 to 30 residues in the C-terminal direction from the dibasic site, i.e., residues 161 to 165. It is of interest that the N-terminus of the four ionic variants of the mature catalyst isolated from bovine aorta has been identified as Asx (aspartic acid or asparagine) in each case, consistent with the presence of the -Gly-Asp-Asp- sequence at residues 162–164 [Sullivan and Kagan, 1982]. It remains possible, however, that the proprotein is subject to more than one, consecutive cleavages to produce the 29 kDa end product seen here. The availability of the expression system described in this report should facilitate further efforts to define the precise site(s) of proteolytic processing as well as other posttranslational modifications critical to its secretion and to the generation of the functional enzyme.

It is also of note that the CHO cells which do not appear to produce endogenous lysyl oxidase can proteolytically process the full-length proenzyme as well as the truncated product and generate active enzyme from each. It is possible that cells such as aortic smooth muscle cells which produce and activate relatively abundant quantities of lysyl oxidase may utilize an extracellular protease for the proteolytic activation which has a more general role in biology and which is not specific to those cells. It also remains to be established whether the CHO cells secrete a protease which processes prolysyl oxidase with a different substrate site specificity.

The generation of apparently identical catalysts from the full length proenzyme and from the markedly truncated version thereof indicates that synthesis of the full propeptide sequence is not necessary for the generation of a processed, functional catalyst. Thus, information required for the proper folding of the protein to generate a functional active site appears to be contained largely and possibly entirely within that N-terminal sequence of which the active, mature catalyst is composed. It is of interest in this regard that the predicted amino acid sequences of the rat [Trackman et al., 1990, 1991], human [Mariani et al., 1992; Hamalainen et al., 1993], mouse [Contente et al., 1993], and chick [Wu et al., 1992] proproteins corresponding to the propeptide region are not nearly as highly conserved as are the sequences corresponding to the mature enzyme region of the proprotein. The apparently nonessential nature of the lysyl oxidase propeptide region deleted in this study to the secretion of the enzyme and the generation of catalytic function is in contrast with other proproteins, such as the precursor form of TGF-β in which the pro region appears to act as a chaperone essential to the proper folding and secretion of the mature protein segment [Lopez et al., 1992]. It is notable that each of the 10 cysteine residues of the rat proenzyme are contained within the segment corresponding to the mature catalyst so that cleaved segments of the propeptide cannot become disulfide-linked to the mature enzyme species. All of the cysteines within the 32 kDa bovine aorta enzyme appear to be involved in intramolecular disulfide bonds [Williams and Kagan, 1985]. This feature is doubtlessly critical to the stabilization of the functional conformation of the mature enzyme and to the unusual thermostability of lysyl oxidase, previously seen to be stable to irreversible denaturation by temperatures up to 90–92°C [Trackman et al., 1981].

While the role of the propeptide in prolysyl oxidase remains to be established, it is conceivable that this peptide sequence suppresses the catalytic function of lysyl oxidase within the cell, thus insuring that collagen and/or elastin precursors do not become crosslinked to high molecular weight polymers prior to secretion. Given the prior observation that lysyl oxidase can oxidize a variety of non-fibrous, globular proteins in assays in vitro [Kagan et al., 1984], such a mechanism might also protect against adventi-

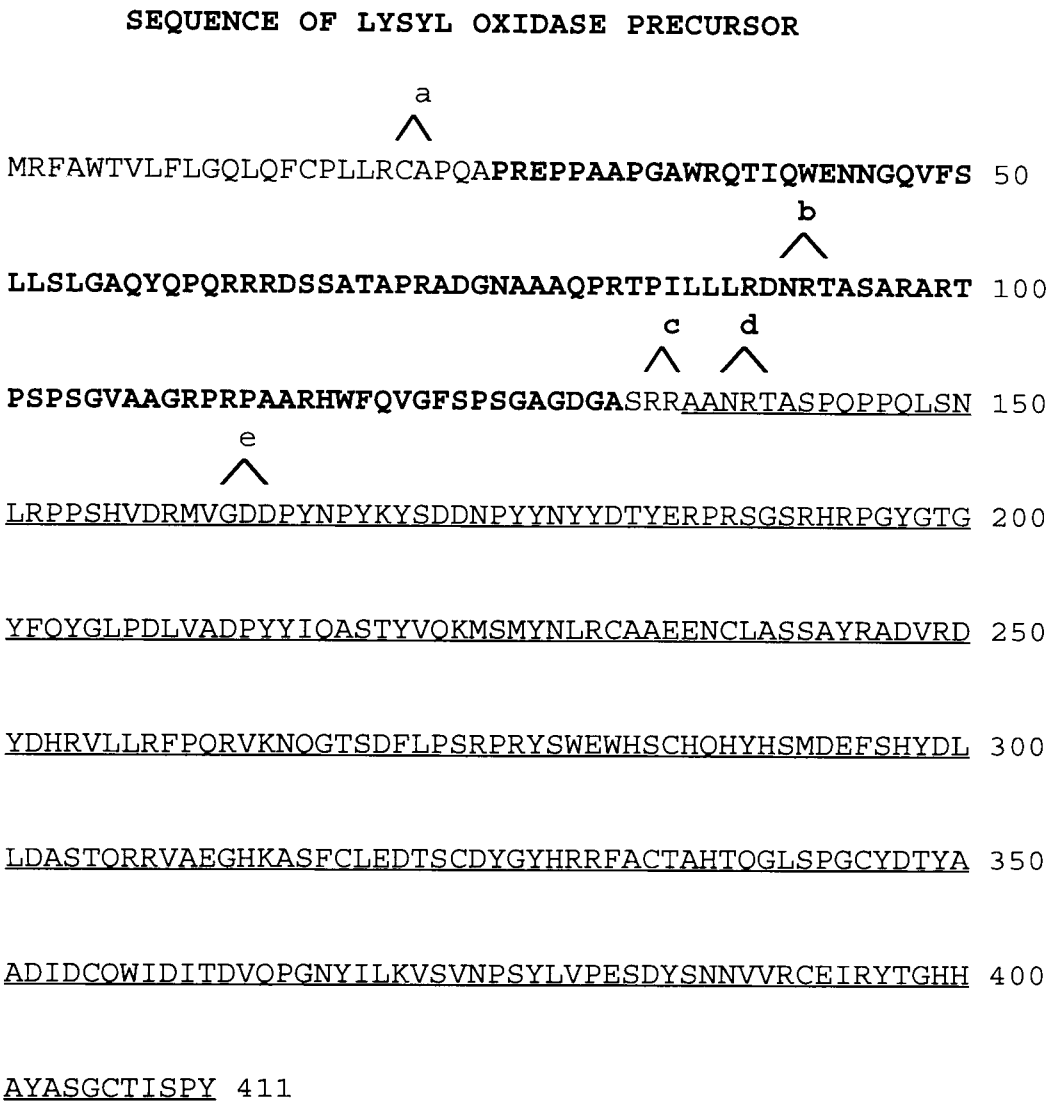


Fig. 5. Amino acid sequence of rat aorta lysyl oxidase predicted from the cDNA sequence [Trackman et al., 1990, 1991]. The portion of the full sequence (residues 26–132) which is deleted in the truncated construct is shown in bold letters. The underlined portion of the sequence would account for a 32 kDa mass of a mature enzyme resulting from proteolysis at site "c." Sites: **a,** consensus signal peptide cleavage site between C21 and A22; **b** and **d,** consensus N-glycosylation sites at NRT sequences; **c** and **e,** possible proteolytic cleavage sites. The active site region contains the metal ion and/or copper-binding consensus sequences at residues 313–333 and 395–401 [Trackman et al., 1990, 1991].

tious posttranslational oxidation of lysine side chains of other, unrelated proteins within the cell, as well. It is also possible that the propeptide directs lysyl oxidase to secretory vesicles which contain or coordinate with elastin and/or collagen precursors in the process of secretion. These and other possibilities remain to be assessed.

ACKNOWLEDGMENTS

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