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Limited proteolysis of *Hansenula polymorpha* yeast amine oxidase: isolation of a C-terminal fragment containing both a copper and quino-cofactor

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Abstract Limited proteolysis of recombinant *Hansenula polymorpha* yeast amino oxidase produces a 48 kDa fragment which corresponds to the C-terminal two-thirds of the protein. The fragment contains both TOPA (2,4,5-trihydroxyphenylalanine) and copper, as well as the histidine ligands implicated in copper binding. The fragment is proposed to be the domain responsible for cofactor production in yeast amine oxidase.

Key words: Topa quinone; Copper protein; Amine oxidase; Cofactor biogenesis; Limited proteolysis

1. Introduction

Recent progress in the study of TOPA-containing amine oxidases has provided evidence for cofactor formation via enzyme-bound copper in a post-translational self-processing event [1–3]. When amino acid sequences from four eukaryotic amine oxidases were aligned, a C-terminal motif consisting of the conserved positioning of various histidines in relation to the cofactor site became apparent [4]. Of particular note is a His-X-His sequence lying 40 to 60 amino acids toward the C-terminus from the tyrosine precursor to TOPA. Several studies have implicated histidines as ligands to the copper in the TOPA-containing amine oxidases [1,4,5]. These findings, together with the frequent use of His-X-His as a copper binding site in proteins [6], has led to the proposal of a domain at the C-terminus of the amine oxidases which contains the structural determinants for TOPA production. This raises the possibility that the more divergent N-terminus of this class of proteins is responsible for the generation of a substrate binding pocket of varying substrate specificity [7 for review] and stereochemistry [8 for review].

In the present study limited proteolysis has been employed to determine whether yeast amine oxidase has regions of enhanced resistance to digestion in the C-terminus, corresponding to the proposed TOPA-biogenesis domain. A stable fragment consisting of the C-terminal two-thirds of the enzyme has been obtained. As described herein, this fragment contains both cofactor and copper, but displays no enzyme activity under assay conditions optimized for full-length enzyme.

2. Materials and methods

2.1 Materials

Elastase (Type II-A: from porcine pancreas, 41 units/mg solid), trypsin (form bovine pancreas, 10,800 units/mg solid), thermolysin (Protease Type X from bacillus thermoproteolyticus rokko, 41 units/mg solid), V8 protease (Type XVII-B from Staphylococcus aureus strain V8, 830 units/mg solid) and immobilized V8 (insoluble enzyme attached to 4% cross-linked agarose, lyophilized powder, stabilized with lactose, 450 units/mg solid) were purchased from Sigma. Enzyme grade HEPES was purchased from Fisher.

2.2 General methods

The yeast amine oxidase used in this study was purified by Danying Cai from a heterologous expression system of the *Hansenula polymorpha* amine oxidase gene in *Saccharomyces cerevisiae* [9]. The enzyme is a dimer with a subunit weight of 78 kDa. The samples of enzyme used for this study varied in activity from 0.06 U/mg to 0.09 U/mg with benzylamine as substrate.

Proteolysis reactions were analyzed on 12% SDS-polyacrylamide gels run under reducing conditions according to the method of Laemmli [10]. Proteins were visualized by incubating the gels for at least 10 min in fixing solution (Sigma) and then staining overnight in Brilliant Blue G-Colloidal (Sigma). Gels were destained in 10% acetic acid/25% methanol for 30 s and then incubated in 25% methanol until the background color was reduced.

Fragment activity was assayed in 100 mM phosphate buffer, pH 7.2 at 37°C with 3.0 mM ethylamine or 1.5 mM methylamine as substrate using a Clark oxygen electrode.

2.3 Limited proteolysis of yeast amine oxidase

Screening of proteases was performed in 100 mM phosphate buffer, pH 7.8 with 0.5–1.0 M urea, 2%–4% (w/w) protease for 2–4 h at 37°C. Time points were quenced by freezing and subsequent boiling in SDS-PAGE sample buffer prior to gel loading. Large-scale limited proteolysis for fragment purification was performed with about 10 units of V8 protease immobilized on agarose beads (Sigma) at 1.0 M urea with stirring for 4 h at 37°C. Upon completion of the digestion, the protease was removed by spinning in a microfuge followed by filtration of the supernatant through a micro-prep disc (Millipore).

2.4 Analyzing for the presence of TOPA in proteolysis fragments

For cofactor analysis gels were blotted onto 0.45 μm nitrocellulose (Bio-Rad) in an Idea Scientific Company blotting apparatus run at 170 mA overnight in a 20% methanol/25 mM Tris/192 mM glycine buffer system. The blot was then stained for quinone moieties by incubating in 0.24 mM Nitroblue tetrazolium (Aldrich) in 0.1 M potassium glycinate buffer, pH 10 for 30–60 min.

2.5. Purification of the C-terminal fragment of yeast amine oxidase

Purification was performed on an FPLC (Pharmacia) fitted with a Mono-Q anion exchange column. A chloride gradient was run from 0 mM to 525 mM at a flow rate of 0.5 ml/min. The C-terminal fragment eluted at about 300 mM chloride. Relevant fractions were then combined, concentrated and dialyzed against 100 mM HEPES, pH 7.0 in a Collodion vacuum concentrator (Schleicher and Schuell).

2.6. N-terminal sequencing of the C-terminal fragment of yeast amine oxidase

The N-terminal sequence was acquired by sequencing directly from a PVDF membrane (Bio-Rad). Electroblotting onto 0.2 μm PVDF was performed as described for the redox stain and the membrane was then stained in Commassie G250 for 10–15 min, followed by destaining in

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10% acetic acid/50% methanol for 30 min to visualize the protein bands. The membrane was then washed extensively in water to minimize the amount of glycine on the membrane. The 48 kDa band was then excised and submitted for sequencing.

2.7. Determination of the Cu content of the C-terminal fragment of yeast amine oxidase

Copper content of the purified C-terminal fragment was assessed on a Perkin Elmer Atomic Absorption machine equiped with a graphite furnace. Purified fragment was first dialyzed against 20–100 mM HEPES buffer, pH 7.0. Attempts were made to minimize the copper content of the buffer by using ultra-pure HEPES and plastic containers. The amount of copper in the fragment was determined both by direct comparison to a standard curve and by the standard addition method. For standard addition the protein concentration was brought to 5–7 μg/ml with copper standard added to 0–0.040 μg/ml copper. 1 mg/ml (1000 ppm ± 1%) copper reference solution in 2% nitric acid was purchased from Sigma.

3. Results and discussion

3.1. Screening of proteases

Limited proteolysis is a technique commonly used to investigate the domain boundaries of proteins for which structural information is limited. It involves digestion under mild proteolytic conditions (short reaction times, low protease concentrations, low detergent concentrations) so that only the most exposed regions of the protein are cut, leaving intact the more tightly folded segments. These areas of enhanced resistance to degradation are often representative of protein domains.

Yeast amine oxidase was subjected to limited digestion with four proteases: elastase, thermolysin, trypsin and V8 protease. 1.0 M urea, 4 h reaction time and 2%–4% (w/w) protease gave the most efficient digestion. Under these conditions, elastase produced many products and thermolysin produced several major bands. The trypsin and V8 digestions gave more promising results with significant accumulation of two products at 39 kDa and 35 kDa for tryptic digestion and one product at 48 kDa for V8 treatment (Fig. 1).

A redox-cycling assay specific for quinone structures [11] was performed on the major products of tryptic and V8 digestion to determine whether these fragments were derived from the C-terminal, TOPA-containing portion of the protein. The positive result is shown in Fig. 1. The standards appear as positives because they are prestained.

3.2. Purification of the fragment produced by V8 digestion

Since V8 gave the largest amount of a single band, purification of the 48 kDa V8 digestion product was undertaken. For large-scale digestion, immobilized V8 was used to ease protease removal.

Table 1
Copper content of the 48 kDa fragment

Protein Subunit (μM)	Copper (μM)	Cu per subunit
0.88	0.68	0.8
0.88	1.11	1.3
2.92	3.23	1.1
		AV: 1.1 ± 0.2

The fragment was purified from the reaction mixture on a Mono Q column with a chloride gradient. A representative preparation gave 143 μg of the 48 kDa band after concentration and dialysis from a starting 750 μg of yeast amine oxidase (30% yield). A gel of purified 48 kDa fragment and its redox stain are shown in Fig. 2. The purified fragment was slightly unstable and tended to precipitate when frozen or when dialyzed into distilled water.

The fragment showed no oxygen consumption in the oxygen electrode when incubated with 1.5 mM methylamine or 3.0 mM ethylamine in 100 mM phosphate buffer, pH 7.2. Comparable amounts of full-length yeast amine oxidase under the same conditions showed appreciable oxygen consumption indicative of substrate turnover and cofactor reoxidation via molecular oxygen.

3.3. Sequencing of the C-terminal fragment from V8 digestion of yeast amine oxidase

Due to solubility problems and lack of protein, sequence information for the purified fragment was obtained by sequencing directly from a PVDF membrane onto which about 100 pmol of fragment had been transferred. The 48 kDa band was shown to be a mixture of two V8 products corresponding to digestion at Glu-230 (the major product) and at Glu-247 (present to a lesser extent). The N-terminal sequence of the major 48 kDa component was Lys-Val-Gly-Met-(Arg)-Pro-Glu which matched the sequence expected from the cDNA of yeast amine oxidase. The Arg is shown in brackets because it was not present in observable quantities in the Edman sequencing.

From the position of digestion (Glu-230) and the size of the fragment (48 kDa), the fragment is concluded to extend to the C-terminus of the protein. In this way it accomodates the conserved histidines and the TOPA-consensus site.

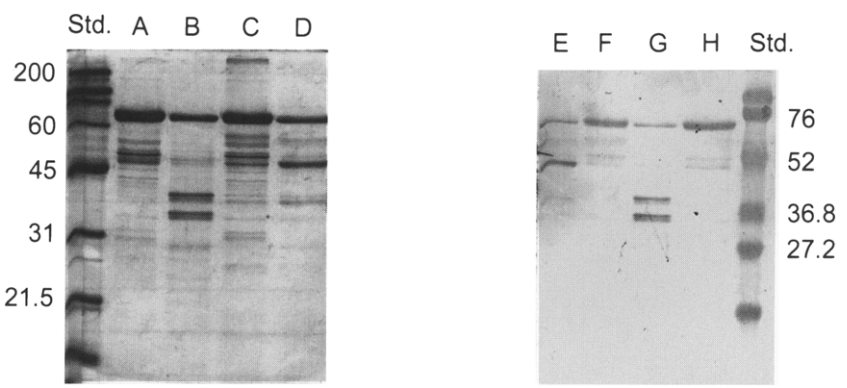

Fig 1. Lanes A–D, Brilliant blue stain; lanes E–F, redox stain. Lane A and H, tryptic digest, $t = 0$; lane B and G, tryptic digest, $t = 2$ h; lane C and F, V8 digest, $t = 0$; lane D and E, V8 digest, $t = 2$ h.

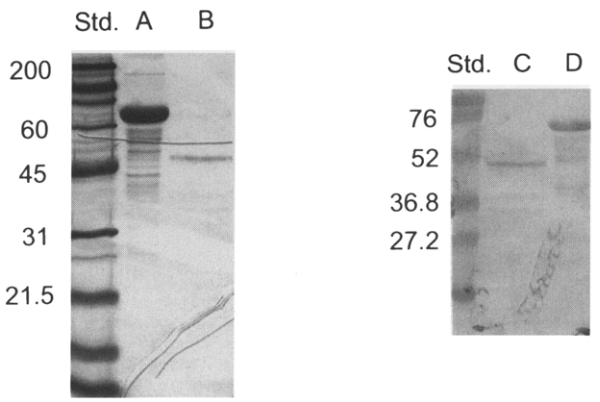

Fig. 2. Lanes A and B, Brilliant blue stain; lanes C and D, redox stain. Lane A and D, full-length yeast amine oxidase; lane B and C, purified 48 kDa fragment.

3.4. Determination of the copper content of the C-terminal fragment from V8 digestion of yeast amine oxidase

Although the 48 kDa fragment includes the histidines implicated in copper binding, it was uncertain whether the trimmed protein would still bind copper.

Dialysis or dilution of the fragment into deionized water for the purpose of atomic absorption analysis proved impracticable due to solubility problems. The analyses were instead obtained in 20–100 mM HEPES buffer, pH 7.0. The amount of copper in the buffer was determined to be 60–100 nM. This was subtracted from the copper content for the enzyme, which was determined both by the direct method and the standard addition method. The variability of the data shown in Table 1 can be attributed to interference caused by HEPES as well as solubility problems with the fragment, problems which became more pronounced upon prolonged storage.

However, the data clearly converge on ca. 1 mol copper per mol of fragment, confirming that the fragment has retained all the putative cofactor-producing machinery of the original enzyme.

3.5. Conclusions and future directions

Limited proteolysis of yeast amine oxidase has yielded a putative TOPA-biogenesis domain, which contains both cofactor and copper but has no activity under turnover conditions optimized for full-length yeast amine oxidase. For the future, we plan to determine if a stable 48 kDa domain can be obtained from the expression system used for production of full length protein [9]. It will be of great interest to see if this domain can catalyze the generation of the mature TOPA cofactor in the absence of the N-terminus of protein.

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