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Copper amine oxidases: current state of knowledge

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Summary. A brief review on the structure and functions of the copper-pyrroloquinoline quinone amine oxidases is presented. Data concerning the metal and organic cofactors are reported, giving special emphasis to their structural relationship with the protein molecule and mechanistic properties. Information on the functional role of these enzymes with respect to polyamine metabolism are also given.

Key words: Amine oxidase(s) (AO) - Copper - Polyamine(s) - Pyrroloquinoline quinone (PQQ)

Introduction

Copper-containing amine oxidases (Cu-AOs) are quinoproteins belonging to a wider class of deaminating oxidases (amine:oxygen oxidoreductases) which also includes several FAD-dependent enzymes, e.g. mitochondrial monoamine oxidase and cytosolic polyamine oxidase as well as several other flavin-containing amine oxidases from plants and microorganisms. Cu-AOs also contain a covalently bound organic prosthetic group which has been isolated and identified as pyrroloquinoline quinone (PQQ) or a closely similar derivative.

Together with the FAD enzymes, Cu-AOs share, within a formal but not mechanistic point of view, the kind of reaction catalysed, i.e. the oxidative deamination of amines with production of hydroperoxide according to the following stoichiometry:

$$R{-}CH_2{-}NH_2 + O_2 + H_2O \longrightarrow R{-}CHO + NH_3 + H_2O_2 \quad (1)$$

(cleavage at a primary amino group)

$$R_1{-}CH_2{-}NH{-}CH_2{-}R_2 + O_2 + H_2O \longrightarrow R_1{-}CHO + H_2N{-}CH_2{-}R_2 + H_2O_2 \quad (2)$$

(cleavage at a secondary amino group)

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Both primary and secondary amino groups are cleaved by AOs, but while the FAD-dependent ones are able to carry out both types of reaction, the Cu-AOs generally oxidize amines only at a primary nitrogen. Physiological substrates of AOs are several naturally occurring aliphatic and aromatic mono-, di- and polyamines: the FAD-AOs are primarily involved in the breakdown of some biogenic monoamine neurotransmitters (monoamine oxidases) and of polyamines (polyamine oxidases) whereas the Cu-AOs oxidize diamines and polyamines as well as histamine. These enzymes lack particularly strict substrate specificity (and often inhibitor specificity also) and, in the case of acetylated amines, altered specificity patterns are observed.

Brief outline on the role of copper AOs in polyamine metabolism

From a physiological point of view and with respect to the metabolism of polyamines, the Cu-AOs should most probably be considered as regulatory enzymes (Mondovi' et al. 1989). In this regard, they exert an inhibitory action on cell proliferation (and on macromolecule biosyntheses in general) which is related to AO catalysis by way of either the metabolic effects of the reaction products, aldehydes and hydroperoxide, or depletion of the intracellular polyamine pool. Evidence is available to support the hypothesis of a regulatory role of Cu-AOs: first, the metabolic functions of the metabolites involved in AO catalysis have to be considered. In addition, the functions of these enzymes appear to be non-essential (as shown by the slight effect of prolonged in vivo administration of inhibitors on the overall condition of the animals), which contrasts with the widespread occurrence of AOs among all living organisms.

As an example of the antiproliferative action of Cu-AOs, a recent report on the large-bowel tumor promotion after mucosal diamine oxidase inhibition in rats (Kusche et al. 1989) may be mentioned.

Structure of the copper amine oxidases

Practically all the Cu-AOs are structured in a very similar manner. The active enzyme molecule consists of a dimer composed of two apparently identical subunits. The presence of disulfide bridges linking the monomers has been reported for some enzymes; other authors did not observe any covalent binding.

Cu-AOs are glycoproteins containing approximately 7%–14% carbohydrate. The molecular masses of Cu-AOs range over 130–250 kDa, the average value for the most commonly investigated enzymes from beef and pig being about 180 kDa. Association-dissociation phenomena were observed with some enzymes depending on the enzyme concentration and environmental characteristics: as an example, the molecular mass of the pig kidney enzyme determined by analytical ultracentrifugation has 130 kDa which is approximately intermediate between those of the monomer (90 kDa) and the dimer (180 kDa).

Further information on the structure of the Cu-AOs was recently provided by differential scanning calorimetry (DSC) performed on the bovine serum AO (Giartosio et al. 1988). This enzyme consists of four distinct domains of different size: two large domains and two small ones, paired in two sets. The four domains show local melting maxima centered at 57°, 67°, 69° and 79° C. The melting temperatures of the larger domains are very similar (67° and 69° C), whereas the two smaller ones greatly differ in thermal stability (57° and 79° C). Results obtained by sequentially heating the enzyme up to the temperature at which each peak exhibits a local maximum indicate that each peak represents a separate and apparently independent transition. Activity determinations carried out after each heating cycle show that the integrity of domain III is related to enzyme catalysis, while such a relation is not found for domains II and IV (Agostinelli et al. 1989a). After heating at 67° C under controlled conditions, a form of the enzyme is obtained which retains nearly 85% of the total activity, although the thermal transition at 57° C is reduced to 50% and the one at 67° C is completely abolished (Agostinelli et al. 1989b). Heating at higher temperature gradually impairs the enzymatic activity: a good agreement was observed between percentage activity retained and the area of the peak at 69° C. The thermal transition at 79° C is unmodified under conditions which almost completely abolish the enzymatic activity. The data suggest that enzymatic activity is connected with the two domains which unfold at higher temperature and should contain Cu and PQQ.

In the context of the thermal properties of bovine serum AO, a peculiar effect of temperature on the enzyme activity was observed (Befani et al. 1989a): bovine serum AO exhibits a non-strictly Arrhenius type of dependence of the reaction rate upon the temperature, probably as a result of some conformational modifications at 38° and 42° C. It is interesting to note a plateau between 38° and 42° C and an increase at higher temperature values, but with a dependence slightly different to that at lower temperatures, suggesting the existence of three different possible conformations of the enzyme molecule.

A role for disulfide bridges related to conformational changes of the pig kidney amine oxidase was recently described (Shah and Ali 1989). This enzyme seems to have two distinct populations of disulfides distinguishable by means of pH-dependent modification. Three disulfides having functional significance in catalytic activity are accessible to reducing agents at pH 7.2; the other three, accessible at pH 8, seem to have a structural role, being involved in conformational changes.

An amine oxidase, probably a Cu-dependent one, was recently cloned in *Hansenula polymorpha* and its amino acid sequence determined. The gene contains an open reading frame of 692 amino acids with a molecular mass of 77435 Da, close to the apparent molecular mass of the purified enzyme determined by SDS-polyacrylamide gel electrophoresis. The derived amino acid sequence was confirmed by sequencing an internal proteolytic fragment of the purified enzyme (Bruinenberg et al. 1989).

Heterogeneity of the pig plasma AO (Falk et al. 1983) is probably due to variable carbohydrate content. Recently, isoelectric forms were also found in the bovine plasma AO (Befani et al. 1989b): an isoelectric form with pI 4.7 appears almost heterogeneous, but AO activity was present in all the isoforms detected. Differences in glycosidic composition or association-dissociation equilibria were suggested.

Properties of the active site of amine oxidase

Cu-AOs belong to the non-blue Cu-Containing proteins. Their EPR parameters are similar to those of small Cu complexes, characteristic of the so-called type-2 copper. The large hyperfine splitting constant indicates a non-covalent binding of the metal ion with tetragonal coordination geometry (Mondovi' et al. 1967). The two Cu atoms of the enzyme are in the cupric state and are not reduced even in the presence of an excess of substrate added in anaerobic conditions. A direct binding between substrate and copper was ruled out, but subtle changes of the EPR spectrum were observed with different substrates, indicating that the environment of Cu is sensitive to the presence of substrate and to the type of substrate added.

Regarding the nature of Cu ligands, EPR spectra indicate the involvement of nitrogen atoms coordinating the metal ion (Mondovi' et al. 1967). Spin-echo experiments indicate the presence of two distinct populations of Cu-coordinated imidazole nitrogens which appear to be in magnetically non-equivalent environments (McCracken et al. 1987) and equally distributed among the two Cu sites (Mondovi' et al. 1987).

Two water molecules, one in axial and one in equatorial position, should be coordinated to Cu. The equatorially bound water molecule appears to be displaced by cyanide or azide and may be involved in the catalytic mechanism.

The role of Cu on the catalytic mechanism of amine oxidation by this class of enzymes is puzzling. It is also not known how many organic prosthetic groups (i.e. PQQ or a closely related compound) are present in the dimeric molecule. The presence of Cu is essential for catalytic activity since this is completely abolished on removal of one or both Cu ions (Morpurgo et al. 1987).

A transient reduction of Cu during amine oxidation cannot be ruled out, although a direct demonstration has not so far been obtained. In fact, indirect evidence was obtained for the production of superoxide anion radicals by the pig kidney diamine oxidase (Rotilio et al. 1970): in this case the binding of oxygen or its reduced intermediate (i.e. superoxide) should be taken in account. In addition, copper reduction was observed by EPR spectroscopy in cyanide-treated amine oxidases: in this case, cyanide should stabilize Cu in the reduced form Cu(I), which in turn would shift the equilibrium towards the one-electron-reduced form of PQQ (Dooley et al. 1987). Actually, an organic free radical was detected in some plant AOs (Finazzi-Agro' et al. 1984).

A possible role of Cu in O_2 activation was suggested by reoxidation of dithionite-reduced AO (Mondovi' et al. 1985). In this context, it should be recalled that a minimal scheme in agreement with a partial reduction of Cu was hypothesized (Mondovi' et al. 1971) but the intermediate Cu(I) species, which should appear during catalysis, cannot be observed because the equilibrium

$$[\mathrm{Cu(II)} * \mathrm{E}^{--}] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [\mathrm{Cu(I)} * \mathrm{E}^{-}]$$

where E represents the enzyme with bound PQQ, is considerably shifted to the left, i.e. $k_2 \gg k_1$. In any case, it should be recalled that a direct observation of the reduction of Cu during catalysis has never been observed. Details and discussion on the role of Cu are presented in the following paper.

The second puzzle, on the presence of one or two organic cofactors, is so far unanswered but some recent experiments provide new information: the purified bovine plasma AO treated with phenylhydrazine gives only one carbonyl-reactive group; this was also formed with enzyme preparations having a specific activity similar to those obtained by Klinman et al. (1988). These discrepancies can be explained by hypothesizing that CuAOs have two reactive carbonyl groups, one of them reacting fast with carbonyl reagents, and the other one only very slowly. Collison et al. (1989) suggest the presence of two PQQ molecules bound/protein molecule in the pig plasma enzyme, but only the more reactive of these PQQ moieties is required for activity.

Recently, in our laboratory we observed that one mole of the bovine plasma AO reacts with two moles of different hydrazine derivatives, suggesting that CuAOs contain two carbonyl groups/molecule (Morpurgo et al., unpublished results). So far we do not have any direct evidence for the presence of two PQQ molecules/enzyme dimer.

Additional experiments are necessary to resolve these problems but the AO dimer appears to contain only one active site. The presence of only one PQQ molecule/dimer was recently confirmed in lentil seedlings AO by reacting the enzyme with antibodies directed against PQQ (Citro et al. 1989).

Last, there is some important evidence about the relationship between Cu and PQQ. A direct binding of Cu to PQQ is not excluded by X-ray absorption and ENDOR experiments (Scott and Dooley 1985), whereas fluorescent studies rule out this possibility (Lamkin et al. 1988). In any case, the possibility of Cu residing in the neighborhood of the carbonyl group of PQQ appears to be ruled out (Mondovi' et al. 1987).

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Note added in proof
On the organic cofactor of copper-AOs

Very recently, Janes et al. (1990) reported the isolation of a cofactor-containing peptide from bovine serum AO. Contrary to expectations, analysis of the proteolytic fragment indicated the presence of 6-hydroxydopa (TOPA) as the cofactor of this Cu-AO instead of PQQ. This new finding, however, does not imply mechanistic properties different from those presented in this paper for Cu-AOs.

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