Kinetic features of ascorbic acid oxidase after partial deglycation

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By means of specific exoglycosidases, sugars have been removed under non-denaturing conditions from ascorbic acid oxidase (AAO), different deglycation schedules being followed. Our results indicate that deglycation clearly affects the kinetic features of AAO, leading to an increase of 'affinity' and 'catalytic ability' of the enzymic forms so generated. A better exposure of the catalytic-site residues could be supposed to occur upon treatment with exoglycosidases. This is supported by the three-dimensional X-ray structure of zucchini (*Cucurbita pepo medullosa*; courgette) AAO.

INTRODUCTION

Ascorbic acid oxidase (AAO) (EC 1.10.3.3) belongs with caeruloplasmin and laccases to the group of 'blue oxidases' [1]. It is a plant copper-containing glycoprotein that catalyses the reaction:

AAO from *Cucurbita pepo medullosa* (zucchini, courgette) is a dimer with a molecular mass of 140 kDa, containing eight copper atoms per molecule. Its high-resolution X-ray structure has been solved very recently [2]. A complete amino acid sequence is available for cucumber (*Cucurbita sativus*) AAO [3] and a partial sequence (about 70%) for zucchini AAO (A. Rossi & R. Petruzzelli, personal communication). The amino acid sequence of cucumber AAO contains three putative sugar-attachment sites. The loci of these attachment sites are covered by the partial amino sequence of zucchini AAO and for one putative attachment site the asparagine residue is replaced by aspartic acid, resulting in only two putative sugar-attachment sites for zucchini AAO. Both identical subunits of zucchini AAO have a globular shape with dimensions of 4.9 nm × 5.3 nm × 6.3 nm and are built up of three domains arranged sequentially on the polypeptide chain and tightly associated in space. The folding of all three domains is of a similar β -barrel type (see Figs. 1a and 1b). It is distinctly related to the blue copper proteins plastocyanin and azurin. Each subunit has four copper atoms bound as mononuclear and trinuclear species. The mononuclear copper is located in the third domain and is supposed to be the binding site of the reducing substrate. The trinuclear copper site is between the first and the third domain and is supposed to be the binding site for O₂. As recently reported [4], the

primary structure of the N-linked carbohydrate chain has been established to be:

with two carbohydrate moieties per subunit.

Taking into account this oligosaccharide chain structure, we have carried out deglycations by enzymic means in order to investigate the influence of the removal of carbohydrate residues on the kinetic features of the deglycated AAO forms.

As expected, electrophoretic patterns obtained under four different sets of conditions do not indicate any difference between the native enzyme and the various deglycated forms. Interestingly enough, kinetic features changed, the deglycation giving rise to an increase of the catalytic efficiency of AAO. The extent of this increase depended upon both the exoglycosidase used and the deglycation schedule applied.

From our results one could argue that catalytic-site residues might be better exposed after carbohydrate removal.

MATERIALS AND METHODS

Coupling procedure

β -Xyl-ase (1 unit), a mixture of α -Man-ase (5 units) and β -Man-ase (5 units) and β -GlcNAc-ase (10 units) were separately coupled to 350 mg of CNBr-activated Sepharose 4B [5].

Abbreviations used: AAO, ascorbic acid oxidase; β -Xyl-ase, β -xylosidase; α -Man-ase, α -mannosidase; β -Man-ase, β -mannosidase; β -GlcNAc-ase, β -N-acetylglucosaminidase; PMSF, phenylmethanesulphonyl fluoride; PAGE, polyacrylamide-gel electrophoresis.

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Fig. 1. Stereo C_{α} -plot of a subunit of zucchini AAO as obtained from the X-ray structure [2]

Included are the four catalytic copper atoms, the ligands to the copper atoms and the asparagine side chains A327 and A442 of the putative sugar-attachment sites. There is a cleft near His-A514, which is the binding site of the reducing substrate. (a) View from the side on to the cleft. The channel for D_2 , leading to the trinuclear copper site, which is the binding site for the O_2 , is at the bottom of the subunit. (b) View from the top on to the cleft (the label A in the Figure refers to subunit A).

Deglycation

The following buffer solutions were used to wash extensively the exoglycosidase-containing columns before loading the samples: 15 bed volumes of 50 mM-sodium acetate, pH 5.0, for the β -Xyl-ase-containing column, 15 bed volumes of 50 mM-potassium phosphate, pH 7.5, for the α - and β -Man-ase-containing column, and 15 bed volumes of 50 mM-potassium phosphate, pH 7.0, for the column that contained β -GlcNAc-ase. PMSF was added to all the buffers at a final concentration of 1 mM, since in preliminary studies we found that AAO processed with β -GlcNAc-ase and subjected to SDS/PAGE analysis gave two bands in the absence of PMSF, whereas in the presence of this proteinase inhibitor, just the expected band could be detected.

AAO purified to homogeneity as described by Avigliano *et al.* [6] was loaded on to each immobilized-exoglycosidase column and incubated for 48 h at room temperature. Elution with 10 bed volumes of washing buffers then followed. The eluted samples, concentrated to a suitable volume, were loaded on to the next column.

An aliquot was taken after each passage for protein and glucide quantification, electrophoresis and kinetic analyses. The following deglycation schedules were carried out in accordance with the composition and sequence of the carbohydrate side chain reported above:

In these schedules 'D' indicates the 'deglycated form' of the enzyme.

Glucide assay

In order to quantify the amount of sugar removed by the action of exoglycosidases, we used a colorimetric assay based on the absorbance at 415 nm of samples containing up to 20 μ g of neutral carbohydrates mixed with L-cysteine hydrochloride in 86 % (v/v) H_2SO_4 (700 mg/l) [7]. Each assay was repeated at least twice and the amount of sugar still present in the AAO after deglycation was evaluated by means of a calibration curve drawn with pure AAO; the difference between the sugar content of untreated AAO (3 %, w/w) and that in the protein after exoglycosidase action allowed us to calculate the amount of sugar removed.

Kinetic analysis

The kinetic parameters of the native enzyme and its deglycated forms were calculated by the Lineweaver-Burk method, recording at 25 °C the decrease of A_{265} related to the ascorbate oxidation ($\epsilon_{265} = 15 \text{ mm}^{-1} \cdot \text{cm}^{-1}$). Assays were performed at least twice in 0.2 M-potassium phosphate buffer, pH 6.0, containing 10 mM-EDTA.

RESULTS AND DISCUSSION

The function of the glycan moiety in glycoproteins has attracted, and still attracts, the interest of many workers [8–10]. Carbohydrate side chains of some glycoproteins may play important roles in the maintenance of protein conformation and solubility [8], proteolytic processing [8] and stabilization against proteolysis [11], activity [10,11], cellular targeting and externalization [14–16] and embryonic development [8, 9].

AAO is a glycoprotein present only in plants and its physiological function is still poorly understood. For a better understanding of the role of the glycan moiety in respect of the kinetics of AAO, different forms of deglycated AAO were obtained and assayed.

Table 1 shows that: (a) up to 50 % (w/w) of sugars can be removed by the sequential use of the three exoglycosidases [bearing in mind that the whole carbohydrate content of the purified AAO amounts to 3 % (w/w) [4]]; (b) the more types of exoglycosidases are used the more sugars are removed, but this depends on the deglycation schedule too. It is noteworthy that removal of xylose makes the attack on the oligosaccharide chain by α - and β -Man-ases easier. The low extent of removal

Table 1. Amounts of sugars removed by different deglycation pathways

Yield refers to protein recovery, estimated by absorbance at 280 nm ($\epsilon_{280} = 240 \text{ mm}^{-1} \cdot \text{cm}^{-1}$). Numbers in parentheses are S.D. values.

Enzyme	Sugars removed (%)	Yield (%)
D.1	23 (0.5)	92 (3)
D.1a	42 (0.7)	91 (3)
D.1b	47 (0.8)	79 (2)
D.2	11 (0.2)	81 (2)
D.2a	20 (0.5)	86 (2)
D.3	9 (0.2)	82 (2)

Table 2. Kinetic parameters calculated by the double-reciprocal method for the native enzyme and its various deglycated forms

The amount of enzyme was determined as described in Table 1. All double-reciprocal plots had a correlation coefficient of more than 99 %. Numbers in parentheses are S.D. values. K_{cat} and K_{cat}/K_m have been calculated from the mean values of V_{max} and K_m respectively.

Enzyme	K_m (μM)	V_{max} ($\mu\text{mol} \cdot \text{min}^{-1}$ mg of enzyme $^{-1}$)	K_{cat} (s^{-1})	K_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)
AAO	262 (22)	3148 (35)	7.6×10^3	29×10^6
D.1	236 (15)	3044 (30)	7.3×10^3	31×10^6
D.1a	159 (11)	5261 (46)	12.7×10^3	80×10^6
D.1b	169 (9)	8482 (58)	20.5×10^3	121×10^6
D.2	103 (6)	4297 (34)	10.4×10^3	101×10^6
D.2a	66 (3)	3483 (30)	8.4×10^3	127×10^6
D.3	127 (8)	4828 (33)	11.6×10^3	91×10^6

of sugars obtained with the use of β -GlcNAc-ase alone upon native AAO is probably due to the presence of contamination in the batch.

Furthermore, from the results in Table 1 one can argue that β -GlcNAc-ase acts to only a small extent, probably because GlcNAc residues are less exposed when the protein is in the native form. Electrophoretic patterns obtained with the various deglycated forms of AAO and obtained under different conditions (i, in the presence of SDS alone; ii, in the presence of β -mercaptoethanol alone; iii, in the presence of both SDS and β -mercaptoethanol; iv, under non-denaturing, non-reducing conditions), do not reveal any particular differences when compared with the native enzyme (results not shown). This is in good agreement with the neutral nature, small size and low amount of the AAO oligosaccharide chains [4]. When assayed for catalytic properties, the different deglycated forms (except for the D.1 form) showed a significant increase in 'affinity' and 'catalytic ability' with respect to the untreated enzyme (Table 2).

In line with the hypothesis of Sairam *et al.* [17], sugar removal might lead to a better exposure of the catalytic sites. This is strongly supported by the localization of one of the putative sugar-attachment sites, Asn-327 and Asn-442, in the three-dimensional structure of zucchini AAO, namely Asn-442. Asn-327 (see Figs. 1a and 1b) is far away from the binding sites of the reducing substrate (cleft near His-514) and the molecular oxygen (channel leading to the trinuclear copper site at the bottom of Fig. 1a). Therefore removal of the sugar moiety attached to Asn-327 should have no influence on the catalytic properties of the enzyme. A different situation is found in the case of Asn-442. It is at the entrance of the binding site for the reducing substrate and will probably decrease the accessibility of this binding site for the reducing substrate. After removal of this sugar moiety, the binding site is better accessible, increasing the catalytic activity of AAO, as observed in the kinetic measurements.

Our findings support the hypothesis that glycan moieties may play a regulatory role on the catalytic properties of enzymic glycoproteins.

Although evidence arose from our experiments *in vitro* that loss of sugars from the glycoprotein improves the

catalytic efficiency, we cannot state that a 'more efficient' enzyme is more suitable for the plant survival.

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