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Co(II)-Regulated Substrate Specificity of Cytosolic α-Mannosidase[1]

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Cytosolic neutral α-mannosidase is a putative catabolic enzyme that produces cytosolic free oligomannosides. Activation of the enzyme by Co(II) treatment has been reported using pyridylamino derivatives of $Man_5GlcNAc$ and $Man_5GlcNAc_2$, and *p*-nitrophenyl α-mannoside as substrates, with the Co(II)-treated enzyme releasing four α-mannose residues from $Man_9GlcNAc$ to give Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc as an end product. When $Man_9GlcNAc$, which is considered to be the actual substrate in the cytosol, was used as a substrate, we found that even before treatment with Co(II) the enzyme was able to cleave a single Manα1-2 residue from $Man_9GlcNAc$ to give Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc as the end product. The K_m value of the Co(II)-treated enzyme for $Man_9GlcNAc$ was found to be 37 μM, which is one-twelfth that of the non-treated enzyme, while the values were V_{max} values were almost the same, indicating that the affinity of the substrate is higher with Co(II). These results indicate that Co(II) regulates the substrate specificity of the enzyme.

Key words: cytosol, α-mannosidase, Co(II), free oligosaccharides.

The first event in *N*-glycosylation is the *en bloc* transfer of $Glc_3Man_9GlcNAc_2$ from the lipid precursor to the asparagine residue of a nascent polypeptide chain, which occurs on the inner surface of the endoplasmic reticulum (ER) (*1*). The newly synthesized glycoproteins are then subjected to various modifications to become mature forms in the lumina of the ER and Golgi apparatus, giving rise to the notion that the processing enzymes for glycans are distributed in the lumina of these organella. However, several lines of evidence indicate the existence of cytosolic oligosaccharides that appear to be derived from *N*-glycans in the cytosol (*2–5*). In earlier work, activity to trim α-mannosides was detected in the cytosol (*6*), and the enzyme, cytosolic α-mannosidase, was partially purified (*7*). More recently, we purified cytosolic α-mannosidase from Japanese quail oviduct (*8*) and found that the enzyme treated with Co(II) hydrolyzed oligomannosides with one GlcNAc residue at the reducing end; the hydrolysis of M5A and M9A with two GlcNAc residues occurred at much reduced rates, while M9A′ was hydrolyzed to M5B′, suggesting that the enzyme is responsible for the hydrolysis of oligomannosides with one GlcNAc residue in the cytosol (*9*). Haeuw *et al.* reported that a rat liver cytosolic extract hydrolyzed M9A′ to M5B′ in the presence of Co(II) and proposed a hydrolysis sequence (*10*). These specific characteristics of the Co(II)-treated enzyme (*i.e.*, hydrolysis of oligomannose with one GlcNAc residue, activation by Co(II), and hydrolysis of 4 specific mannose residues from M9A′) are common among cytosolic α-manosidases from Japanese quail oviduct, bovine liver and hen oviduct (*8, 9, 11, 12*).

We previously purified cytosolic α-mannosidase from hen oviduct (*11*) and found that the enzyme, comprising four subunits, is gradually activated through incubation with Co(II). We also showed that the enzyme not treated with Co(II) contains two cobalt cations per molecule and incorporates two additional cobalt cations upon incubation with Co(II) to become the activated form (*11*). All the cobalt cations are removed by incubation with EDTA, and the activity is lost after incubation with EDTA for 8 h. Although intracellular concentrations of Co(II) are submicromolar, the enzyme is activated most efficiently at 1 mM Co(II). However, even under these conditions of excess Co(II), the activation of the enzyme occurs gradually. M9A′ is considered to be the native substrate for cytosolic α-mannosidase as the majority of free oligosaccharides detected in the cytosol have structures in the series from M9A′ to Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc, the end product of M9A′ with purified cytosolic α-mannosidase (*2–5*).

The substrates used in past investigations of Co(II) activation were M5A-PA, M5A′-PA, and *p*-nitrophenyl α-mannoside (*8, 9, 11, 12*). As the precise substrate specificity relevant to Co(II) activation has never been analyzed using M9A′ as a substrate, we re-analyzed the Co(II) activation of cytosolic α-mannosidase using M9A′.

MATERIALS AND METHODS

Materials—*p*-Nitrophenyl α-mannoside and rice α-glucosidase were purchased from Sigma (St. Louis, MO), and endo-β-*N*-acetylglucosaminidase H was supplied by Seikagaku (Tokyo). M9A-PA was prepared from G1M9A-PA ob-

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Abbreviations: PA, pyridylamino. The structures and abbreviations of the sugar chains are listed in Table I.

tained from hen egg yolk IgY by digestion with α-glucosidase. M9A′, M8A′, and M8C′ were prepared by endo-β-*N*-acetylglucosaminidase H digestion of M9A-PA, M8A-PA, and M8C-PA, respectively. The preparations of M8A-PA, M8C-PA, and M5B′ have been described previously (*11, 13*). M3B-PA was prepared according to the reported method (*14*). A Shodex Asahipak NH2P-50 column (6.0 × 100 mm) was purchased from Showa Denko (Tokyo), and a Cosmosil 5C18-P column (1.5 × 250 mm) from Nacalai Tesque (Kyoto).

Cytosolic α-mannosidase was purified from hen oviduct according to the procedure described previously, employing sequential ammonium sulfate precipitation, DEAE-Sephacel chromatography, octyl-Sepharose chromatography, Sephacryl S-300 gel filtration, mannan-Sepharose affinity chromatography, hydroxyapatite chromatography, and Mono Q chromatography (*11*). The purity of part of the end pool was tested by SDS-PAGE according to Laemmli (*15*). The purified material gave a single band (data not shown).

HPLC—PA-sugar chains were separated by size-fractionation HPLC on a Shodex Asahipak NH2P-50 column at a flow rate of 1.0 ml/min at 25°C using two eluents, A and B; Eluent A was 0.3% (v/v) acetic acid in a 200:800 (v/v) mixture of acetonitrile:water adjusted to pH 7.0 with 12.5% ammonia water, and Eluent B was 0.3% (v/v) acetic acid in a 800:200 (v/v) mixture of acetonitrile:water adjusted to pH 7.0 with 12.5% ammonia water. The column was equilibrated with Eluent B. After injection of a sample, the proportion of Eluent B was linearly decreased from 100 to 50% in 60 min. The excitation and emission wavelengths were 310 and 380 nm, respectively. Isomeric structures were identified by reversed-phase HPLC on a Cosmosil 5C18-P column at a flow rate of 150 μl/min at 25°C with two eluents, C and D; Eluent C was 100 mM ammonium acetate buffer, pH 6.0, and Eluent D was 100 mM ammonium acetate buffer, pH 6.0, containing 1.0% (v/v) of 1-butanol. The column was equilibrated with 5% Eluent D. After injection of a sample, the proportion of Eluent D was linearly increased from 5 to 52% in 51 min, and then from 52 to 100% in an additional 12 min. The excitation and emission wavelengths were 310 and 380 nm, respectively. An L-6200 pump (Hitachi, Tokyo) equipped with a Hitachi F-1050 fluorescence detector was used for size-fractionation HPLC, and a Nanospace SI-1 liquid chromatograph (Shiseido, Tokyo) for the reversed-phase HPLC.

TABLE I. **Sugar chain structures and abbreviations.**

Abbreviation	Structure
M9A′	Manα1−2Manα1−6 Manα1−2Manα1−3 Manα1 Manα1−2Manα1−2Manα1 6 3 Manβ1−4GlcNAc
M8A′	Manα1−2Manα1−6 Manα1−3 Manα1 Manα1−2Manα1−2Manα1 6 3 Manβ1−4GlcNAc
M8C′	Manα1−6 Manα1−2Manα1−3 Manα1 Manα1−2Manα1−2Manα1 6 3 Manβ1−4GlcNAc
M5A′	Manα1−6 Manα1−3 Manα1 Manα1 6 3 Manβ1−4GlcNAc
M5B′	Manα1 6 3 Manβ1−4GlcNAc Manα1−2Manα1−2Manα1
G1M9A	Manα1−2Manα1−6 Manα1−2Manα1−3 Manα1 Glcα1−3Manα1−2Manα1−2Manα1 6 3 Manβ1−4GlcNAcβ1−4GlcNAc
M9A	Manα1−2Manα1−6 Manα1−2Manα1−3 Manα1 Manα1−2Manα1−2Manα1 6 3 Manβ1−4GlcNAcβ1−4GlcNAc
M8A	Manα1−2Manα1−6 Manα1−3 Manα1 Manα1−2Manα1−2Manα1 6 3 Manβ1−4GlcNAcβ1−4GlcNAc
M8C	Manα1−6 Manα1−2Manα1−3 Manα1 Manα1−2Manα1−2Manα1 6 3 Manβ1−4GlcNAcβ1−4GlcNAc
M5A	Manα1−6 Manα1−3 Manα1 Manα1 6 3 Manβ1−4GlcNAcβ1−4GlcNAc
M3B	Manα1 6 3 Manβ1−4GlcNAcβ1−4GlcNAc Manα1

Enzyme Assay—The initial rate of hydrolysis was measured using M9A′ as a substrate. Mixtures of 10 μl enzyme solution (1.7 mU) and 20 μl 200 mM HEPES buffer, pH 7.0, with [referred to as Co(II)-treated] and without [Co(II)-non-treated] 1.5 mM $CoCl_2$ were pre-incubated at 37°C for 45 min. To these solutions was added 20 μl of 25 μM M9A′ and the mixtures were further incubated at 37°C. The enzymatic reaction was stopped by heating at 100°C for 3 min. After the digests were pyridylaminated as described previously (*16*), PA-sugar chains were first separated by size-fractionation HPLC on a Shodex Asahipak NH2P-50 column. Each fraction collected was then subjected to reversed-phase HPLC. The structures were analyzed by comparing their elution times with those of standard PA-sugar chains. M3B-PA was used as an internal standard since cytosolic α-mannosidase is unable to hydrolyze this oligosaccharide (*11*). The peak area of M9A′-PA relative to that of M3B-PA was calculated, and the decrease in the relative area was taken as the hydrolysis rate. One unit of α-mannosidase activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol from *p*-nitrophenyl α-D-mannoside per min at 37°C.

EDTA Treatment—A mixture of 10 μl enzyme solution (1.7 mU) and 20 μl 200 mM HEPES buffer, pH 7.0, with or without 25 mM EDTA was pre-incubated at 37°C for 8 h. Then, 20 μl of 25 μM M9A′ was added to the preincubated enzyme solution, and the mixture was further incubated at 37°C for 1 h. The reaction was stopped by heating at 100°C for 3 min.

RESULTS

Alteration of Substrate Specificity of Cytosolic α-Mannosidase by Co(II)— M9A′, considered to be the natural substrate of the cytosolic enzyme, was incubated with Co(II)-treated or Co(II)-non-treated enzyme. The products were pyridylaminated, and the derivatives were analyzed by HPLC (Fig. 1). M9A′ was hydrolyzed in the exo-manner by the Co(II)-treated enzyme and converted to its end product,

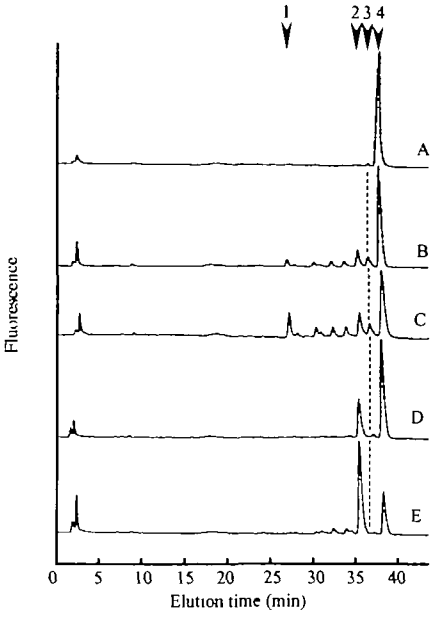

Fig. 1. **Effect of Co(II) on the substrate specificity of α-mannosidase.** Digests of M9A′ hydrolyzed by cytosolic α-mannosidase were pyridylaminated prior to analysis by size-fractionation HPLC. A, digestion with α-mannosidase for 0 min; B, with Co(II)-treated α-mannosidase for 10 min; C, with Co(II)-treated α-mannosidase for 30 min; D, with Co(II)-non-treated α-mannosidase for 10 min; E, with Co(II)-non-teated α-mannosidase for 30 min. Arrowheads indicate the following elution positions: 1, M5B′-PA; 2, M8C′-PA; 3, M8A′-PA; 4, M9A′-PA.

TABLE II. **Kinetics parameters of the enzyme.** The substrate used was M9A′.

Enzyme	K_m (μM)	V_{max} (pmol/min/μg)
Co(II)-non-treated	460	250
Co(II)-treated	37	98

M5B′, through M8A′ or M8C′ as intermediates as described previously (*11*) (Fig. 1, B and C). Upon 3-h incubation all the substrate was converted to M5B′ (data not shown). On the other hand, the Co(II)-non-treated enzyme hydrolyzed a mannose residue from M9A′, converting it to a single isomer, M8C′. In contrast to the Co(II)-treated enzyme, the Co(II)-non-treated enzyme did not digest this product further (Fig. 1, D and E).

Kinetic Analysis—The kinetic parameters of the two states of the enzyme were determined from Lineweaver-Burk plots. The apparent K_m value of the Co(II)-non-treated enzyme was 460 μM and that of the Co(II)-treated enzyme was 37 μM when $Man_9GlcNAc$ was used as a substrate (Table II).

Elimination of Co(II) from Cytosolic α-Mannosidase—Since purified α-mannosidase already carries two Co(II) ions, the enzyme treated with Co(II) carries four (11). Hence, we examined whether the two Co(II) ions in the non-treated enzyme have a role in the activity that produces M8C′ from M9A′. The enzyme is known to lose all its cobalt cations when treated with EDTA (*9*). Two forms of cytosolic α-mannosidase [with two Co(II) ions or without Co(II) using EDTA] were prepared as described in "MATERIALS AND METHODS." The substrate M9A′ was then hydrolyzed with each form of the enzyme to produce M8C′ as the end product (Fig. 2). The results indicate that the activity of the Co(II)-non-treated enzyme to produce M8C′ from M9A′ is independent of Co(II).

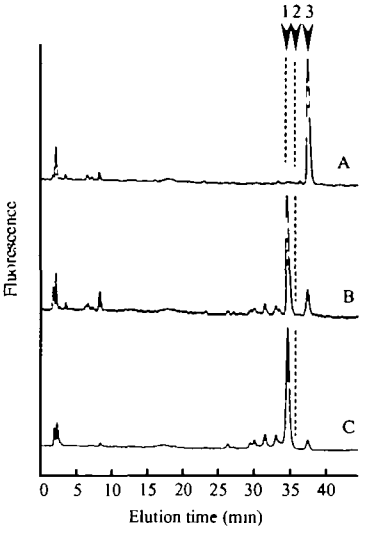

Fig. 2. **Effect of EDTA on the substrate specificity of α-mannosidase.** Digests of M9A′ hydrolyzed by cytosolic α-mannosidase were pyridylaminated prior to analysis by size-fractionation HPLC. A, digestion with Co(II)-non-treated α-mannosidase for 0 min; B, with the EDTA-treated α-mannosidase for 60 min; C, with the Co(II)-non-treated α-mannosidase for 60 min. Arrowheads indicate the following elution positions: 1, M8C′-PA; 2, M8A′-PA; 3, M9A′-PA.

DISCUSSION

We previously detected cytosolic α-mannosidase by measuring the hydrolysis rate, which increased 11- to 26-fold when the enzyme was treated with Co(II) using M5A-PA, M5A′-PA, and *p*-nitrophenyl α-mannoside as substrates (*8, 9, 11*). It was found that Co(II)-treated cytosolic α-mannosidase prefers M5A′ and M9A′ as substrates to M5A and M9A, and that this exo-type enzyme recognizes not only the non-reducing ends of substrates, but also the reducing ends. Thus we proposed that *in vivo* the enzyme hydrolyzes high-mannose type oligosaccharides with a single GlcNAc residue at the reducing end, rather than those with two GlcNAc residues, in a Co(II)-dependent manner (*9*). Furthermore, structural analyses of cytosolic free oligosaccharides have shown them to be of the high-mannose type with a single GlcNAc residue, with M5B′ the most abundant high-mannose sugar chains among them (*3–5*). The fact that cytosolic α-mannosidase hydrolyzes M9A′ to M5B′ suggests that the former is the substrate in the cytosol. We studied the behavior of cytosolic α-mannosidase toward M9A′ under different states of cobalt ion capture. While the Co(II)-treated enzyme hydrolyzed four Manα1-2 residues from M9A′, the non-treated enzyme trimmed a specific Manα1-2 residue to M8C′. The apparent K_m value of the Co(II)-treated enzyme is 37 μM, which is one-twelfth that of the non-activated enzyme, while the V_{max} value of the Co(II)-non-treated enzyme is only 2.6 times greater than that of the treated mannosidase. These results indicate that Co(II)-treatment alters the tertiary structure of the

enzyme so that substrate specificity is largely changed, and also the ES complex is stabilized, which agrees with our previous data that Co(II)-treatment requires incubation for at least 45 min, and that the enzyme gradually loses activity over several hours following treatment with EDTA (*11*). The effects of Co(II) on the cytosolic α-mannosidase are to alter the substrate specificity rather than the hydrolyzing efficiency. Weng and Spiro (*17*) reported that the initial hydrolysis rate of M9A′ with cytosolic α-mannosidase (rat liver) increases fivefold in the presence of Co(II), and proposed that each stage of hydrolysis is accelerated. They did not, however, describe the isomeric structures of $Man_{7\text{-}8}$GlcNAc and Man_5GlcNAc. The fact that the Co(II)-treated enzyme hydrolyzes four mannose residues from M9A′ in a spatial location–specific rather than a linkage-specific manner suggests that the active site of the enzyme is highly restricted to the substrate and that the cytosolic α-mannosidase structure is changed during Co(II)-treatment under an extremely precise mechanism.

The presence of M5B′ as the main cytosolic free oligosaccharide, together with M8A′ and M8C′, has been reported (*5*), and these oligosaccharides are the products obtained by digestion of M9A′ with purified Co(II)-treated cytosolic α-mannosidase (*11*) and not obtained by digestion with the Co(II)-non-treated enzyme. Additionally, our previous data showed that the purified enzyme has two cobalt cations (*11*) and that among various metal ions tested Co(II) activates the enzyme the most (*8, 11*). This suggests that the functional cytosolic α-mannosidase is the Co(II)-treated form in the cytosol. It can be speculated that some mechanism in the eukaryotic cytosol enables cytosolic α-mannosidase with Co(II) to be activated more efficiently, since the intracellular Co(II) concentration is quite low (*18*).

ER α-mannosidase II has catalytic and immunological properties similar to those of cytosolic α-mannosidase, suggesting that the ER α-mannosidase II is a post-translationally translocated and modified product of cytosolic α-mannosidase (*17*). In the present study, we show that the hydrolysis of a specific Manα residue by the Co(II)-non-treated enzyme is similar to that generated by ER α-mannosidase II (*17, 19*), although the activity of cytosolic α-mannosidase depends on Co(II) and the enzyme produces M8A′ in addition to M8C′ from M9A′ in the presence of Co(II). However, the relationship between ER α-mannosidase II and cytosolic α-mannosidase remains obscure because the structures of the two enzymes have yet to be elucidated.

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