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Note

On the configuration of the transfer product formed by the action of α-D-mannosidase on *p*-nitrophenyl α-D-mannopyranoside in the presence of methanol

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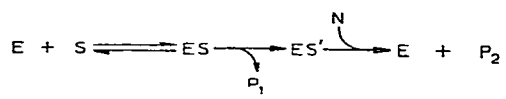
In a recent communication[1] on the mode of action of α-D-mannosidase from *Medicago sativa* L., the following two-step mechanism was proposed.



After the formation of the Michaelis–Menten complex ES, the aglycon group (P_1) is split off with simultaneous formation of an enzyme–D-mannosyl complex ES′. In the second step, the complex ES′ reacts with water or an added nucleophile (*e.g.*, an alcohol), yielding D-mannose or an alkyl D-mannoside, respectively. The mechanism is thus similar to that proposed, for example, for β-D-galactosidase[2] and α-D-galactosidase[3]. We now report on the identification and anomeric configuration of the transfer product formed when methanol was added to the enzymic reaction mixture containing *p*-nitrophenyl α-D-mannopyranoside.

EXPERIMENTAL

The purification of α-D-mannosidase from *Medicago sativa* L., the synthesis of *p*-nitrophenyl α-D-mannopyranoside (PNPM), as well as the definition of the unit of enzymic activity have been described[1,4]. Methyl tetra-*O*-acetyl-β-D-mannopyranoside was a gift from Dr. G. Ekbörg (Stockholm).

T.l.c. was performed on Silica Gel G, using acetic acid–water–ethyl acetate (1:1:3) (*A*) for mannosides, and ethyl acetate–benzene (3:7) (*B*) for acetates. Detection was effected with 5% sulphuric acid in ethanol (10 min at 120°).

Evaluation of reaction products was performed by t.l.c. (solvent *A*) of aliquots (40 μl) of a reaction mixture (30 ml) containing *p*-nitrophenyl α-D-mannopyranoside (0.18 mmol), methanol (90 mmol), and α-D-mannosidase (74 munits) in 50mM McIlvane buffer (pH 4). On the basis of a comparison with authentic samples, D-mannose and methyl D-mannopyranoside were identified. After complete hydrolysis of the substrate (3 h), the reaction mixture was boiled with charcoal, filtered, and concentrated *in vacuo*, and the residue was dried (P_2O_5).

Acetylation of the residue was performed for 30 min at 4° with pyridine (5 ml) and acetic anhydride (5 ml). The acetates were extracted with chloroform in the usual way.

Analysis by g.l.c. was carried out after trifluoroacetylation[5] of the transfer product. A Varian 1200 Aerograph was used, and *myo*-inositol was added as an internal standard. Since free mannose complicated the analysis by g.l.c., it was removed from the reaction mixture by preparative t.l.c. (solvent *A*).

Spectra of free mannosides were recorded with a Varian HA-100 spectrometer at room temperature for solutions in D_2O with hexamethyldisiloxane (HMDS) as the external standard. δ Values are expressed as p.p.m.

RESULTS AND DISCUSSION

Analysis of the products formed by the action of α-D-mannosidase on *p*-nitrophenyl α-D-mannopyranoside in the presence of methanol revealed methyl α-D-mannopyranoside. No methyl α-D-mannopyranoside was formed when the enzyme was omitted from the reaction mixture, or when *p*-nitrophenyl α-D-mannopyranoside was replaced by D-mannose. T.l.c. of the acetylated products revealed penta-*O*-acetyl-α-D-mannopyranose (R_F 0.41) and methyl tetra-*O*-acetyl-α-D-mannopyranoside (R_F 0.38); methyl tetra-*O*-acetyl-β-D-mannopyranoside (R_F 0.30) could not be detected. When D-mannose was trifluoroacetylated and analyzed by g.l.c., three peaks were detected, the first of which had the same retention time as trifluoroacetylated methyl α-D-mannopyranoside. Consequently, mannose had to be removed before g.l.c. When the D-mannose-free reaction products were trifluoroacetylated, and analyzed by g.l.c., only one peak, having the same retention time as the methyl α-D-mannopyranoside derivative, was detected.

The resonances for H-2 in the p.m.r. spectra {D_2O, internal sodium 2,2,3,3-tetrakis[tri(methyl-d_3)silyl]propionate (TSP)} of α- and β-D-mannopyranose and methyl α-D-mannopyranoside occurred at δ 3.93. Using HMDS as external standard, the resonance for H-2 of the transfer product occurred at δ 4.39. Thus, conversion of the TSP into the HMDS scale requires the addition of 0.46 p.p.m. The H-1 resonance for methyl α-D-mannopyranoside, found at δ 4.77 with TSP, was thus expected at δ 5.23 with HMDS; the value obtained for the transfer product was δ 5.22. There was also a peak at δ 3.83 (HMDS) for OMe. There was no resonance having the chemical shift [δ 5.16 (HMDS)] predicted[6] for H-1 of methyl β-D-mannopyranoside.

Determination of *p*-nitrophenol[1] and D-mannose[7] in the transfer experiments

gave a D-mannose/methyl D-mannoside ratio of 1:3, which indicates methanol to be 7 times as reactive as water towards the D-mannosyl–enzyme complex.

The fact that the transfer product (methyl α-D-mannopyranoside) has the same configuration as the starting material indicates that the enzymic reaction does not proceed through a single S_N2 displacement reaction (nucleophilic attack on C-1 of the substrate), as inversion of configuration would then have been expected. A simple addition of methanol to an unshielded oxonium–carbonium ion (in the D-mannosyl–enzyme complex ES′) is also ruled out, as both anomers of methyl D-mannopyranoside should then be formed. If, however, the oxonium–carbonium ion is stabilized (ion-pair) by a nucleophilic enzyme group (*e.g.*, carboxylate) attacking the rear side of C-1 in the *C1*(D) conformation, the incoming alcohol can approach only from the same side as the leaving aglycon group. The retention of configuration can also be explained by assuming a double-displacement mechanism. A first inversion of configuration occurs when the nucleophilic group of the enzyme attacks C-1, in the *C1*(D) conformation, from the rear-side and replaces the aglycon group, yielding a mannosyl–enzyme complex in which D-mannose is covalently bound to the enzyme in, for example, the $^{1,4}B$(D) conformation. The second inversion occurs when the alcohol attacks C-1 of the enzyme-bound glycon group, yielding methyl α-D-mannopyranoside. The equilibrium mechanism proposed by Sinnott[8] for β-D-galactosidase is also possible.

The retention of configuration at C-1 of the D-mannopyranose ring during the enzyme-catalyzed transmannosylation thus clearly indicates that the reaction proceeds by a pathway involving the formation of a D-mannosyl–enzyme complex. However, further experimental work is necessary to decide whether the D-mannose residue is stabilized by ion-pair formation, or by covalent binding.

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