Maltokinase (ATP:maltose 1-phosphotransferase) from *Actinoplanes* sp.: demonstration of enzyme activity and characterization of the reaction product

Anne Drepper, Reinhard Peitzmann, Hermann Pape*

Institut für Mikrobiologie, Westfälische Wilhelms-Universität, Corrensstr. 3, D-48149 Münster, Germany

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Abstract Cell free extract of the acarbose producer *Actinoplanes* sp. catalyzes ATP-dependent phosphorylation of maltose. This was shown by two different assays. The product was purified and its structure determined to be α-maltose-1-phosphate by chemical analysis and NMR spectroscopy.

Key words: Maltose; Kinase; Maltose-1-phosphate; NMR; *Actinoplanes*

1. Introduction

Disaccharides are used as carbon and energy sources by bacteria usually after cleavage to monosaccharides, followed by an ATP-dependent phosphorylation of a monosaccharide and its introduction into the central catabolic pathways. With the exception of some disaccharides like lactose, cellobiose, maltose or trehalose which are transported and phosphorylated via the phosphoenol pyruvate-dependent phosphotransferase system (PTS) in certain facultatively anaerobic bacteria [1], the phosphorylation of disaccharides appears to be an unknown principle of their metabolization. The ubiquitous disaccharide maltose (4-*O*-α-D-glucopyranosyl-D-glucose) is taken up by bacteria usually via non-PTS disaccharide transport systems and cleaved intracellularly either by α-glucosidases into two mol of glucose or by phosphorylases into glucose and glucose-1-phosphate. The actinomycete *Actinoplanes* sp. uses maltose (among other sugars) as a carbon and energy source for growth. In addition, maltose is needed in the biosynthesis of the secondary metabolite acarbose produced by this organism [2]. We want to report on an ATP dependent formation of a maltose phosphate catalyzed by an enzyme activity present in the cytoplasm of this organism. To our knowledge, such a reaction has not been described so far.

2. Material and methods

2.1. Fermentation

The acarbose producer *Actinoplanes* sp. SN 223/29 was fermented submersed in a two stage complex medium. Preculture (72 h): defatted soy flour (Henselwerk, Magstadt, Germany) 2%; glycerol 2%; $CaCO_3$ 0.2%; tap water. pH was adjusted to 7.2 before sterilisation. Main culture (120 h): maltzin (Diamalt, Munich) 7.5%; STV yeast extract (Ohly, Hamburg) 0.7%; NZ amine (Sigma) 0.3%; $CaCO_3$ 0.3%; K_2HPO_4 0.3%; tap water (Drepper, A. and Pape, H., submitted).

2.2. Determination of enzyme activity

Crude extract was obtained by sonication and centrifugation as previously described (Drepper, A. and Pape, H., submitted) and used in enzyme assays. Maltose kinase activity can be measured by one of the following assays. (a) Photometric assay (Drepper, A. and Pape, H., submitted): activity was measured photometrically coupled to the oxidation of NADH with pyruvate kinase and lactate dehydrogenase in 65 mM Tris/HCl buffer (pH 7.8), 3.3 mM $MgCl_2$, 1.1 mM ATP, 1.6 mM PEP (potassium salt) and 0.5 mM NADH. The reaction was started with maltose (7.5 mM). Activity was measured as a decrease in absorption at 340 nm. (b) Radioassay: desalted crude extract (gel filtration, Sephadex G 25) (25 μl) was incubated for 60 min at 50°C in 0.2 M Tris/HCl pH 7.6, 15 mM ATP pH 8.0, 60 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 10 mM [U-^{14}C]maltose (containing 81 000 dpm) and demineralised water in a total volume of 62.5 μl. After addition of the same volume of ethanol, proteins were removed by centrifugation. The supernatant (110 μl) was shaken with approximately 400 μl of Amberlite IRA-93/H_2O (1:1). Gel was washed three times (H_2O). Anionic substances were eluted with (0.5 M) NH_4HCO_3 and radioactivity measured by scintillation counting.

Protein concentration was determined according to Bradford [3].

2.3. Enzyme purification

100 ml crude extract were desalted by gel filtration (Sephadex G-25). After acid precipitation with 50 ml 0.5 M HAc/NaAc pH 4.75 the supernatant was adjusted to pH 6.0, concentrated by ultrafiltration and desalted again (Drepper, A. and Pape, H., submitted). The sample was applied to a DEAE-Sephacel column (2×9.9 cm) and equilibrated with 10 mM Tris/HCl pH 7.5, 1 mM β-mercaptoethanol. After washing with 90 ml of the same buffer proteins were eluted with a gradient from 0 to 0.75 M NaCl. Maltose kinase activity eluted at 0.5 M NaCl.

2.4. Thin-layer chromatography

Silica gel plates (Merck, Darmstadt) were used with solvent A (ethanol/H_2O 7/3 (v/v)) [4] and solvent B (*n*-butanol/isopropanol/H_2O 3/5/2 (v/v/v)) [5]. Sugars and sugar phosphates were detected by molybdate reagent [6] or ceric sulfate reagent: phosphomolybdic acid×H_2O (25 g), cer(IV)-sulfate×4 H_2O (10 g), H_2SO_4 conc. (60 ml), demineralised water (940 ml). R_f values were compared with those of maltose, glucose and synthetic maltose-1-phosphate (Sigma, St. Louis, MO, USA).

2.5. Purification of enzymatically formed maltose phosphate

The reaction mixture for enzymatic synthesis of maltose phosphate contained 20 ml of desalted crude extract, 0.2 M Tris/HCl pH 7.6, 50 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 10 mM maltose, 15 mM ATP pH 7.7 and demineralised water in a total volume of 50 ml. After incubation for 1 h at 50°C and adding 50 ml ethanol, precipitated protein was removed by centrifugation (20 min, 30 000×*g*). The supernatant was freeze dried. The product was purified by chromatography on QAE-Sephadex A-25 (2.5×16.5 cm) using a gradient of NH_4HCO_3 (0.002–0.5 M). Maltose phosphate eluted prior to adenylates and was located by TLC. For NMR spectroscopy metal ions were removed by treatment with Dowex Chelating Resin A-1 (0.8×10 cm). The resulting aqueous solution was evaporated to dryness.

2.6. Chemical analysis

Up to 1.4 mg maltose phosphate was incubated for 30 min at 37°C with alkaline phosphatase (1 U) (Boehringer, Mannheim), 0.15 M diethanolamine pH 9.0, 1 mM $MgCl_2$ and double distilled water in a total volume of 50 μl. The resulting products phosphate and maltose were quantified by phosphate [7] and colorimetric maltose assay [8].

*Corresponding author. Fax: (49) (251) 83 83 88.

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Table 1
Natural abundance ^{13}C-NMR signals (H-decoupled) of maltose and maltose-phosphate obtained by ATP-dependent phosphorylation

C-atom	Chemical shift values (ppm) of maltose (first column) and maltose-phosphate (second column)								
	C-1–C-6			C-1′–C-6′		C-1–C-6		C-1′–C-6′	
	(α-configuration)		Δ δ[a]	(α-configuration)		(β-configuration)		(β-configuration)	
1	92.18	95.12 (d)[b]	+2.9	99.86	99.91	96.06	–	–	–
2	71.59	71.37 (d)[b]	−0.3	72.06	72.02	74.31	–	71.96	–
3	73.51	73.30	−0.2	73.17	73.15	76.49	–	–	–
4	77.32	76.87	−0.5	69.65	69.70	77.11	–	–	–
5	70.27	71.54	+1.3	72.97	72.93	74.86	–	–	–
6	60.82	60.75	−0.1	60.82	60.82	61.07	–	–	–

[a]Δ δ = change of chemical shift values; in agreement with data for α-L-fucose, α-D-galactose, α-D-glucose and α-D-mannose and their C-1-phosphorylated compounds [10].
[b]Doublet: C-1 (J = 5.0 Hz); C-2 (J = 8.0 Hz).

2.7. NMR spectroscopy

Maltose phosphate (approx. 100 mg) was dissolved in D_2O (1 ml). To localise the phosphorylated OH group H-decoupled ^{13}C (natural abundance) and ^{1}H-NMR spectroscopy of maltose and maltose phosphate in D_2O were registered with a WM 300 Bruker NMR Spectrometer using 3-(trimethylsilyl)propanesulfonic acid as external standard.

3. Results and discussion

Cell free extract of *Actinoplanes* sp. SN 223/29 contains an enzyme which catalyzes ATP-dependent phosphorylation of maltose. This enzymatic activity can be measured by two independent assay methods.

In a standard coupled photometric assay for ATP-dependent kinases crude extract leads to a decrease of NADH when maltose is added as substrate. Activity (typically between 0.3 and 1.0 nkat/mg protein) is strongly dependent on ATP and PEP. Since desalted crude extract of *Actinoplanes* sp. contains both an α-glucosidase/maltase activity (0.5–0.7 nkat/mg) and hexo/glucokinase (1.0–2.0 nkat/mg), the combined activities could theoretically mimic a 'maltokinase' activity. This possibility was excluded by using maltase inhibiting assay conditions (with 50 mM Tris buffer no maltase activity was detectable) and by separation of hexokinase and 'maltokinase' activities. Crude extract contains hexo/glucokinase and 'maltokinase' (0.31 nkat/mg) activities, enriched 'maltokinase' (18.4 nkat/mg) is free of hexo/glucokinase activity after acid precipitation and ion exchange chromatography.

Additionally crude extract was tested for 'maltokinase' activity by a radioassay. After incubation of desalted crude extract with [U-^{14}C]maltose and ATP the mixture was treated with Amberlite IRA-93 and the anionic fraction was analyzed by thin-layer chromatography (solvent A). Results show ATP-dependent formation of an anionic product formed in 83.1% radiochemical yield. Because adenylation is excluded by photometric assay, this indicates a phosphorylation reaction catalyzed by a kinase.

The purified anionic reaction product (solvent A: $R_f = 0.40$; B: 0.12) was treated with alkaline phosphatase. TLC analyses of cleavage product indicated the formation of maltose (A: 0.61; B: 0.40) and not glucose (A: 0.60; B: 0.50). Separation of glucose from maltose with solvent B enabled us to identify the modified product after treatment with alkaline phosphatase as maltose. This again excluded formation of glucose phosphate during the incubation of maltose with crude extract.

Quantitative determination of maltose and inorganic phosphate after alkaline phosphatase treatment showed a stoichiometric relation of maltose:orthophosphate = 1:0.96, indicating a monophosphorylated disaccharide.

The localization of the phosphorylated OH in maltose-phosphate was achieved by both ^{13}C-NMR spectroscopy (Table 1) and ^{1}H-NMR (Table 2).

The ^{13}C-NMR spectrum of maltose was similar to the one described previously by Heyraud et al. [9]. The α- and β-configuration gave different signals for the reducing ring and for one atom in the second ring. In the spectrum of maltose-phosphate no signals of β-configuration were detectable indicating the stereospecific modification at C-1 of maltose. The spectrum showed a significant downfield shift of the signal for C-1_α (+2.9 ppm) and for C-5_α (+1.3). Doublets at C-1 (J = 5.0 Hz) and C-2 (J = 8.0 Hz) can be explained by ^{31}P-^{13}C coupling. Comparison of these data with ^{13}C-NMR spectra of other α-glycopyranosyl-1-phosphates and their non-phosphorylated compounds (α-L-fucose, α-D-galactose, α-D-glucose and α-D-mannose) [10] showed similar shifts and coupling constants.

Data indicate a phosphorylation at C-1 of α-configured maltose.

Data of ^{1}H-NMR spectroscopy confirmed this interpretation (Table 2). Signals were partly identified by comparison with previously described spectra from de Bruyn et al. [11] and Waard and Vliegenthart [12]. Signals for H-1_β and H-2_β are not present in the spectrum of maltose phosphate. The signal of H-1_α shows a downfield shift of +0.268 ppm compared to the signal of H-1_α in the spectrum of maltose. A similar downfield shift (+0.264 ppm) was reported for the pair glucose-1-phosphate/glucose [10,13]. A coupling between H-1_α and phosphorus is clearly visible in the spectrum of

Table 2
^{1}H-NMR signals of maltose and maltose-phosphate obtained by ATP-dependent phosphorylation

H-atom	Chemical shift values and coupling constants			
	maltose		maltose-phosphate	
	ppm	Hz	ppm	Hz
H-1_α	5.135		5.393	
	5.148	3.7	5.404	
			5.415	
			5.427	3.2
H-1_β	4.552		–	
	4.579	8.0	–	
H-2_β	3.160		–	
	3.187		–	
	3.218	8.0	–	

maltose phosphate ($^3J_{P-H-(1)} = 6.7$ Hz). A coupling constant of 7.0 Hz ($^3J_{P-H-(1)}$) was reported for glucose-1-phosphate.

These data are in agreement with the conclusion drawn from the ^{13}C-NMR analysis described above. Together with our results from enzymatic and chemical examinations, the spectral data allow the identification of the reaction product as α-maltose-1-phosphate. The enzymatic activity catalyzing the formation of sugar phosphate from maltose and ATP therefore is a maltokinase (ATP:maltose 1-phosphotransferase).

To our knowledge, an enzyme of this specificity has not been described previously. It is probably the first known example of an enzyme catalyzing specifically the ATP-dependent phosphorylation of a disaccharide. At present the physiological function of this enzyme is still unknown.

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