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STERIC COURSE OF THE HYDROLYSIS OF α,α-TREHALOSE AND α-D-GLUCOSYL FLUORIDE CATALYZED BY PIG KIDNEY TREHALASE*

MASATOSHI NAKANO†, CURTIS F. BREWER, TAKAFUMI KASUMI‡, AND EDWARD J. HEHRE**
Departments of Microbiology and Immunology, and of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461 (U.S.A.)

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ABSTRACT

We are unable to confirm the report of Labat *et al.*[3] that pig kidney trehalase hydrolyzes α,α-trehalose to form solely α-D-glucose. Highly purified trehalase from pig renal cortex was found, in reactions monitored by ^{1}H-n.m.r. spectra, to hydrolyze α,α-trehalose with the formation of both α- and β-D-glucose. That the β anomer constitutes the enzymically mobilized glucosyl residue is indicated by the further finding that β-D-glucose is the product formed on hydrolysis of α-D-glucosyl fluoride by the enzyme. Present results show the stereochemical behavior of pig kidney trehalase in hydrolyzing α,α-trehalose to be indistinguishable from that reported by ourselves and others for trehalase preparations from a range of biological sources including rabbit renal cortex.

INTRODUCTION

Studies in the past decade have brought a new understanding of the catalytic capabilities of trehalase. This widely distributed enzyme was long regarded as being strictly specific for hydrolyzing α,α-trehalose or close analogs, as lacking transglycosylative ability, and as acting with retention of configuration[1]. Newer findings contradict each of these formerly accepted general characterizations, but the earlier reported finding of Labat *et al.*[2,3] that pig kidney trehalase hydrolyzes α,α-trehalose to form only α-D-glucose — though now known to be exceptional — raises a fundamental question. Is the pig enzyme a unique type of trehalase, distinguished by the steric course of its hydrolytic reactions, much as papaya lysozyme is a unique type of lysozyme? Trehalase from the flesh fly (*Sarcophaga barbata*) was shown by Clifford to hydrolyze trehalose to form equimolar α- and β-D-glucose[4,5]. Rabbit

*Supported by Grant GM 25478 (to E.J.H.) from the National Institutes of Health.
†Present address, Institute for Medical Science of Aging, Aichi Medical College, Nagakute-cho, Aichi 480-11, Japan.
‡Research Associate in Microbiology and Immunology, Albert Einstein College of Medicine, on leave from the National Food Research Institute, Tsukuba, Ibaraki 305, Japan.
**Author for correspondence.

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kidney and *Candida tropicals* trehalase likewise were shown to produce both α- and β-D-glucose from trehalose[6]; also to hydrolyze α-D-glucosyl fluoride with inversion of configuration, and to catalyze glucosyl transfer from β-D-glucosyl fluoride (in the presence of α-D-glucose) to form α,α-trehalose[6]. The enzyme from *Trichoderma reesei* also was found to hydrolyze trehalose with inversion[7] and to transfer the glucosyl residue of β-D-glucosyl fluoride to α-D-xylose, forming α-D-glucopyranosyl α-D-xylopyranoside[8]. Trehalase from the May bug, *Melolontha vulgaris*, likewise, was clearly shown to hydrolyze trehalose to form equimolar α- and β-D-glucose[9].

The discrepancy between the reported steric course of trehalose hydrolysis by pig kidney trehalase[2,3] and that of the reaction catalyzed by the enzyme from other sources, especially rabbit kidney[6], prompted us to examine purified pig kidney trehalase with respect to the manner in which it catalyzes trehalose and α-D-glucosyl fluoride hydrolysis, using ^{1}H-n.m.r. spectroscopy to judge product configuration.

RESULTS

A pig kidney trehalase (A_2 form) preparation, purified to apparent homogeneity by a recently described multi-step procedure[10], was found to catalyze the hydrolysis of 30mM α-D-glucopyranosyl fluoride at an initial rate of 19.1 μmol $min^{-1}mg^{-1}$ at 30°, approximately 2.3 times faster than its hydrolysis of 30mM α,α-trehalose under identical reaction conditions. To examine the anomeric configura-

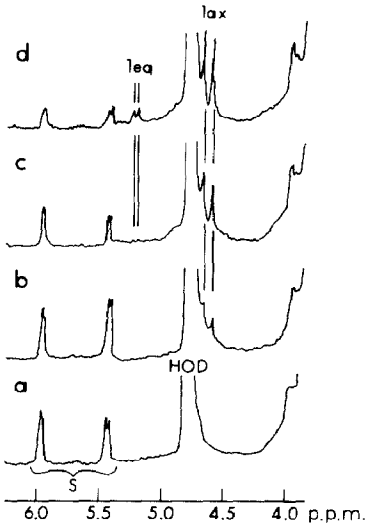

Fig. 1. Fourier-transform ^{1}H-n.m.r. spectra at 100 MHz. Digest of 40mM α-D-glucosyl fluoride and 66 μg mL^{-1} pig kidney trehalase (A_2 form) in 0.1M acetate-d_4–D_2O buffer, pD 5.6, incubated at 23° *a*, 3 min; *b*, 20 min; *c*, 50 min; *d*, 150 min. *S*, H-1 resonances of the substrate; *leq*, H-1 resonance of α-D-glucose; *lax*, H-1 resonance of β-D-glucose. Chemical shifts (p.p.m.) refer to sodium 4,4-dimethyl-4-silapentanesulfonate.

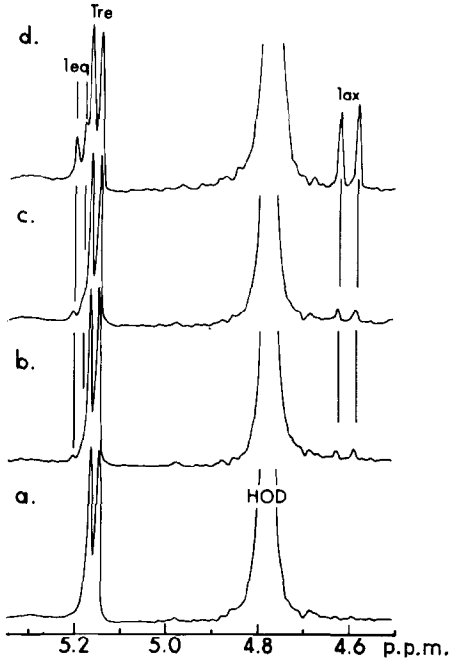

Fig. 2. Fourier-transform ^{1}H-n.m.r. spectra at 200 MHz. Digest of 30mM α,α-trehalose·2 H_2O and 300 μg mL^{-1} pig kidney trehalase (A_2 form, Con A stage) in 0.1M acetate-d_4–D_2O buffer of pD 6.0 incubated at 23° *a*, 3 min; *b*, 12 min; *c*, 24 min; *d*, 130 min. *Tre*, H-1, H-1′ resonance of substrate; *leq*, H-1 resonance of α-D-glucose; *lax*, H-1 resonance of β-D-glucose.

tion of the D-glucose produced from α-D-glucosyl fluoride, the enzyme was exhaustively dialyzed at 8° against 0.1M acetate-d_4–D_2O buffer of pD 5.6) to exchange its ^{1}H atoms for ^{2}H atoms. The dialyzed enzyme (40 μg in 0.6 mL of pD 5.6 buffer) was added to 24 μmol of pure α-D-glucosyl fluoride freshly dried from solution in methanol-d_1. The digest was immediately transferred to a 5-mm n.m.r. tube and the reaction at 23° monitored by Fourier-transform ^{1}H-n.m.r. spectra recorded at 100 MHz. As shown in Fig. 1, the enzymically produced glucose was exclusively the β-anomer. Spectrum *a* (3 min incubation) shows the H-1 resonance of the substrate, a pair of doublets centered at 5.69 ppm with $J_{1,2}$ 3 Hz and $J_{1,F}$ 53.1 Hz*. Spectra *b–d*, recorded at 20, 50, and 150 min at 23°, show the progressive decline of the substrate H-1 signals and increasing presence of a new doublet at 4.62 p.p.m. ($J_{1,2}$ 8 Hz) assignable to the axial anomeric proton of β-D-glucose (detected at 8 min; spectrum not illustrated). A second doublet, at 5.24 p.p.m. ($J_{1,2}$ 3.8 Hz), due to the equatorial anomeric proton of α-D-glucose, is just detectable

*In spectra recorded at 100 MHz, the downfield (5.95 p.p.m.) doublet shows a smaller coupling constant than the doublet at 5.43 p.p.m.; however, we have shown[11] that, at 220 MHz, this anomalous *J* splitting pattern disappears. The different transition levels in the downfield doublet of H-1 appear to be the result of a frequency-dependent kinetic-exchange process[11].

after 50 min incubation (spectrum *c*) when the *1ax* resonance of β-D-glucose is prominent. The *1eq* resonance is still small, relative to the *1ax* signal, after 150 min (spectrum *d*). The low level of α-D-glucose found at this late stage is attributable to non-enzymic anomerization of the primary reaction product, and attests to the absence of D-glucose 1-epimerase in the trehalase preparation.

The steric course of the hydrolysis of α,α-trehalose by pig kidney trehalase was examined with a second highly purified preparation of the enzyme of A_2 form which had been exhaustively dialyzed at 8° against 0.1M acetate-d_4–D_2O buffer of pD 6.0. A solution containing 18 μmol of α,α-trehalose·2 H_2O in 1 mL deuterium oxide (99.8 atom% D) was evaporated to dryness under vacuum at 45° immediately before use. The trehalose was treated with 190 μg of the dialyzed enzyme in 0.6 mL of pD 6.0 buffer, and the reaction course at 23° monitored by Fourier-transform ^{1}H-n.m.r. spectra recorded at 200 MHz.

As illustrated in Fig. 2, the hydrolysis of trehalose by the pig kidney trehalose yielded both α- and β-D-glucose as products. Spectrum *a* (3 min) shows a large doublet (*Tre*) at 5.17 p.p.m., $J_{1,2}$ 3.8 Hz, representing the H-1 and H′-1 resonance of the α-D-glucopyranosyl residues of the substrate[12,13]. Spectra *b* (12 min) and *c* (24 min) at 23° show both the *1ax* resonance doublet of β-D-glucose at 4.61 p.p.m. ($J_{1,2}$ 8.1 Hz) and the *1eq* doublet of α-D-glucose at 5.22 p.p.m. ($J_{1,2}$ 4.0 Hz); the upper field resonance of the latter doublet is occluded by the downfield slope of the *Tre* resonance. After 130 min (spectrum *d*), the *1ax* resonance of β-D-glucose has become substantially larger than the *1eq* resonance of α-D-glucose, consistent with the occurrence of some nonenzymic anomerization of the α-product during incubation.

DISCUSSION

Present findings do not substantiate the conclusion of Labat *et al.*[2,3] that pig kidney trehalase hydrolyzes, α,α-trehalose to form exclusively α-D-glucose. Instead, they show that the pig kidney enzyme behaves like the trehalase from rabbit kidney cortex[6], flesh fly[4,5], May bug[9], *Candida tropicalis*[6], and *Trichoderma reesei*[7] — all of which hydrolyze trehalose with configurational inversion*. Our results show further that pig kidney trehalase, like that from other sources (rabbit kidney, rat intestinal mucosa, May bug, and *T. reesei*)[6,8], hydrolyzes α-D-glucosyl fluoride faster than α,α-trehalose. Finally, as with trehalase from rabbit kidney cortex and *C. tropicalis*[6], the demonstration that the pig kidney enzyme acts on α-D-glucosyl fluoride to form β-D-glucose (Fig. 1) illustrates in an easily understandable way that it is the mobilized (transferred) glucosyl residue which undergoes configurational inversion.

*In a study reported since completion of the present work, the hydrolysis of trehalose by honeybee trehalase was found to proceed with inversion of configuration[14].

It is to be noted that the ^{1}H-n.m.r. spectra recorded by Labat *et al.*[2,3] for the hydrolysis of α,α-trehalose by the pig kidney enzyme show no resonance that unequivocally indicates the presence of either free α- or β-D-glucose in the incubated reaction mixture. The spectra, therefore, never were evidence for the formation of α-D-glucose. Indeed, it would appear likely that the particular enzyme used in the reaction mixture may have been inactive — perhaps denatured during lyophilization from solution in deuterium oxide. The authors state that, under the conditions used, 80% of the trehalose substrate (100 mg mL^{-1}) would be hydrolyzed in 15 min. If so, ~150mM glucose should have been present after 5 min and, even if this were all α-D-glucose, anomerization during the next 10 min of incubation at 40° should have produced sufficient β-D-glucose for its H-1 resonance to be seen in the spectrum recorded at 15 min. In fact, no such signal was present.

We are unable to evaluate the results reported by Labat *et al.* using procedures other than n.m.r. spectroscopy, but note that the glucose oxidase method used to evaluate the configuration of glucose formed on the hydrolysis of trehalose failed to differentiate between a control of β-D-glucose and a mixture of equal parts of α- and β-D-glucose.

Although trehalase preparations of different origins, including pig kidney cortex, have been found to hydrolyze α,α-trehalose and α-D-glucosyl fluoride with inversion of configuration of the mobilized glucosyl residue, it should be noted that trehalase is not limited to acting as an "inverting enzyme". We have recently demonstrated[15] the ability of *T. reesei* trehalase to catalyze the hydration of (*Z*)-3,7-anhydro-1,2-dideoxy-D-*gluco*-oct-2-enitol, K_m 11.7mM, and to create from this prochiral substrate 1,2-dideoxy-D-*gluco*-octulose of the same (β-) configuration as that of the glucose mobilized on hydrolyzing α,α-trehalose. Trehalase is not alone in showing such behavior. More than twenty reactions catalyzed by various glycosylases with prochiral (enolic) substrates have been found, without exception, to yield products of the same anomeric configuration as that of products formed from chiral (glycosidic) substrates by the same enzyme[15–17]. For trehalase and other glycosylases that have been examined, these findings show that the stereochemical outcome of an enzyme's reactions is more strictly conserved than the structure of its substrate(s). That structures as remote from α,α-trehalose as α- and β-D-glucosyl fluoride and (*Z*)-3,7-anhydro-1,2-dideoxy-D-*gluco*-oct-2-enitol are acted upon by trehalase[6,8,15] suggests that natural substrates other than trehalose may well exist. It is unlikely that the function of trehalase in the renal cortex of certain mammals, including man, has to do with hydrolyzing α,α-trehalose.

EXPERIMENTAL

Enzyme. — Two samples of highly purified pig kidney trehalase (A_2 form) were examined. These were prepared by the procedure[10] used to purify and separate the forms of rabbit kidney trehalase. This involves solubilization of the enzyme from the membrane fraction of homogenized renal cortical tissue; succes-

sive fractionation on columns of Sephacryl S-300, DEAE-Sephacel, and phenyl-Sepharose CL-4B; followed by separation of the A_1, A_2, B_1, and B_2 forms of the enzyme on Con-A–Sepharose. One of the pig kidney trehalase-A_2 samples was further subjected to chromatofocusing on PBE-94 (Pharmacia). It was homogeneous on polyacrylamide gel electrophoresis and initially hydrolyzed 30mM α,α-trehalose at the rate of 65.7 μmol D-glucose formed $min^{-1}mg^{-1}$. The second A_2 sample, purified through the Con-A Sepharose step, initially produced 12.8 μmol D-glucose $min^{-1}mg^{-1}$ from 30mM trehalose. Both highly purified samples suffered considerable loss of activity in transit between laboratories; however, their activity for trehalose (16.3 and 3.0 μmol glucose released $min^{-1}mg^{-1}$, respectively) at the time of use proved sufficient for the present study.

Substrates. — Pure crystalline α-D-glucopyranosyl fluoride was prepared and characterized as previously described[11]. Accompanying free fluoride ion was less than 0.1% of total fluorine. Stock solutions of known concentration in dry methanol were kept at $-20°$, and required amounts of the compound were dried under vacuum in small plastic tubes immediately before use. Fluoride ion concentrations were measured with a specific ion electrode and meter (Orion). α,α-Trehalose·2 H_2O was the crystalline (Sigma) product.

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