Biophys. Res. Commun. 51, 620–625.
Holbrook, J. J., & Ingram, V. A. (1973) *Biochem. J. 131*, 729–738.
James, M. N. G., Sielecki, A. R., Brayer, G. D., Delbaere, L. T. J., & Bauer, C.-A. (1980) *J. Mol. Biol. 144*, 43–88.
Koehler, K. A., & Hess, G. P. (1974) *Biochemistry 13*, 5345–5350.
Kolattukudy, P. E. (1980a) *Science (Washington, D.C.) 208*, 990–1000.
Kolattukudy, P. E. (1980b) in *The Comprehensive Biochemistry of Plants* (Stumpf, P. K., Ed.) Vol. 4, p 571, Academic Press, New York.
Kolattukudy, P. E. (1981) *Annu. Rev. Plant Physiol. 32*, 539–567.
Köller, W., Allan, C. R., & Kolattukudy, P. E. (1982) *Physiol. Plant Pathol. 20*, 47–60.
Kraut, J. (1977) *Annu. Rev. Biochem. 46*, 331–358.
Krisch, K. (1971) *Enzymes, 3rd Ed. 5*, 43–69.
Krupka, R. M. (1966) *Biochemistry 5*, 1988–1998.
Kurzer, F., & Douraghi-Zadeh, K. (1967) *Chem. Rev. 67*, 107–152.
Lin, T. S., & Kolattukudy, P. E. (1980) *Physiol. Plant Pathol. 17*, 1–15.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Maiti, I. B., & Kolattukudy, P. E. (1979) *Science (Washington, D.C.) 205*, 507–508.
Meyer, S. E., & Cromartie, T. H. (1980) *Biochemistry 19*, 1874–1881.
Miles, E. W. (1977) *Methods Enzymol. 47*, 431–443.
Narita, K. (1980) in *Frontiers of Biorganic Chemistry and Molecular Biology* (Anachenko, S. N., Ed.) pp 135–143, Pergamon Press, Oxford.
Paterson, A. K., & Knowles, J. R. (1972) *Eur. J. Biochem. 31*, 510–517.
Purdy, R. E., & Kolattukudy, P. E. (1973) *Arch. Biochem. Biophys. 159*, 61–69.
Purdy, R. E., & Kolattukudy, P. E. (1975a) *Biochemistry 14*, 2824–2831.
Purdy, R. E., & Kolattukudy, P. E. (1975b) *Biochemistry 14*, 2832–2840.
Rosenberry, T. L. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 103–218.
Schneider, F. (1978) *Angew. Chem., Int. Ed. Engl. 17*, 583–592.
Semeriva, M., & Desnuelle, P. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 48*, 319–370.
Shaykh, M., Soliday, C. L., & Kolattukudy, P. E. (1977) *Plant Physiol. 60*, 170–172.
Sielecki, A. R., Hendrickson, W. A., Broughton, C. G., Delbraere, L. T. J., Brayer, G. D., & James, M. N. G. (1979) *J. Mol. Biol. 134*, 781–804.
Soliday, C. L., & Kolattukudy, P. E. (1976) *Arch. Biochem. Biophys. 176*, 334–343.

Trehalase: Stereocomplementary Hydrolytic and Glucosyl Transfer Reactions with α- and β-D-Glucosyl Fluoride†

Edward J. Hehre,* Teruo Sawai,‡ Curtis F. Brewer, Masatoshi Nakano,§ and Takahisa Kanda⊥

ABSTRACT: A new understanding has been obtained of the catalytic capabilities of trehalase, an enzyme heretofore held to be strictly specific for hydrolyzing α,α-trehalose and devoid of transglycosylative ability. Highly purified rabbit renal cortical trehalase and a partly purified *Candida tropicalis* yeast trehalase were found to utilize both α- and β-D-glucosyl fluoride as substrates. In each case, the reactions were competitively inhibited by α,α-trehalose. Both enzymes catalyzed rapid hydrolysis of α-D-glucosyl fluoride to form β-D-glucose (also, of α,α-trehalose to form equimolar α- and β-D-glucose). In addition, digests of β-D-glucosyl fluoride plus α-D-[^{14}C]-glucopyranose with either trehalase (but not controls of enzyme with α-D-[^{14}C]glucopyranose alone) yielded small amounts of radioactive trehalose (α-D-glucopyranosyl α-D-[^{14}C]glucopyranoside) which does not accumulate since it is rapidly hydrolyzed. Trehalase thus catalyzes two stereocomplementary types of glycosylation reactions: (I) α-D-glucosyl fluoride (or α,α-trehalose) + H_2O → β-D-glucose + HF (or α-D-glucose); (II) β-D-glucosyl fluoride + α-D-glucopyranose → α,α-trehalose + HF. Such behavior shows that the catalytic groups of trehalase, as recently found for other glycosylases, are functionally flexible. The results illustrate the inadequacy of conventional views of carbohydrase specificity and the rigor, as a basic guiding principle, of the concept that glycoside hydrolases and glycosyltransferases form a class of glycosylases effecting glycosyl/proton interchange.

The enzyme trehalase, elaborated by a wide range of living forms, has long been considered strictly specific for catalyzing the hydrolysis of α,α-trehalose and certain close analogues (Bourquelot, 1893; Kalf & Rieder, 1958; Saito, 1960; Dahlqvist, 1960; Courtois et al., 1962; Avigad et al., 1965; Guilloux et al., 1968; Nisizawa & Hashimoto, 1970; Sacktor, 1972; Labat et al., 1973; Defaye et al., 1981). From the susceptibility (or lack thereof) of differently modified analogues, Defaye et al. (1981) recently affirmed that trehalase requires a substrate with the steric bulk of α-D-glycopyranosyl α-D-glucopyranoside. Apart from virtual restriction to utilizing α,α-trehalose, trehalase also has invariably been viewed as a strict hydrolase, devoid of the ability to catalyze glycosyl transfer other than to water (Dahlqvist, 1960; Courtois et al., 1962; Avigad et al., 1965; Guilloux et al., 1968; Nisizawa &

† From the Department of Microbiology and Immunology and the Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461. *Received December 4, 1981.* This study was supported by U.S. Public Health Service Research Grant GM-25478 from the National Institute of General Medical Sciences.
‡ Present address: Department of Biology, Aichi Kyoiku University, Kariya, Aichi 448, Japan.
§ Present address: Department of Pathology, Aichi Medical College, Nagakute, Aichi 480-11, Japan.
⊥ Research Associate in Microbiology and Immunology, Albert Einstein College of Medicine. Permanent address: Faculty of Engineering, Shinshu University, Nagano, Japan.

0006-2960/82/0421-3090$01.25/0

Hashimoto, 1970; Sacktor, 1972; Labat et al., 1972; Defaye et al., 1981). With such long accepted characteristics established by studies based on traditional notions of carbohydrase actions, trehalase offered an unparalleled opportunity to examine a different general concept of carbohydrase capabilities (Hehre et al., 1971, 1973). This latter concept considers glycoside hydrolases and glycosyltransferases as forming an inclusive class of glycosylases potentially able to utilize, as glycosyl donors, compounds having no more than the ability to bind appropriately at an active site and to yield a glycosyl residue on protonation.

In the present investigation, α- and β-D-glucosyl fluorides were used to probe the catalytic capabilities of trehalase preparations from rabbit renal cortex and *Candida tropicalis* yeast. Previous studies of glycosylation reactions proceeding without glycosidic bond cleavage (Hehre et al., 1971, 1973, 1979, 1980; Kitahata et al., 1981, and cited references) have brought new insight into the scope and mechanism of a variety of well-known carbohydrases through the use of glycosyl fluorides, glycals, and other enolic glycosyl donors as substrates. The two anomers of D-glucosyl fluoride were chosen for initial examination with trehalase since studies with such substrates have recently brought a new understanding of the mechanism of several inverting exo-α-glucanases (Hehre et al., 1979; Kitahata et al., 1981). Trehalase appeared to have much in common with these enzymes which also were long held to hydrolyze only certain strictly specified linkages (of glucans and derived oligosaccharides) and to be limited to catalyzing hydrolysis or its reversal. We saw a further parallel to the inverting exo-α-glucanases in the recent finding (Clifford, 1980) that flesh fly trehalase catalyzes the hydrolysis of α,α-trehalose with inversion of configuration of the mobilized glucosyl residue.

Both rabbit kidney and *Candida* yeast trehalase have in fact been found to hydrolyze α-D-glucosyl fluoride to form β-D-glucose, as well as to catalyze glucosyl transfer from β-D-glucosyl fluoride (with α-D-glucose as acceptor) forming α,α-trehalose as a transient (rapidly hydrolyzed) intermediate. On the basis of the stereochemistry of these reactions, a description is presented of the complementary mechanisms effecting the observed catalysis of hydrolysis and glycosyl transfer by trehalase. Finally, the broad potentiality of the glycosylase concept which led to this different view of the catalytic abilities of trehalase than that afforded by the conventional representation of carbohydrase specificity is discussed.

Materials and Methods

Reaction Components. Rabbit renal cortical trehalase was a preparation of the A-1 form purified to near homogeneity according to a recently developed method (Nakano, 1980). Rabbit kidney cortical tissue was homogenized in 10 mM phosphate buffer, pH 7.2, containing 0.9% NaCl, 5 mM EDTA,[1] and 1 mM $MgCl_2$. The homogenate was squeezed through gauze and centrifuged at 500*g* for 10 min. The precipitate was washed twice with the buffer and centrifuged as above. The combined supernatant fluids were centrifuged at 110000*g* for 60 min, and the pelleted membrane fraction was resuspended in buffer. This suspension was incubated with 0.02% papain (activated by dithiothreitol) at 37 °C for 30 min, and then recentrifuged at 110000*g* for 60 min. The papain-digested membranes, suspended in 5 mM phosphate buffer (pH 7.2) containing 0.9% NaCl, 5 mM EDTA, and 1 mM $MgCl_2$, were solubilized by a mixture of 0.5% Triton X-100 and 0.5% sodium deoxycholate (25 °C, 30 min) and pelleted at 110000*g* for 60 min. The pellet was treated again with the detergent mixture and recentrifuged. The combined supernatant fluids (solubilized membrane fraction) were applied to a 2.6 × 90 cm column of Sephacryl S-300 that was equilibrated with 5 mM Tris-HCl buffer (pH 7.2) containing 0.2% Tween 80 and eluted with this buffer. The active trehalase fraction was then applied to a 2.6 × 30 cm column of DEAE-Sephacel which had been equilibrated with the same buffer. Trehalase was eluted with a linear gradient of 0–0.5 M NaCl in the same buffer. The active fraction was then subjected to hydrophobic interaction chromatography by using a 2.6 × 10 cm column of phenyl-Sepharose CL-4B which was equilibrated with 5 mM Tris-HCl, pH 7.2, buffer containing 0.8 M ammonium sulfate. Elution was with a linear gradient of 0.8 M ammonium sulfate without detergent to 0 M ammonium sulfate with 0.2% Tween 80 in 5 mM Tris-HCl buffer, pH 7.2. This step separated two peaks of trehalase activity, A and B. These were further subdivided on a 1.6 × 10 cm column of Con A–Sepharose into A-1, A-2, B-1, and B-2 forms of trehalase, the interrelationships of which are under investigation (M. Nakano, unpublished results). The A-1 fraction used in the present study was homogeneous on 7.5% polyacrylamide gel electrophoresis (Williams & Reisfeld, 1964) except for a minor band. Its specific activity was extremely high, originally 118 μmol min^{-1} (mg of protein)$^{-1}$, or approximately 150 times that of the crude membranes. Although this value was much reduced in transit between laboratories, the preparation retained high activity for trehalose and was free from detectable activity for maltose, isomaltose, and sucrose; it also showed no β-glucosidase activity in tests with methyl β-D-glucoside and salicin.

The yeast trehalase preparation was derived from a large sample of *C. tropicalis* α-glucosidase which had been extensively purified from cell autolysates (Sawai, 1967) yet retained some trehalase activity. When this sample was chromatographed on Sepharose 6B (two developments on a 2 × 115 cm column) with 0.1 M acetate buffer of pH 5.0, a slowly migrating fraction rich in trehalase activity but carrying appreciable α-glucosidase activity was obtained. Pure α-glucosidase, essentially free of hydrolytic activity for α,α-trehalose and showing a single protein band on disc gel electrophoresis, was obtained as a faster migrating fraction. The catalytic characteristics of this α-glucosidase have been described previously (Hehre et al., 1980, and cited references). The fraction with activity for both trehalose and maltose showed two bands, one migrating at the same rate as the α-glucosidase. As efforts to free the trehalase from the accompanying α-glucosidase were unsuccessful, the partly purified preparation was used in the present study; the purified α-glucosidase was examined concurrently to identify the reactions catalyzed by this component. The specific activities of both preparations for various substrates are described under Results.

Highly purified α- and β-D-glucosyl fluorides were prepared and characterized as recently described (Kitahata et al., 1981). Stock solutions of known concentration, in dry methanol, were kept at −20 °C protected from moisture. Just before use, desired amounts of either compound were obtained by drying aliquots of stock solution in a Rotovap vacuum evaporator below 30 °C. α-D-Glucopyranose, $[\alpha]^{22}_D$ +112° (*c* 1, methyl sulfoxide), was made according to the procedure of Hudson & Dale (1917) from chromatographically pure glucose. α-D-[^{14}C]Glucopyranose was made from D-[U-^{14}C]glucose (110

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

μCi/μmol, Calbiochem) diluted with unlabeled glucose to 3.2 μCi/μmol. This sample was purified by paper chromatography and further diluted with chromatographically pure glucose to 0.16 μCi/μmol. The purified [^{14}C]glucose (165 mg) was then dissolved in 0.85 mL of water, momentarily heated to 80 °C, and treated with 0.33 mL of glacial acetic acid (Hudson & Dale, 1917). After 10 days at 25 °C, the α-D-[^{14}C]glucopyranose crystals were separated by centrifugation, washed with several changes of absolute ethanol, and dried in a vacuum oven at 35 °C: yield 85.5 mg; $[\alpha]^{23}_{D}$ +111° (*c* 0.5, methyl sulfoxide). Crystalline α,α-trehalose dihydrate was purchased from Sigma; maltose monohydrate was a purified laboratory preparation; isomaltose was kindly furnished by Dr. Allene Jeanes.

General Methods. Paper chromatography (descending) was carried out with Whatman No. 1 paper and 1-butanol/pyridine/water (6:4:3). Staining was by a silver nitrate dipping method with papers hung in air for 12 min following application of the alkali. Solvent evaporations were performed in a rotary vacuum apparatus at or below 30 °C unless otherwise noted. Total glucose was determined by the phenol–sulfuric acid method; free glucose was determined by the glucose oxidase/chromogen method. Both procedures were standardized concurrently with glucose. Protein was determined (Lowry et al., 1951) against bovine serum albumin. Fluoride ion was measured with a combination fluoride electrode (Orion Model 96-09) and specific ion meter (Model 407A), standardized with sodium fluoride. All determinations were made in the presence of TISAB buffer [1 M sodium acetate buffer, pH 5.2, with 1 M sodium chloride and 0.4% 1,4-cyclohexanebis(dinitrilotetraacetic acid) monohydrate] 45 s after electrode immersion. Radioactivity was measured with a Beckman LS 233 liquid scintillation counter. Test samples (0.10 mL) were shaken with 4.8 mL of Aquasol (New England Nuclear) in plastic minivials. All counts per minute values were corrected for background.

Enzyme Assays. Rates of enzymic utilization of 10 or 40 mM α- or β-D-glucosyl fluoride, in the presence or absence of 25 mM α-D-glucopyranose, were determined in test mixtures (150 μL) comprising equal volumes of 30 or 120 mM D-glucosyl fluoride freshly dissolved in ice-cold 0.05 M acetate buffer of pH 5.6, 75 mM α-D-glucopyranose, also freshly dissolved in the cold buffer (or buffer alone), and appropriately diluted enzyme. Individual mixtures were set up at 2-min intervals and incubated at 30 °C for 14.0 min. Each was then treated with 0.80 mL of TISAB buffer and the fluoride ion concentration immediately determined. Incubated substrate/buffer mixtures without enzyme were included in each experiment, and correction was made for the fluoride concentration found in these controls (generally ~0.2% of the α-D-glucosyl fluoride or ~3.2% of the β anomer).

^{1}H NMR Spectra. Fourier transform ^{1}H NMR spectra were recorded at 100 MHz by using a Jeol PFT 100 spectrometer interfaced with a Nicolet 1080 series computer.

Enzymes Used in Transfer Product Characterization. Purified rabbit kidney trehalase was used in a final concentration of 3 μg/mL. Rat intestinal mucosal trehalase of A-1 form, purified to near homogeneity by the method described above for the rabbit kidney enzyme, assayed 2 μmol min^{-1} (mg of protein)$^{-1}$ and was free from activity for maltose, isomaltose, sucrose, and cellobiose; it was used in a final concentration of 39 μg/mL. Rice α-glucosidase (50 IU/mg, maltose hydrolysis) was purchased from Makor Chemicals Ltd., Jerusalem, Israel, and used at 200 μg/mL. Purified β-glucosidase from sweet almonds, 40 IU/mg (salicin hydrolysis), was from Boehringer-Mannheim Corp.; it was used at 100 μg/mL.

α- and β-D-Glucosyl Fluoride Utilization in the Presence of α,α-Trehalose. Experiments were carried out to determine the effect of trehalose on the rate of release of fluoride ion from 10 or 40 mM α- or β-D-glucosyl fluoride catalyzed by the rabbit kidney and *Candida* trehalase preparations. For each experiment, four or five replicate 1.8-μmol (or 7.2-μmol) samples of α- or β-D-glucosyl fluoride were dried under vacuum at 30 °C from methanolic solution immediately before use. The 1.8-μmol samples of α-D-glucosyl fluoride were treated with 60 μL of freshly prepared 6 mM α-D-glucose[2] in ice-cold 0.05 M acetate buffer (pH 5.6), 60 μL of buffer (or of 1.8–12 mM α,α-trehalose in buffer), and 60 μL of rabbit trehalase (1.2 μg/mL) or *Candida* trehalase (6 μg/mL). The 7.2-μmol α-D-glucosyl fluoride samples were treated similarly except that a lower concentration of the rabbit kidney enzyme (0.48 μg/mL) was used. The β-D-glucosyl fluoride samples were treated with 60 μL of freshly prepared 150 mM α-D-glucopyranose in ice-cold buffer, 60 μL of buffer (or 9–36 mM α,α-trehalose in buffer), and 60 μL of rabbit trehalase (30 μg/mL) or *Candida* trehalase (150 μg/mL). Individual digests in each series were set up at 2-min intervals, incubated at 30 °C for 10.0 min, then treated with 0.77 mL of TISAB buffer, and immediately examined for fluoride ion concentration. Concurrently incubated α- or β-D-glucosyl fluoride/α-D-glucose/buffer controls, included in each experiment, showed fluoride concentrations corresponding to 0.2% of the α-D-glucosyl fluoride (2.7% of the β-D-glucosyl fluoride) present. Correction for the nonenzymatically released fluoride allowed calculation of the specific initial rates of C–F bond cleavage of either substrate in the absence or presence of α,α-trehalose in different concentrations.

Results

The ability of trehalase to use both α- and β-D-glucosyl fluoride as substrates is shown by the following observations made with highly purified rabbit kidney trehalase and a *Candida tropicalis* trehalase plus α-glucosidase preparation (which was examined in comparison with the purified α-glucosidase component). As the first step, the enzymes were assayed for initial rates of fluoride ion release catalyzed with 10 and 40 mM α- and β-D-glucosyl fluoride in the presence and absence of 25 mM α-D-glucose. Rates of α,α-trehalose and maltose hydrolysis were also determined for comparison. As shown in Table I, the purified rabbit trehalase, as well as the *Candida* trehalase plus α-glucosidase preparation, caused rapid cleavage of the C–F glycosylic bond of α-D-glucosyl fluoride. Indeed, the kidney enzyme which was free from α-glucosidase activity utilized 10 mM α-D-glucosyl fluoride several times faster than 10 mM α,α-trehalose. That *Candida* trehalase also rapidly utilizes this compound is not unequivocally shown by the results but is indicated on taking account of the probable contribution to hydrolysis of the α-glucosidase component of the trehalase-rich preparation, i.e., ca. 7.5 of the 14.4 μmol min^{-1} mg^{-1} rate observed with the trehalase-rich preparation.[3] Other findings described below confirm that

[2] Incorporation of α-D-glucose (2 mM final concentration) in the experiments with α-D-glucosyl fluoride provided a reasonably uniform (slightly inhibitory) level of α-D-glucose in all digests of a series, whether or not these contained trehalose which would be partly hydrolyzed to yield 0.03–0.4 mM α-D-glucose under the assay conditions.

[3] Roughly estimated as (rate of maltose hydrolysis with the trehalase-rich preparation/rate with purified α-glucosidase) × rate of hydrolysis of 10 mM α-D-glucosyl fluoride catalyzed by the α-glucosidase, i.e., (2.6/6.7) × 19.3 = 7.5 μmol min^{-1} mg^{-1}.

Table I: Rates of Reactions Catalyzed by Trehalase Preparations with α- and β-D-Glucosyl Fluoride[a]

substrate	concn (mM)	purified rabbit kidney trehalase (μmol min^{-1} mg^{-1})	*Candida tropicalis* trehalase + α-glucosidase (μmol min^{-1} mg^{-1})	*Candida tropicalis* α-glucosidase control (μmol min^{-1} mg^{-1})
α-D-glucosyl fluoride	10	35.6 (3.9)[b]	14.4 (6.6)	19.3 (14.3)
α-D-glucosyl fluoride	40	88.7 (21.0)	15.6 (10.9)	21.5 (18.8)
β-D-glucosyl fluoride	10	0.51 (2.3)	0.10 (0.58)	0.04 (0.02)
β-D-glucosyl fluoride	40	2.15 (5.6)	0.36 (1.21)	0.06 (0.01)
α,α-trehalose	10	14.3	9.2	0.04
maltose	5	<0.01	2.6	6.7

[a] Rates represent μmol of substrate utilized min^{-1} (mg of protein)$^{-1}$, corrected for controls incubated without enzyme. None of the enzymes was active (<0.001 μmol min^{-1} mg^{-1}) in tests with 25 mM methyl β-D-glucoside or salicin. [b] Rates in parentheses are for digests containing 25 mM added α-D-glucopyranose.

α-D-glucosyl fluoride is rapidly hydrolyzed by *Candida* trehalase. In contrast, β-D-glucosyl fluoride in the concentrations tested was utilized only very slowly by either the rabbit or *Candida* trehalase preparation. Moreover, as shown by the figures in parentheses in Table I, the presence of 25 mM α-D-glucose dramatically increased the rates of fluoride release from 10 or 40 mM β-D-glucosyl fluoride by each of the (β-glucosidase-free) trehalase preparations, whereas 25 mM α-D-glucose had an inhibitory effect on the fluoride release from α-D-glucosyl fluoride.

The stereochemistry of the reactions was investigated to elucidate the basis of the differences between the reactions with α- and β-D-glucosyl fluoride catalyzed by the trehalase preparations. The high rates of enzymatic hydrolysis of the α anomer allowed the use of ^{1}H NMR spectroscopy to learn the anomeric configuration of the glucose formed. To this end, the rabbit and yeast enzymes were dialyzed at 6 °C vs. 0.1 M acetate-d_4/deuterium oxide buffer of pD 5.6 to exchange their labile ^{1}H atoms for ^{2}H atoms. At separate zero times, test mixtures were prepared by adding 0.60 mL of dialyzed *Candida* trehalase (96 μg), *Candida* α-glucosidase (48 μg), or rabbit kidney trehalase (12 μg) to 24 μmol of α-D-glucosyl fluoride, freshly dried from methanolic solution. Similar digests also were made by adding 0.60 mL of the dialyzed rabbit or yeast trehalase to 18 μmol of α,α-trehalose which had been dried under vacuum at 55 °C from solution in deuterium oxide to exchange its labile ^{1}H atoms. Each mixture was transferred to a 5-mm NMR tube, and ^{1}H NMR spectra were recorded at intervals during incubation at 25 °C. Each spectrum consisted of 64 free induction decays using 3-s repetition times.

As shown in Figure 1A, the hydrolysis of 40 mM α-D-glucosyl fluoride catalyzed by the *Candida* trehalase plus α-glucosidase preparation produced both α- and β-D-glucose in roughly equal proportions. Spectra A-1 to A-3, recorded between 2 and 42 min of incubation, show the concurrent development of doublets centered at 5.23 ppm ($J_{1,2}$ = 3.4 Hz) and at 4.64 ppm ($J_{1,2}$ = 8.1 Hz) due respectively to the anomeric protons of α- and β-D-glucose. Both resonances first appear after 11–14 min (spectrum A-2) and both are prominent at 39–42 min (spectrum A-3). In contrast (Figure 1B), the reaction catalyzed by the *Candida* α-glucosidase with 40 mM α-D-glucosyl fluoride yields α-D-glucose exclusively. Spectra B-1 to B-3, recorded between 7 and 45 min of incubation, show an increasingly prominent resonance doublet centered at 5.23 ppm ($J_{1,2}$ = 3.4 Hz) due to the equatorial H-1 proton of α-D-glucose. Resonance of the H-1 proton of β-D-glucose appeared after longer incubation and nonenzymic anomerization. These results leave no doubt that *Candida* trehalase catalyzes the rapid hydrolysis of α-D-glucosyl fluoride to produce β-D-glucose. They also strongly suggest that the

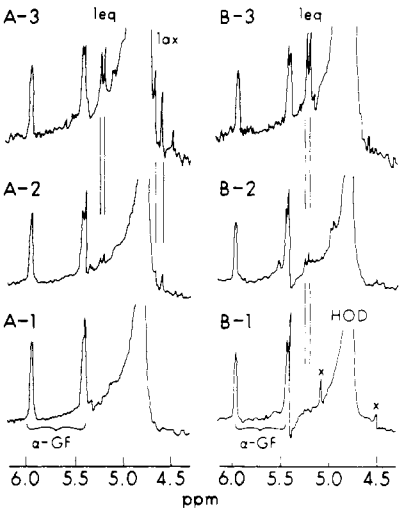


FIGURE 1: ^{1}H NMR spectra at 100 MHz recorded in 0.1 M acetate-d_4/D_2O buffer, pD 5.6. (A-1, A-2, A-3) 40 mM α-D-glucosyl fluoride incubated at 25 °C with *Candida* trehalase plus α-glucosidase for 2–5, 11–14, and 39–42 min, respectively. (B-1, B-2, B-3) 40 mM α-D-glucosyl fluoride incubated at 25 °C with purified *Candida* α-glucosidase for 7–10, 16–19, and 45–48 min, respectively. α-GF, resonance of the equatorial anomeric proton of α-D-glucosyl fluoride; leq, resonance of the equatorial anomeric proton of α-D-glucose; lax, resonance of the axial anomeric proton of β-D-glucose; x, spinning side band; ppm, parts per million from 3-(trimethylsilyl)propanesulfonic acid sodium salt.

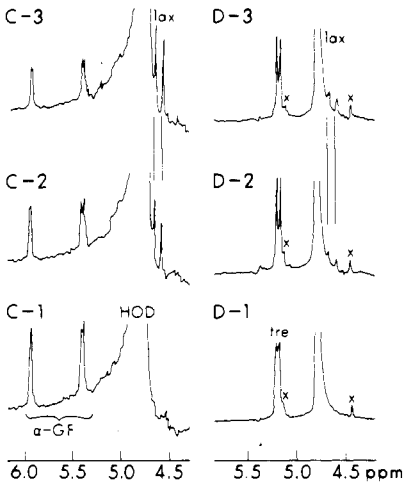


FIGURE 2: ^{1}H NMR spectra at 100 MHz recorded in 0.1 M acetate-d_4/D_2O buffer, pD 5.6. (C-1, C-2, C-3) 40 mM α-D-glucosyl fluoride incubated at 25 °C with rabbit kidney trehalase for 2–5, 18–21, and 32–35 min, respectively. (D-1, D-2, D-3) 30 mM α,α-trehalose incubated at 25 °C with rabbit kidney trehalase for 9–12, 20–23, and 36–39 min, respectively. tre, resonance of the equatorial anomeric protons of the α-D-glucosyl residues of α,α-trehalose; other notations as in Figure 1.

Table II: Radioactive Transfer Product Formed in Enzymic Digests of 40 mM β-D-Glucosyl Fluoride plus 75 mM α-D-[^{14}C]Glucopyranose[a]

substrates plus	components eluted from chromatograms of 30-μL digest as: glucose (cpm)	trehalose: cpm	trehalose: %[b]
rabbit kidney trehalase	655 000	1960	0.25
Candida trehalase plus α-glucosidase	582 000	5580	0.90
Candida α-glucosidase	646 000	570	0.04
buffer only	621 000	280	

[a] Digests incubated (pH 5.6) at 30 °C for 15 min. [b] % of total radioactivity migrating as trehalose, corrected for 0.045% of counts in the trehalose region of the buffer control.

Table III: Enzymic Degradation of the ^{14}C-Labeled Transfer Product[a]

enzyme source of transfer product	radioactivity found as [^{14}C]glucose after treatment[b]: rabbit trehalase (%)	rat trehalase (%)	α-glucosidase (%)	β-glucosidase (%)
rabbit kidney	73.5	76.9	1.3	2.5
Candida yeast	93.6	84.0	4.3	2.1

[a] Samples (in 40 μL) of transfer product from the rabbit kidney trehalase (~1330 cpm, 0.004 μmol) or from the yeast trehalase (~5400 cpm, 0.016 μmol) were incubated at pH 5.6 (30 °C, 4 h) with enzyme or buffer (40 μL); then 50 μL of each digest was chromatographed. Materials migrating as glucose and trehalose were eluted and subjected to scintillation counting (30 min). [b] Values corrected for the percent radioactivity migrating as glucose in mixtures of the product with buffer.

α-D-glucose evident in Figure 1A is formed entirely by the α-glucosidase component.

Figure 2C shows that the reaction catalyzed by purified rabbit kidney trehalase with 40 mM α-D-glucosyl fluoride results in the exclusive formation of β-D-glucose. Spectra C-1 to C-3, recorded between 2 and 35 min of incubation, show the development of the doublet at 4.64 ppm ($J_{1,2}$ = 8.1 Hz) due to the axial anomeric proton of β-D-glucose. No resonance indicative of the presence of α-D-glucose is observed in any of the spectra. Figure 2D illustrates spectra recorded with an incubated mixture of rabbit kidney trehalase with 30 mM α,α-trehalose. Resonance at 4.64 ppm ($J_{1,2}$ = 8 Hz), reflecting the formation of β-D-glucose, is evident after 20–23 and 36–39 min of incubation (spectra D-2 and D-3). The concomitant release of α-D-glucose is apparent only as a slightly increased height of the lowest field resonance peak and as a small shoulder downfield on that peak (D-3); resolution at 100 MHz is insufficient to separate the resonance of the anomeric proton of free α-D-glucose from that of the two anomeric protons of the α,α-trehalose substrate. However, spectra of a similar digest recorded at 300 MHz (C. F. Brewer, unpublished results) showed that both α- and β-D-glucose are formed in equivalent proportions. Comparable results were obtained with a digest of 30 mM α,α-trehalose with the *Candida* trehalase preparation (spectra not shown). Both trehalases thus catalyze the hydrolysis of trehalose and α-D-glucosyl fluoride with configurational inversion of the mobilized D-glucosyl residue.

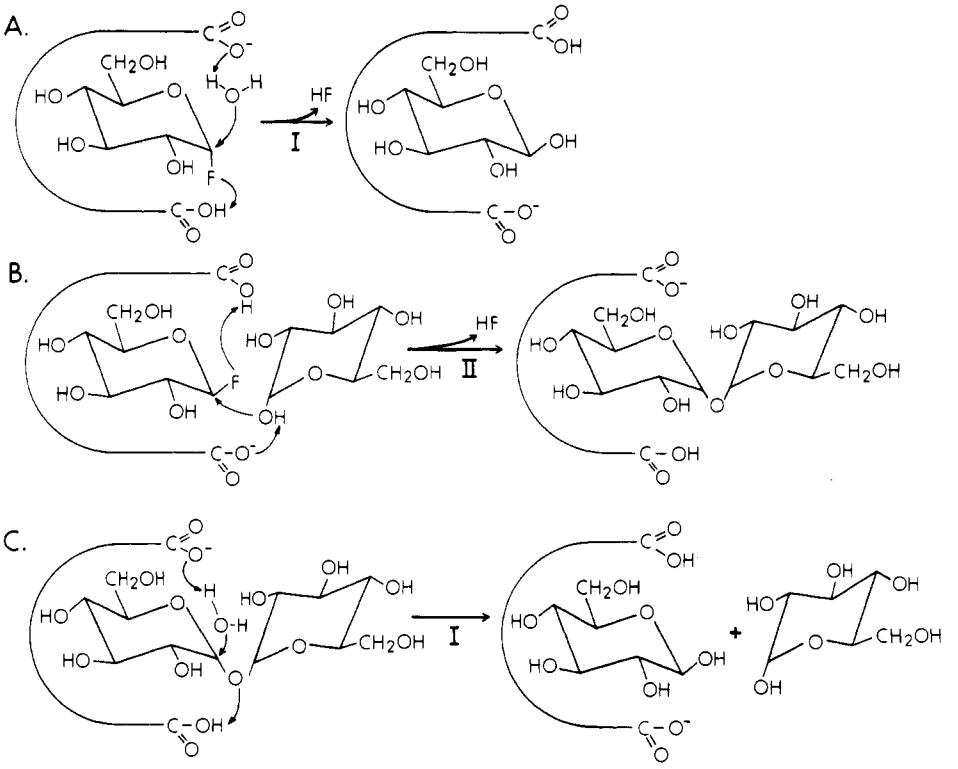
A different technique was used to study the reactions catalyzed with β-D-glucosyl fluoride. Test mixtures (90 μL) containing 0.08 mg/mL enzyme, 40 mM β-D-glucosyl fluoride, 75 mM α-D-[^{14}C]glucopyranose, and 0.05 M acetate buffer (pH 5.6) were incubated at 30 °C for 15 min. Samples (30 μL) were then chromatographed as 3-cm bands, along with adjacent glucose and trehalose markers. The latter, when stained, served as guides for eluting glucose and material migrating as trehalose from the chromatographed digests. Scintillation counting of the eluates (Table II) revealed that a small but reproducible proportion of the radioactivity in the digests with the rabbit and yeast trehalase preparations migrated as trehalose. Insignificant amounts were produced by the *Candida* α-glucosidase (or in control trehalase/α-D-[^{14}C]glucose digests), indicating that the product arises from a trehalase-catalyzed glucosyl transfer reaction with β-D-glucosyl fluoride as the donor, and not by condensation (reversal of hydrolysis) from the α-D-[^{14}C]glucose.

Small amounts of the radioactive product migrating as trehalose were isolated from similar digests (0.4–0.8 mL) prepared with each trehalase and examined for susceptibility to attack by specific glucosidases. The results (Table III) show that the isolated product in each case consisted mainly, if not entirely, of α,α-trehalose. The *Candida* trehalase product, purified by rechromatography, was 84–94% hydrolyzed by purified rat intestinal trehalase and rabbit kidney trehalase; it was not attacked by rice α-glucosidase or sweet almond β-glucosidase. Material from the digest with rabbit kidney trehalase (which had not been rechromatographed and which provided only 5 × 10^{-5} M solutions for the enzymatic analysis) was 73–77% hydrolyzed by the purified trehalases and not attacked by the α- or β-glucosidase. The α,α-[^{14}C]trehalose from the *Candida* trehalase digest with β-D-glucosyl fluoride plus α-D-[^{14}C]glucose was also directly compared as to specific radioactivity with the ^{14}C-labeled glucose used in its preparation. Solutions of each were concurrently analyzed for total glucose (phenol–sulfuric acid) and for radioactivity. The enzymically synthesized α,α-[^{14}C]trehalose showed a specific activity of 0.078 μCi/μmol of glucose; the [^{14}C]glucose used as acceptor gave a value of 0.161 μCi/μmol. This finding confirms that one of the two glucose residues of the isolated α,α-[^{14}C]trehalose transfer product was derived from the unlabeled β-D-glucosyl fluoride and the other from the α-D-[^{14}C]glucose.

Evidence that the presently observed reactions with α- and β-D-glucosyl fluoride (apart from the α-D-glucosyl fluoride hydrolysis catalyzed by the yeast α-glucosidase) are catalyzed at the active center of trehalase was obtained through their inhibition by α,α-trehalose. Digests with either rabbit kidney or yeast trehalase preparation, containing 10 or 40 mM α-D-glucosyl fluoride and 2 mM α-D-glucose[2] (or 10 and 40 mM β-D-glucosyl fluoride and 50 mM α-D-glucose), were incubated (30 °C, 10.0 min) in the absence or presence of different concentrations of α,α-trehalose. Specific rates of enzymically catalyzed fluoride release, v, were then determined after correction for fluoride release in control mixtures incubated without enzyme.

Dixon (1953) plots of $1/v$ vs. trehalose concentration (Figure 3A,B) show the inhibitory effect of α,α-trehalose on α-D-glucosyl fluoride utilization by rabbit kidney and *Candida* trehalase, respectively. Similarly, parts C and D of Figure 3 show the effect of trehalose on the use of β-D-glucosyl fluoride by the rabbit and yeast trehalase, respectively. In each case the linear curves obtained at the two substrate concentrations (s_1 = 10 mM, s_2 = 40 mM) intersect above the abscissa. This result, and the finding that plots of s_1/v and s_2/v vs. α,α-trehalose concentration (Cornish-Bowden, 1974) yielded essentially parallel linear curves, provides unambiguous evidence that trehalose is a competitive inhibitor of α- and β-D-glucosyl fluoride utilization by both rabbit and *Candida*

Scheme I



trehalase preparations. The data of Figure 3 indicate k_i (α,α-trehalose) to be ca. 2–2.5 mM with each enzyme; assays of α,α-trehalose hydrolysis (at pH 5.6) by the rabbit and *Candida* trehalase preparations yielded k_m values similar to the k_i observed. α,α-Trehalose caused no detectable inhibition in comparable tests made with α-D-glucosyl fluoride and *Candida* α-glucosidase.

Discussion

Trehalase is shown for the first time in its long history to utilize substrates half the size of α,α-trehalose, to utilize a β-D-glucosyl substrate, and to have a nonhydrolytic mode of action in addition to (and stereocomplementary to) its hydrolytic action. Highly purified rabbit renal cortical trehalase and partly purified *Candida tropicalis* trehalase were found to effect the rapid hydrolysis of α-D-glucosyl fluoride to form β-D-glucose and to catalyze D-glucosyl transfer from β-D-glucosyl fluoride to the C-1 hydroxyl position of α-D-glucopyranose to form α,α-trehalose. The effective competitive inhibition of these reactions by α,α-trehalose confirms that they are catalyzed by trehalase at the same active center responsible for the hydrolysis of α,α-trehalose. Independent evidence for the same point comes from the finding, with both rabbit and yeast trehalase preparations, that configurational inversion marks the hydrolysis of α,α-trehalose as well as the reactions with each glucosyl fluoride.

Mechanisms consistent with the actions of the two trehalase preparations on α- and β-D-glucosyl fluoride and trehalose are illustrated in Scheme I. The observed reactions with these α- and β-D-glucosyl substrates correspond respectively to the stereocomplementary type I (hydrolytic) and type II (nonhydrolytic) glucosylation reactions recently observed for the actions of β-amylase with α- and β-maltosyl fluoride (Hehre et al., 1979) and of glucoamylase and glucodextranase with α- and β-D-glucosyl fluoride (Kitahata et al., 1981). Defaye et al. (1981) have reported evidence for the presence, in the active site of cockchafer trehalase, of carboxyl groups and an imidazole residue which were considered to be catalytic

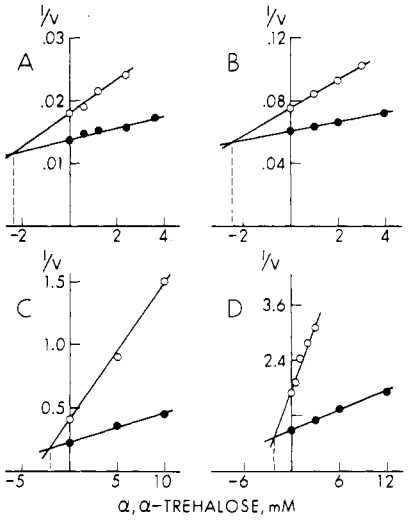


FIGURE 3: Inhibition by α,α-trehalose of α-D-glucosyl fluoride utilization by rabbit kidney (A) and by *C. tropicalis* (B) trehalase. (○) Digests with 10 mM α-D-glucosyl fluoride plus 2 mM α-D-glucose; (●) digests with 40 mM α-D-glucosyl fluoride plus 2 mM α-D-glucose. Inhibition by α,α-trehalose of β-D-glucosyl fluoride utilization by rabbit kidney (C) and by *C. tropicalis* (D) trehalase. (○) Digests with 10 mM β-D-glucosyl fluoride plus 50 mM α-D-glucopyranose; (●) digests with 40 mM β-D-glucosyl fluoride plus 50 mM α-D-glucopyranose. Dixon (1953) plots of $1/v$ (μmol of fluoride released min^{-1} mg^{-1}) vs. α,α-trehalose concentration (μmol/mL). Dotted line projected on abscissa indicates K_i for α,α-trehalose.

functional groups. No evidence is available on the nature of the functional groups in the active sites of rabbit kidney trehalase or *Candida* trehalase. However, a reasonable model for the active site functional groups of both enzymes incorporates a pair of cooperatively interacting carboxyl groups located above and below the glycosidic linkage of bound trehalose (Scheme IC), in much the same manner as in the active site of hen's egg white lysozyme. Substitution of an imidazole group for one of the carboxyls is also possible. The catalytic mechanism proposed for the hydrolysis of α,α-trehalose by the

rabbit and yeast trehalase (Scheme IC) is shown with inversion of configuration as observed in the present study. In this mechanism, the protonated carboxyl group acts as a general acid to aid cleavage of the glycosidic oxygen bond while the other carboxyl group, as an anion, acts as a general base in aiding attack of solvent water. A concerted mechanism is shown for reasons discussed below.

Scheme IA illustrates the proposed mechanism of hydrolysis of α-D-glucosyl fluoride by the two trehalase preparations; this is consistent with the observed inversion of configuration of the product and is similar to the mechanism proposed for the hydrolysis of trehalose in Scheme IC. The role of the functional groups in giving rise to the formation of β-D-glucose and elimination of HF is similar to that envisioned for the hydrolysis of trehalose.

Scheme IB illustrates the postulated mechanism for the reaction catalyzed with β-D-glucosyl fluoride. The mechanism is consistent with the observed formation of α,α-trehalose as the product of the reaction of one molecule of β-D-glucosyl fluoride with one molecule of α-D-glucopyranose. It is also consistent with the observed enhancement effect of added α-D-glucopyranose on the rate of β-D-glucosyl fluoride utilization by the enzymes. This transfer step corresponds to the type II (nonhydrolytic) reaction previously mentioned in which the roles of the two active site functional groups are reversed in a concerted displacement mechanism that requires both substrate molecules to be simultaneously bound at the active site. This probable concerted displacement reaction suggests, but certainly does not prove, that the hydrolysis of trehalose to give α- and β-D-glucose may also occur by a concerted mechanism as shown. It is also possible that trehalose hydrolysis may occur by a stepwise reaction involving a carbonium ion intermediate. Indeed, our studies (Kitahata et al., 1981) emphasize that different reaction mechanisms can occur for different substrates with a given enzyme. It is of interest to note that the postulated mechanisms for the reactions catalyzed with α- and β-D-glucosyl fluoride and trehalose by the two enzymes studied here do not involve ring opening, in contrast to the mechanism proposed by Defaye et al. (1981) for trehalose hydrolysis by cockchafer trehalase. Regardless of the mechanistic details, the results show clearly that the catalytic groups of trehalase, like those of other glycosylases (Hehre et al., 1979, 1980; Kitahata et al., 1981), are functionally flexible beyond the requirements of the principle of microscopic reversibility.

It is tempting to speculate that all trehalases may have catalytic properties similar to those herein described. In preliminary tests, purified rat intestinal mucosal trehalase has shown similar behavior; it hydrolyzed α-D-glucosyl fluoride faster than α,α-trehalose and utilized β-D-glucosyl fluoride in the presence of α-D-glucose. There is also an obvious similarity between flesh fly trehalase (Clifford, 1980) and the *Candida* and rabbit kidney enzymes in that all hydrolyze α,α-trehalose with configurational inversion. Pig kidney trehalase, on the other hand, has been reported to hydrolyze this substrate with formation of two molecules of α-D-glucose (Labat et al., 1973). Further examination of its catalytic activities along the lines of the present study would be of special interest in view of the proposed possible role of trehalase in the active transport of glucose in mammals (Sacktor, 1972).

The present demonstration that trehalase utilizes substrates structurally remote from α,α-trehalose (α- and β-D-glucosyl fluoride), and also has a nonhydrolytic mode of action (with β-D-glucosyl fluoride), contrasts with the failure to detect such capabilities in past studies using glycosidic substrates. When appropriate glycosyl donors of nonglycosidic type were employed, comparably broad activities were recently found for several other "strict hydrolases" originally held to act only on narrowly specified glycosidic linkages, viz., β-amylase, glucoamylase, and glucodextranase (Hehre et al., 1979, 1980; Kitahata et al., 1981). In each case the new findings furnish clear evidence against the widely accepted notions that carbohydrases are specific for one anomer of a given substrate and that donors with a glycosidic bond are needed to effect nonhydrolytic reactions. With each enzyme, including trehalase, new reaction patterns were found despite high apparent odds by experiments based on the concept that glycoside hydrolases and glycosyltransferases form a class of enzymes whose reactions effect a simple chemical change, glycosyl/proton interchange (Hehre et al., 1971, 1973). This concept, which is consistent with all known enzymic glycosylation reactions including those with truncated glycosyl donors, thus merits wider use as a guiding principle in place of conventional views of carbohydrase specificity.

Since completion of this work, Bar-Guilloux et al. (1981) have reported evidence obtained by ^{13}C NMR spectroscopy that cockchafer trehalase catalyzes the hydrolysis of α,α-[1-^{13}C]trehalose with inversion of configuration of one glucose residue. Further, a sample of partly purified cockchafer enzyme, kindly furnished by Drs. Bar-Guilloux and Defaye, has been found to hydrolyze α-D-glucosyl fluoride faster than α,α-trehalose and to catalyze fluoride release from β-D-glucosyl fluoride (with the rate enhanced in the presence of α-D-glucose). These observations are in complete accord with the presently described results.

References

Avigad, G., Ziv, O., & Neufeld, E. (1965) *Biochem. J. 97*, 715–722.

Bar-Guilloux, E., Defaye, J., Driguez, H., & Henrissat, B. (1981) Abstracts of the First European Symposium on Carbohydrate Glycoconjugates, Vienna, Austria, Sept 14–17.

Bourquelot, E. (1893) *C. R. Hebd. Seances Acad. Sci. 116*, 826.

Clifford, K. H. (1980) *Eur. J. Biochem. 106*, 337–340.

Cornish-Bowden, A. (1974) *Biochem. J. 137*, 143–144.

Courtois, J.-E., Petek, F., & Kohali-Zanouzi, M.-A. (1962) *Bull. Soc. Chim. Biol. 44*, 735–743.

Dahlqvist, A. (1960) *Acta Chem. Scand. 14*, 9–16.

Defaye, J., Driguez, H., & Henrissat, B. (1981) *Mechanisms of Saccharide Polymerization and Depolymerization* (Marshall, J. J., Ed.) pp 331–353, Academic Press, New York.

Dixon, M. (1953) *Biochem. J. 55*, 170–171.

Guilloux, E., Courtois, J.-E., & Percheron, F. (1968) *Bull. Soc. Chim. Biol. 50*, 1915–1931.

Hehre, E. J., Genghof, D. S., & Okada, G. (1971) *Arch. Biochem. Biophys. 142*, 382–393.

Hehre, E. J., Okada, G., & Genghof, D. S. (1973) *Adv. Chem. Ser. No. 117*, 309–333.

Hehre, E. J., Brewer, C. F., & Genghof, D. S. (1979) *J. Biol. Chem. 254*, 5942–5950.

Hehre, E. J., Brewer, C. F., Uchiyama, T., Schlesselmann, P., & Lehmann, J. (1980) *Biochemistry 19*, 3557–3564.

Hudson, C. S., & Dale, J. K. (1917) *J. Am. Chem. Soc. 39*, 320–328.

Kalf, G. F., & Rieder, S. V. (1958) *J. Biol. Chem. 230*, 691–698.

Kitahata, S., Brewer, C. F., Genghof, D. S., Sawai, T., & Hehre, E. J. (1981) *J. Biol. Chem. 256*, 6017–6026.

Labat, J., Baumann, F., & Courtois, J.-E. (1973) *Carbohydr. Res. 26*, 341-349.
Lowry, H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Nakano, M. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1919.
Nisizawa, K., & Hashimoto, Y. (1970) *The Carbohydrates* (Pigman, W., & Horton, D., Eds.) 2nd ed., Vol. 2, pp 241-300, Academic Press, New York.
Sacktor, B. (1972) *Biochemistry of the Glycosidic Linkage* (Piras, R., & Pontis, H. G., Eds.) pp 281-289, Academic Press, New York.
Saito, S. (1960) *J. Biochem. (Tokyo) 48*, 101-109.
Sawai, T. (1967) *Proceedings of the Amylase Symposium, 1967* (Society of Amylase Researchers, Eds.) pp 111-117, Osaka, Japan.
Williams, D. E., & Reisfeld, R. A. (1964) *Ann. N.Y. Acad. Sci. 121*, 373-381.

Formation of a Single Phosphodiester Bond by RNA Polymerase B from Calf Thymus Is Not Inhibited by α-Amanitin†

Alain C. Vaisius* and Theodor Wieland

ABSTRACT: The template-directed synthesis of a single phosphodiester bond by highly purified calf thymus RNA polymerase B is not inhibited by high concentrations of α-amanitin (10^{-6} M). However, a subsequent internucleotide bond is not synthesized in the presence of α-amanitin. These results suggest that translocation of the nascent RNA and RNA polymerase B along the DNA template is the enzymatic process inhibited by α-amanitin. It is also shown that the formation of a single phosphodiester bond by RNA polymerase B results in a stable ternary transcription complex, i.e., between the enzyme, the DNA, and the nascent RNA. Under reaction conditions which normally favor the elongation of RNA, the transcriptional process is arrested at initiation by α-amanitin. Such ternary initiation complexes have been isolated by agarose gel electrophoresis.

The cytotoxin α-amanitin is a bicyclic octapeptide occurring in high concentrations in the deadly, poisonous mushroom *Amanita phalloides*. The primary cytopathogenicity of the amatoxins is the inhibition of RNA polymerase B (ribonucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) which is the enzyme that transcribes the precursor of mRNA. RNA polymerases B from higher eukaryotes are typically half-maximally inhibited by α-amanitin at a concentration of 5×10^{-9} M [for a review, see Wieland & Faulstich (1978)].

In a very detailed study, Cochet-Meilhac & Chambon (1974) demonstrated that the amatoxins bind to RNA polymerase B with a 1:1 stoichiometry, that the affinity of the toxin for the enzyme is very strong with an equilibrium association constant of 3.5×10^8 M^{-1} at 30 °C, and that the dissociation is very slow and highly dependent upon temperature and ionic strength. Subsequently, it was shown by Brodner & Wieland (1976), using a carbodiimide condensation reaction of labeled amatoxin to calf thymus RNA polymerase B, that the 140-kilodalton subunit was a binding site for the amatoxins. Thus, it has been clearly shown that inhibition by α-amanitin is the result of a direct interaction of the toxin with RNA polymerase B.

The process of RNA synthesis catalyzed by DNA-dependent RNA polymerase is quite complex and may be described in several steps as follows: (a) formation of a stable binary complex with the DNA template; (b) RNA chain initiation; (c) translocation and elongation of the nascent RNA; (d) termination and dissociation of the RNA chain. Hence, there are a number of enzyme mechanisms which could possibly be specifically disrupted by α-amanitin. It has been shown that the binding of α-amanitin does not cause a dissociation of the DNA–RNA polymerase B binary complex or the dissociation of the DNA–enzyme–RNA ternary complex (Cochet-Meilhac & Chambon, 1974). In the same study, direct evidence was given that the binding of α-amanitin was responsible for the inhibition of RNA chain elongation as catalyzed by RNA polymerase B. The inhibition of chain initiation was suggested by indirect experimentation, i.e., by a failure to detect pyrophosphate exchange which is presumably the reverse reaction of phosphodiester bond formation (Krakow & Fronk, 1969). Therefore, it was concluded by Cochet-Meilhac & Chambon (1974) that the binding of amatoxin to RNA polymerase B prevents the formation of phosphodiester bonds.

In this paper, we report that calf thymus RNA polymerase B will catalyze a template-directed synthesis of a single phosphodiester bond in the presence of a high concentration of α-aminitin. However, we were unable to detect the subsequent synthesis of a second internucleotide bond. Our results suggest that translocation may be the enzymatic process blocked by α-amanitin. A preliminary report of these results was presented earlier (Vaisius & Wieland, 1981).

Materials and Methods

Biochemicals. Amanitin was a preparation from our laboratory. Ribonucleases and alkaline phosphatase (minimal nuclease) were commercial preparations from P-L Biochemicals. Nucleoside triphosphates, purified by high-performance liquid chromatography, were purchased from ICN Biochemicals. Radioactively labeled nucleoside triphosphates were from NEN and Amersham International. Dinucleoside (3′→5′) monophosphates and trinucleoside (3′→5′) diphosphates were from P-L Biochemicals. The synthetic templates poly(dA-dT) and poly(dI-dC) were purchased from Boehringer, Mannheim.

† From the Max-Planck-Institut für medizinische Forschung, Abteilung Naturstoffchemie, D-69 Heidelberg, West Germany. *Received January 14, 1982.*

* Address correspondence to this author at the Molecular Genetics Group, National Research Council Laboratories, Ottawa, Canada K1A 0R6.

0006-2960/82/0421-3097$01.25/0