Active Site Similarities of Glucose Dehydrogenase, Glucose Oxidase, and Glucoamylase Probed by Deoxygenated Substrates

Michael R. Sierks,[‡] Klaus Bock, Susanne Refn,[§] and Birte Svensson*

Department of Chemistry, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK 2500, Copenhagen Valby, Denmark

Received March 18, 1992; Revised Manuscript Received June 12, 1992

ABSTRACT: The specificity constants, k_{cat}/K_M, were determined for glucose oxidase and glucose dehydrogenase using deoxy-D-glucose derivatives and for glucoamylase using deoxy-D-maltose derivatives as substrates. Transition-state interactions between the substrate intermediates and the enzymes were characterized by the observed k_{cat}/K_m values and found to be very similar. The binding energy contributions of individual sugar hydroxyl groups in the enzyme/substrate complexes were calculated using the relationship $\Delta(\Delta G) = -RT \ln [(k_{cat}/K_M)_{deoxy}/(k_{cat}/K_M)_{hydroxyl}]$ for the series of analogues. The activity of all three enzymes was found to depend heavily on the 4- and 6-OH groups (4′- and 6′-OH in maltose), where changes in binding energies from 10 to 18 kJ/mol suggested strong hydrogen bonds between the enzymes and these substrate OH groups. The 3-OH (3′-OH in maltose) was involved in weaker interactions, while the 2-OH (2′-OH in maltose) had a very small if any role in transition-state binding. The three enzyme–substrate transition-state interactions were compared using linear free energy relationships (Withers, S. G., & Rupitz, K. (1990) *Biochemistry 29,* 6405–6409) in which the set of k_{cat}/K_M values obtained with substrate analogues for one enzyme is plotted against the corresponding values for a second enzyme. The high linear correlation coefficients (ρ) obtained, 0.916, 0.958, and 0.981, indicate significant similarity in transition-state interactions, although the three enzymes lack overall sequence homology. A short amino acid sequence, however, which is critical for glucoamylase activity (Clarke, A. J., & Svensson, B. (1984) *Carlsberg Res. Commun. 49,* 559–566; Sierks, M. R., Ford, C., Reilly, P. J., & Svensson, B. (1989) *Protein Eng. 2,* 621–625) was recognized in glucose oxidase. Whether this area also plays a crucial role in that enzyme is not yet known.

Comparison of active site substrate interactions for distantly related enzymes is a difficult task. The relevant tertiary structures are rarely available, and it is not likely that inspection of the primary structures could reveal information on active site similarities. Therefore, a nonstructural approach that compares active sites of related enzymes on the basis of kinetic properties can be a very useful tool. Linear free energy relationship (LFER[1]) analysis has recently been used to show that two phylogenetically separate but highly conserved α-glucan phosphorylases have essentially identical transition-state interactions (Withers & Rupitz, 1990). This procedure utilizes a molecular recognition approach to measure the binding contribution of specific substrate atoms in the transition state (Street et al., 1986). The free energy change resulting from substitution of a substrate group can be determined ($\Delta(\Delta G) = -RT \ln [(k_{cat}/K_M)_{substituted}/(k_{cat}/K_M)_{unsubstituted}]$) (Fersht, 1985), leading to an energetic fingerprint of the active site that depicts the contribution of individual substrate structural elements to transition-state stabilization.

Enzymes acting on glucose or glucosidic substrates present an excellent case for comparison of distintly related enzymes by use of LFER, since sugar OH groups generally hydrogen-bond with side chains in the enzyme active site in ground-state and transition-state complexes (Johnson et al., 1988; Quiocho, 1989; Strynadka & James, 1991). Three such enzymes, glucose dehydrogenase (EC 1.1.1.47), glucose oxidase (EC 1.1.3.4), and glucoamylase (EC 3.2.1.3), are examined in the present work. The first two are oxidoreductases acting on D-glucose, while the third hydrolyzes α-1,4- and α-1,6-D-glucosides. Besides the specificity differences, they also possess very different structural features, as seen from their amino acid sequences (Frederick et al., 1990; Heilmann et al., 1988; Svensson et al., 1983), molecular sizes, and oligomeric states. Glucose dehydrogenase (GDH), which catalyzes the oxidation of β-D-glucose to gluconic acid using the coenzyme $NADP^+$ (Pauly & Pfeiderer, 1975), is a homotetramer containing 261 amino acid residues per subunit (Heilmann et al., 1988). Glucose oxidase (GO) in turn catalyzes the oxidation of β-D-glucose to D-glucono-1,5-lactone along with the reduction of molecular oxygen to hydrogen peroxide (Müller, 1928). GO from *Aspergillus niger* is a homodimer (Pazur & Kleppe, 1964) with 583 amino acid residues per subunit (Frederick et al., 1990). Finally, glucoamylase (GA) releases β-D-glucose by catalyzing the hydrolysis of α-1,4- and α-1,6-bonds at the nonreducing ends of oligomeric and polymer substrates (Hiromi et al., 1966). GA can also utilize glucose as a substrate in condensation reactions to form the disaccharides maltose and isomaltose, although at a much slower rate than the hydrolytic reaction (Fujimoto et al., 1988). GA from *A. niger* is a monomer produced in two forms which have identical catalytic domains and contain 512 (GA2) or 616 (GA1) amino acid residues, respectively (Svensson et al., 1982, 1983, 1986). The active site of GA contains seven consecutive binding subsites accommodating D-glucosyl residues, with the catalytic groups located between subsites 1 and 2 (Hiromi et al., 1983; Meagher et al., 1989).

* Author for correspondence and proofs; fax, +45 33274708; phone, +45 31221022/ext 5221 or direct +45 33275345.

‡ Present address: Department of Chemical and Biochemical Engineering, University of Maryland Baltimore County, Baltimore, MD 21228.

§ Department of Organic Chemistry, The Technical University of Denmark, Building 201, DK-2800 Lyngby, Denmark.

[1] Abbreviations: GA, glucoamylase; GDH, glucose dehydrogenase; GO glucose oxidase; LFER, linear free energy relationship; TAA, Taka amylase A.

0006-2960/92/0431-8972$03.00/0

D-Glucono-1,5-lactone binds specifically to subsite 1 (Hiromi et al., 1983) and resembles the carbonium ion like intermediate postulated to exist during GA catalysis (Matsui et al., 1989; Konstantinidis & Sinnott, 1991). Since subsite 1 of GA and the active site of GO both recognize D-glucono-1,5-lactone, they may likely have certain common structural features. Early work with *A. niger* GO, however, indicated that the reaction rates on 3- and 4-deoxy-D-glucose were much lower than on D-glucose, and 1-deoxyglucose (1,5-anhydroglucitol) was not a substrate (Pazur & Kleppe, 1964), while the 4′- and 6′-OH groups of maltose which bind at subsite 1 of GA were found to be crucial for *A. niger* GA activity (Bock & Pedersen, 1987).

The transition-state binding interactions of GDH, GO, and GA can be compared using LFER (Withers & Rupitz, 1990) by plotting the log (k_{cat}/K_M) values obtained with a series of substrate analogues for one enzyme versus the corresponding values for another. This procedure indicates how well the binding energetics for the different enzymes resemble each other. Two enzymes with identical active site transition-state interactions thus yield an LFER with slope and correlation coefficient (ρ) both equal to 1 (Withers & Rupitz, 1990), whereas unrelated enzymes will have very poor correlation coefficients. Related enzymes can yield slopes diverging from 1 (Withers & Rupitz, 1990) if the dependence on the electronic structure of the transition states differs.

The binding energy contributions of the individual OH groups of D-glucose with GDH and GO were calculated from k_{cat}/K_M values determined on a series of deoxy-D-glucose analogues. Equivalent data were collected for GA to quantify its interactions with the nonreducing end ring OH groups of maltose using a series of deoxy-D-maltose analgoues. The series of calculated $\Delta(\Delta G)$ values or binding energy contributions are then compared pairwise for the three enzymes using LFERs. The similarities of their active sites will be discussed.

MATERIALS AND METHODS

Substrate Preparation. Syntheses of methyl β-D-maltoside, methyl 2′- and 3′-deoxy-β-D-maltoside (Bock & Pedersen, 1987), and 3-, 4-, and 6-deoxy-D-glucose (Bock et al., 1983) have been described previously. 2-Deoxy-D-glucose was purchased from Fluka AG. The syntheses of 4′- and 6′-deoxy-D-maltose are reported below. Melting points are uncorrected. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. NMR spectra were obtained on Bruker WH-90, AC-250, and AM-500 NMR instruments. The spectra of protected compounds were measured in $CDCl_3$. Unprotected compounds were measured in D_2O relative to the internal reference acetone (δ 2.22) for ^{1}H-NMR spectra and dioxane (67.4 ppm) for ^{13}C-NMR spectra. TLC was performed on silica gel coated plates (Merck F-254). Preparative TLC was performed on 20 × 40 cm plates coated with 1 mm of silica gel.

6-Deoxymaltose (**4**). A solution of methyl 2,3,6,2′,3′-penta-*O*-acetyl-4′-*O*-benzoyl-6′-deoxy-β-maltoside prepared in analogy with the corresponding 4′-acetate **1** (Scheme I) (Bock & Pedersen, 1987) (700 mg, 1.07 mmol) in dichloromethane (10 mL) was treated with anhydrous zinc bromide (60 mg, 0.2 mmol) and dichloromethyl methyl ether (1 mL, 10 mmol). After being stirred for 4 h, the mixture was concentrated and toluene (10 mL) was coevaporated. Workup of the reaction mixture as described above gave 2,3,6,2′,3′-penta-*O*-acetyl-4′-*O*-benzoyl-6′-deoxy-α-maltosyl chloride (**2**) (δ 6.2 ppm; J_{12} = 4 Hz for the anomeric proton) as a syrup, which was sufficiently pure for further reaction as judged by a ^{1}H-NMR spectrum. Compound **2** (755 mg, ~1.07 mmol) was treated, with stirring, with acetic acid (6 mL) and silver acetate (250 mg, 1.5 mmol) for 20 h. Filtration and concentration of the reaction mixture gave 772 mg of a syrup, which was purified by preparative TLC with ethyl acetate–hexane, 1:1, as eluant, yielding 1,2,3,6,2′,3′-hexa-*O*-acetyl-4′-*O*-benzoyl-6′-deoxy-β-(α)-maltose (**3**) (542 mg, 0.79 mmol, 74%), predominantly as the β-anomer as seen from ^{1}H- and ^{13}C-NMR data. De-O-acylation of **3** (542 mg, 0.79 mmol) with 0.1 M NaOMe in methanol (5 mL) gave 6′-deoxymaltose (**4**) (223 mg, 0.68 mmol, 86%) as a mutarotated syrup, which was characterized by its ^{1}H- and ^{13}C-NMR spectral data (see supplementary material).

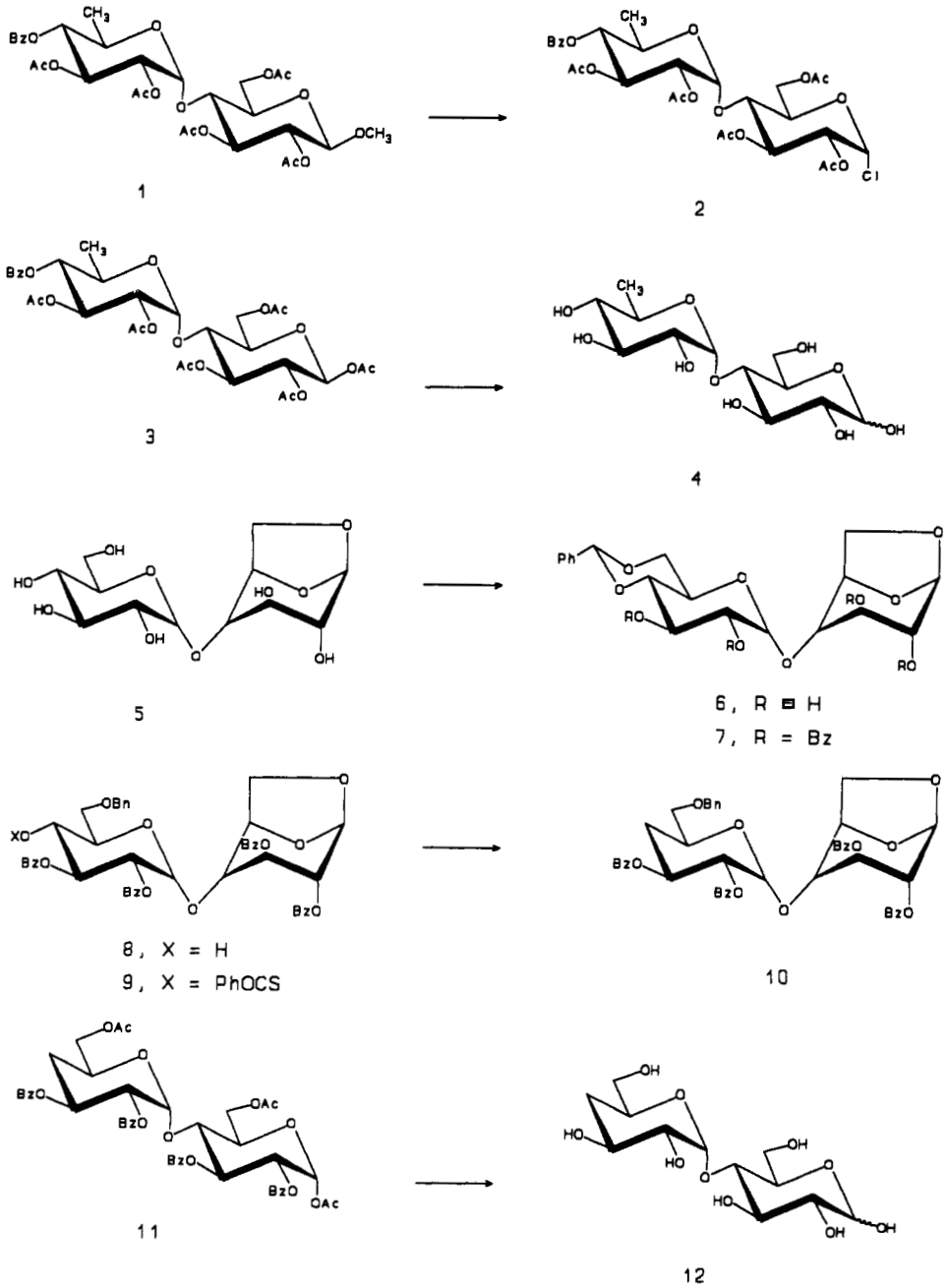
4′-Deoxymaltose (***12***). **5** (Bock & Pedersen, 1988) (2 g, 6.17 mmol), α,α-dimethoxytoluene (1.3 mL), and *p*-TsOH,-H_2O (0.2 g) in anhydrous *N*,*N*-dimethylformamide (15 mL) were placed in a round-bottomed flask, which was attached to a rotary evaporator and rotated for 2 h at a bath temperature of ~50 °C under diminished pressure (~30 Torr). The mixture was cooled, neutralized with triethylamine, and concentrated, and toluene was coevaporated. The crude product 1,6-anhydro-4′,6′-*O*-benzylidene-β-maltose (**6**) was treated with benzoyl chloride (5 mL) in pyridine (50 mL) for 16 h. The benzoate was isolated as a crude syrup (5.30 g), and part (2 g) of the syrup was purified on a silica gel column by flash chromatography using ethyl acetate–hexane (1:2) as eluant. This gave 1,6-anhydro-2,3,2′,3′-tetra-*O*-benzoyl-4′,6′-*O*-benzylidene-β-maltoside (**7**) as a syrup (1.43 g, corresponding to 69%), characterized by its NMR data (see supplementary material).

To a solution of **7** (1.43 g, 1.72 mmol) and sodium cyanoborohydride (1.33 g, 20 mmol) in dry tetrahydrofuran (50 mL), protected from moisture with 4-Å molecular sieves and a calcium chloride tube, was added diethyl ether containing HCl until the evolution of gas ceased. After 5 min the mixture was filtered, concentrated, and purified by flash chromatography using ethyl acetate–hexane (1:2) as eluant. This gave 1,6-anhydro-2,3,2′,3′-tetra-*O*-benzoyl-6′-*O*-benzyl-β-maltose (**8**) (1.2 g, 1.22 mmol, 71%) as a syrup, characterized by its NMR data (see supplementary material). To compound **8** (500 mg, 0.6 mmol) in dichloromethane (20 mL) and pyridine (5 mL) was added phenoxythiocarbonyl chloride (0.2 mL), and the mixture was stirred for 16 h under protection from moisture and light. Workup as described previously (Bock & Pedersen, 1987) gave 653 mg, which was purified by preparative TLC using hexane–dichloromethane–diethyl ether, 2:1:1, as eluant to give 1,6-anhydro-2,3,2′,3′-tetra-*O*-benzoyl-4′-*O*-(phenoxythiocarbonyl)-6′-*O*-benzyl-β-maltose (**9**) (396 mg, 0.41 mmol, 69%), characterized by its NMR data (see supplementary material).

To compound **9** (700 mg, 0.73 mmol) dried in toluene (30 mL) with 4-Å molecular sieves were added tri-*n*-butyltin hydride (0.6 mL, 2.25 mmol) and α,α-azobis(isobutyronitrile) (20 mg, 0.12 mmol). The mixture was refluxed for 1 h and was then left for 16 h at room temperature under nitrogen. Purification by preparative TLC using ethyl acetate–hexane (1:2) as eluant gave 1,6-anhydro-2,3,2′,3′-tetra-*O*-benzoyl-4′-deoxy-6′-*O*-benzyl-β-maltose (**10**) as a syrup (519 mg, 0.64 mmol, 88%), characterized by its NMR data (see supplementary material).

To a solution of **10** (400 mg, 0.49 mmol) in acetic acid (8 mL) were added acetic anhydride (8 mL) and concentrated sulfuric acid (20 drops). After being stirred for 50 min, the

Scheme I



mixture was worked up (Bock & Pedersen, 1987), yielding a syrup (423 mg), which was purified by preparative TLC using ethyl acetate–hexane (1:2) as eluant. This gave syrup **11** (320 mg, 0.37 mmol, 75%) as an anomeric mixture, α/β = 6:1, characterized by its ^{13}C-NMR data for the α-anomer. Anal. Calcd for $C_{51}H_{48}O_{16}$: C, 63.40; H, 5.19. Found: C, 63.59; H, 5.10. De-O-acylation of **11** (150 mg, 0.17 mmol) in 0.1 M NaOMe in methanol (5 mL) yielded 4′-deoxymaltose (**12**) (64 mg), which was purified by chromatography on Sephadex G-15 using methanol–water (1:1) as eluant, giving **12** (56 mg, 0.7 mmol, 100%), characterized by its NMR data (see supplementary material).

Enzyme Assays. GDH from *Bacillus* sp. (Sigma, Lot 59F-0159) was assayed at varying substrate concentrations by spectrophotometry at 35 °C in 50 mM sodium phosphate, pH 7.0, containing 0.4 mM $NADP^+$ and using a GDH concentration of 3.0×10^{-5} mg/mL. The $NADP^+$ concentration was sufficiently high that saturating conditions are expected with the different substrates (Hones et al., 1987). The substrate concentrations used were 0.045 and 0.090 mM for D-glucose and between 0.09 and 0.72 mM for the deoxy-D-glucose analogues. Initial rates were measured from the absorbance at 340 nm (Smith et al., 1989) and the specific activity expressed by micromoles of NADPH produced per second per milligram of enzyme assuming a GDH molecular weight of 118 000 (Pauly & Pfleiderer, 1975).

GO from *A. niger* (Sigma (Type VII), Lot 17F3869) was assayed spectrophotometrically at 35 °C using a modified procedure of Lockridge et al. (1972) as described by Frederick et al. (1990). D-Glucose and deoxy-D-glucose analogues were tested at concentrations between 0.10 and 2.0 mM using a GO concentration of 4.0×10^{-2} mg/mL, assuming a GO molecular weight of 150 000 (Pazur & Kleppe, 1964). In all assays 1 mM KCN was added to inhibit any contaminating catalase activity (Bright & Appelby, 1965). Dissolved oxygen concentrations were assumed to be saturating with all substrates tested since initial rates were determined and the oxygen binding constant decreases slightly with the 2-deoxy

Table I: Kinetic Parameters Determined for Glucose Dehydrogenase, Glucose Oxidase, and Glucoamylase with a Series of Glucose, Maltose, or Methyl β-Maltoside Substrate Analogues[a]

	glucose dehydrogenase		glucose oxidase		glucoamylase	
substrate	k_{cat}/K_M (mM^{-1} s^{-1})	$\Delta(\Delta G)$ (kJ/mol)	k_{cat}/K_M (mM^{-1} s^{-1})	$\Delta(\Delta G)$ (kJ/mol)	k_{cat}/K_M (mM^{-1} s^{-1})	$\Delta(\Delta G)$ (kJ/mol)
glucose (maltose)	1.03		8.58		7.3	
2-deoxy (2′-deoxy)	1.62	−1.2	3.57	3.1	6.8[b]	0.19
3-deoxy (3′-deoxy)	8.10×10^{-2}	6.5	1.62	4.3	0.23[b]	9.1
4-deoxy (4′-deoxy)	1.37×10^{-3}	17.0	0.17	10.0	5.87×10^{-3}	18.8
6-deoxy (6′-deoxy)	1.50×10^{-2}	10.8	0.19	9.8	1.02×10^{-2}	17.4

[a] Parameters determined as described under Materials and Methods. [b] k_{cat}/K_M values for the methyl β-D-maltoside analogue substrates were corrected for the 4% difference with the D-maltose and methyl β-D-maltoside substrates. This enabled comparison of all substrate analogues relative to maltose.

analogue (Frederick et al., 1990).

Glucoamylase G1 from *A. niger* was expressed in *Saccharomyces cerevisiae* and isolated as previously described (Sierks et al., 1989). GA activity at 45 °C was determined from the amount of released D-glucose or D-glucose analogue as measured by a glucose oxidase assay (Sierks et al., 1989). In reactions with D-maltose and methyl β-D-maltoside, only D-glucose was detected, since methyl β-D-glucose does not react with glucose oxidase under the assay conditions used. GA activity on 4′- and 6′-deoxy-D-maltose was determined relative to a standard containing equimolar amounts of D-glucose and either 4- or 6-deoxy-D-glucose, respectively. GA activity on methyl 2′- and 3′-deoxy-β-D-maltosides utilized either 2- or 3-deoxy-D-glucose as standards. In assays for 2- and 3-deoxy-D-glucose the glucose oxidase concentration of 5 U/mL was supplemented by 165 U/mL to ensure adequate color development as both glucose derivatives are relatively poor GO substrates. The GA concentration was 3.0×10^{-4} mg/mL for D-maltose, methyl β-D-maltoside, and methyl 2′-deoxy-β-D-maltoside; 6.0×10^{-4} mg/mL for methyl 3′-deoxy-β-D-maltoside; and 9.3×10^{-3} mg/mL for 4′- and 6′-deoxy-D-maltose, respectively. GA concentrations were determined spectrophotometrically using $\epsilon_{280} = 1.37 \times 10^5$ M^{-1} cm^{-1} (Clarke & Svensson, 1984) and a GA molecular weight of 95 000 (Sierks et al., 1989). Substrate concentrations varied from 0.10 to 1.0 mM.

The individual kinetic parameters, k_{cat} and K_M, were not determined as several of the substrate analogues were available only in limited amounts. However, the second-order rate constant, k_{cat}/K_M, was determined for at least two different concentrations of substrate varying by a factor of 2 or more to ensure that a linear dependence of activity on substrate concentration was fulfilled. The error in k_{cat}/K_M determination was ≤5% with GO and GDH and ≤10% with GA.

Sequence Comparison. Amino acid sequence similarities between GDH, GO, and GA were searched with the aid of the program RELATE (Dayhoff et al., 1983) using a "window" length of 15 residues. For protein structural predictions the program COMBINE (Biou et al., 1988) and hydrophobic cluster analysis (Gaboriaud et al., 1987) have been employed.

RESULTS AND DISCUSSION

The specificity constants, k_{cat}/K_M, determined for GDH, GO, and GA with the different substrate analogues are given in Table I. Changes in activation energy ($\Delta(\Delta G)$) due to substitution of individual hydroxyl groups were calculated using the relationship $\Delta(\Delta G) = -RT \ln [(k_{cat}/K_M)_{deoxy}/(k_{cat}/K_M)_{hydroxyl}]$ (Wilkinson et al., 1983; Street et al., 1986), where the subscripts deoxy and hydroxyl refer to the analogue and the corresponding parent compound, respectively (Table I). Assuming that removal of the individual OH groups does not lead to a change in mechanism or rate-limiting step, and that the α:β ratio is about 4:6 for all analogues, the measured $\Delta(\Delta G)$ values reflect changes in hydrogen-bonding interactions. For simplicity the 2′-, 3′-, 4′-, and 6′-OH groups representing the OH groups at the nonreducing end ring of maltose will be referred to as the 2-, 3-, 4-, and 6-OH groups here. The largest increases in activation energy occurred with the 4- and 6-deoxy-D-glucose (maltose) substrates for all three enzymes. The 3-OH group, while important for transition-state binding interactions, had significantly lower $\Delta(\Delta G)$ values than the 4- and 6-OH groups. The 2-OH group had much less effect, slightly improving activity with GDH, slightly decreasing activity with GO, and barely influencing the GA activity (Table I). The calculated $\Delta(\Delta G)$ values for the three enzymes (Table I) show a similar pattern in the involvement of the substrate OH groups for transition-state stabilization. The effect of the 2-OH group on GO activity was noted earlier (Gibson et al., 1964). In contrast to the present findings, GO was earlier reported to have lowest activity toward 3-deoxy-D-glucose (Pazur & Kleppe, 1964). However, the different assay system used by these authors may account for the discrepancy.

The change in free energy resulting from removal of a hydrogen-bonding group interacting with an uncharged or charged protein side chain has been shown to be 2–6 and 15–19 kJ/mol, respectively (Fersht et al., 1985; Street et al., 1986). The 4-OH group therefore likely binds to a charged side chain of GDH as do the 4- and 6-OH groups to GA. The 4- and 6-OH for GO, the 6-OH for GDH, and the 3-OH groups for all three enzymes may form hydrogen bonds to one or more uncharged side chains. The 2-OH group, however, seems to hydrogen bond only with GO. The binding contribution of each hydroxyl group may be over- or underestimated if there is an altered reaction rate for the noncatalyzed reaction of the analogue compared to the parent compound (Wolfenden & Kati, 1991).

The similarity of the transition-state interactions for these enzymes can be evaluated using LFER plots (Withers & Rupitz, 1990), where the log (k_{cat}/K_M) values generated on a set of substrate analogues with one enzyme are compared to the equivalent set generated with a second enzyme. Comparison of GO and GDH yields an LFER plot of slope 0.558 having a correlation coefficient, ρ, of 0.916. Similarly, comparison of GDH and GA yields a slope of 0.801 and ρ of 0.981, while comparison of GO and GA gives a slope of 0.447 and ρ of 0.958 (Figure 1). The high correlation coefficients indicate a strong similarity between these three active sites. The slopes from the LFER plots comparing GO with either GA or GDH are significantly less than 1 (Table II). This may illustrate a weaker binding energy furnished by the substrate hydroxyl group in interactions with GO as compared to either GA or GDH. Alternatively, this may reflect a difference in the electronic structure of the GO transition

Table II: Linear Regression Analyses Comparing Transition-State Interactions of Glucose Oxidase, Glucose Dehydrogenase, Glucoamylase, Rabbit Muscle Glycogen Phosphorylase *b*, and Potato Glycogen Phosphorylase[a]

	GO vs GDH	GDH vs GA	GO vs GA	R-GP vs GDH	P-GP vs GDH	R-GP vs GO	P-GP vs GO	R-GP vs GA	P-GP vs GA	R-GP vs P-GP
slope	0.558	0.801	0.477	1.02	0.950	1.590	1.514	0.958	0.913	0.996
ρ	0.916	0.981	0.958	0.558	0.516	0.625	0.593	0.661	0.629	0.999

[a] GO = glucose oxidase, GDH = glucose dehydrogenase, GA = glucoamylase. Values for R-GP = rabbit muscle glycogen phosphorylase *b* and P-GP = potato glycogen phosphorylase are from Withers & Rupitz (1990). ρ = correlation coefficient. Glucose and 2-, 3-, 4-, and 6-deoxyglucose were substrates for GO and GDH. Glucose and 3-, 4-, and 6-deoxy-D-glucose 1-phosphate were substrates for P-GP and R-GP. Maltose, methyl β-D-maltoside, methyl 2- and 3-deoxy-β-D-maltoside, and 4- and 6-deoxy-D-maltose were substrates for GA.

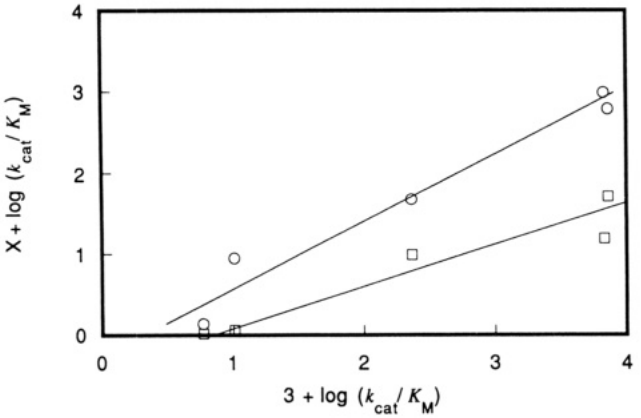


FIGURE 1: Logarithmic plot of the k_{cat}/K_M values measured for a series of deoxy-D-glucose or maltoside analogues with glucoamylase (GA), glucose oxidase (GO), and glucose dehydrogenase (GDH). GDH vs GA = ○; GO vs GA = □. X = −1 for GO and 1 for GDH. Conditions were as described under Materials and Methods.

state as compared to GA and GDH.

LFER plots of potato and rabbit muscle α-glucan phosphorylases were previously reported to have a slope of 1.0 and ρ of 0.999 (Withers & Rupitz, 1990). The two evolutionarily related phosphorylases, however, are highly homologous, having over 50% identical residues and a much greater conservation in their active site regions (Palm et al., 1985; Nakano & Fukui, 1986). Since the study on the α-glucan phosphorylases utilized 3-, 4-, and 6-fluoro-D-glucose 1-phosphates (Withers & Rupitz, 1990), the phosphorylases may also be compared with the present enzymes. The $\Delta(\Delta G)$ values obtained with the phosphorylases varied from 3.4 to 6.2 kcal/mol (Withers & Rupitz, 1990), and the LFER plots clearly illustrate that little if any similarities exists between the transition-state interactions of α-glucan phosphorylases and GDH, GO, or GA, having ρ-values between 0.516 and 0.661 (Table II). This supports, however, that the active site interactions of GDH, GO, and GA are indeed related. GDH, GO, and GA have no overall sequence homology and are at present not recognized as being evolutionarily related, yet all LFER combinations yield high ρ-values. The closer the value of ρ to 1.0, the stronger the correlation that the enzymes are related.

This likely similarity in transition-state binding interactions for the three enzymes prompted a search for related amino acid sequences. A significant resemblance between a short region of GA and GO was revealed using the program RELATE. This same sequence in GA was previously detected to be similar to the α-amylase, Taka amylase A (TAA) (Clarke & Svensson, 1984; Svensson, 1988). The related sequences are shown in Figure 2. Interestingly, this region in GA encompasses Trp120, previously shown to be involved in activity (Clarke & Svensson, 1984) and essential for stabilization of the transition-state complex as suggested by mutagenic studies on Trp120 (Sierks et al., 1989). Since GO and GA both have

```
Taka amylase A          73- CAYFDAYTGYWQTDIY SLNENYGTAD
Glucoamylase           110- NVDETAYTGSWGRPQR DGPALRATAM
Glucose oxidase        101- GGSTLVNGGTWTRPHK AQVDSWETVF
Glucose dehydrogenase   89- VMINNAGMENPVSSHE MSLSDWNKVI
```

FIGURE 2: Sequence similarity between glucoamylase from *A. niger*, glucose oxidase from *A. niger*, glucose dehydrogenase from *Bacillus* sp., and Taka amylase A, an α-amylase from *Aspergillus oryzae*. Shaded residues are conserved or closely related in at least two enzymes, priority being given to GA.

transition-state structures resembling D-glucono-1,5-lactone, the area in question may play similar roles in both enzymes. The GA Trp120 residue and the matching Trp83 of TAA, however, have both been located in a subsite distant from the catalytic site (Clarke & Svensson, 1984; Matsuura et al., 1984). The 3D structure of TAA indicates that the sequence Tyr75–Trp83 creates a large part of the binding cleft that accommodates the nonreducing end of the substrate maltodextrin (Matsuura et al., 1984). The equivalent residues in GA are 112–120. However, it is GA residues 117–125 that align best with GO, and that are also most highly conserved in fungal glucoamylases (Itoh et al., 1987). Therefore, GA residues 121–125 and their counterparts in GO likely have a critical role in activity in contrast to the corresponding residues in TAA which are on the surface of the enzyme (Matsuura et al., 1984). The GDH segment indicated in Figure 2 contains several invariant residues found among the four known GDH sequences (Makino et al., 1989), but has previously been predicted to belong to the coenzyme binding domain (Jörnvall et al., 1984). Structure predictions using COMBINE (not shown) and hydrophobic cluster analysis (not shown) of GA, GDH, and GO suggest that their respective aligned sequences occur in the middle of a long loop region similar to that seen in TAA. Neither GO nor GDH, however, has activity on substrates longer than a single glucosyl residue.

In summary, the energetics of transition-state interactions of the active sites of GDH, GO, and GA have been explored using molecular recognition to indicate key roles for the 4- and 6-OH groups of glucose and the corresponding nonreducing end D-glucosyl residue of maltose, respectively. The LFER plots indicate that the active sites of the three enzymes have important structural features in common, but they are not nearly as similar as previously seen for two α-glucan phosphorylases by using the same procedure (Withers & Rupitz, 1990). A regional sequence similarity with GO around the critical GA Trp120 raises the interesting possibility of structure/function relationship similarities among these enzymes in their active sites. The rather widespread occurrence of this sequence recognized in TAA, cyclodextrin glucanotransferases (Svensson, 1988; MacGregor & Svensson, 1989), *exo*-α-amylases, and debranching enzymes (Jespersen, MacGregor, Sierks, & Svensson, unpublished) suggests that it has a general function in activity on glucosyl units. Whether this function is in binding or in direct protein conformational changes as evidenced with GA (Svensson and Sierks, 1992)

needs to be addressed in further studies.

ACKNOWLEDGMENT

We are grateful to Dr. Hans M. Jespersen for performing the COMBINE, RELATE, and hydrophobic cluster analyses and to Cetus Corporation for the gifts of the glucoamylase gene, the yeast expression vector pGAC9, and the *S. cerevisiae* strain, C468.

SUPPLEMENTARY MATERIAL AVAILABLE

Table containing ^{1}H and ^{13}C NMR data for compounds **3β**, **4α**, **4β**, **7–10**, **11α**, **12α**, and **12β** (1 page). Ordering information is given on any current masthead page.

REFERENCES

Biou, V., Gibrat, J. F., Levin, J. M., Robson, B., & Garnier, J. (1988) *Protein Eng. 2*, 185–191.

Bock, K., & Pedersen, H. (1987) *Acta Chem. Scand., Ser. B B41*, 617–628.

Bock, K., & Pedersen, H. (1988) *Acta Chem. Scand., Ser. B B42*, 75–85.

Bock, K., Meldal, M., Meyer, B., & Wiebe, L. (1983) *Acta Chem. Scand., Ser. B B37*, 101–108.

Bright, H. J., & Appleby, M. (1969) *J. Biol. Chem. 244*, 3625–3634.

Clarke, A., & Svensson, B. (1984) *Carlsberg Res. Commun. 49*, 559–566.

Dayhoff, M. O., Schartz, R. M., & Orcott, L. (1983) in *Atlas of Protein Sequence and Structure* (Dayhoff, M. O., Ed.) Vol. 5, pp 345–352, National Biomedical Research Foundation, Washington, DC.

Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, 2nd ed., pp 302–303, W. H. Freeman and Company, New York.

Fersht, A. R., Shi, J., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M. Y., & Winter, G. (1985) *Nature 314*, 235–238.

Frederick, K. R., Tung, J., Emerick, R. S., Masiarz, F. R., Chamberlain, S. H., Vasavada, A., & Rosenberg, S. (1990) *J. Biol. Chem. 265*, 2793–3802.

Fujimoto, H., Hishida, H., & Ajisaka, K. (1988) *Agric. Biol. Chem. 52*, 1345–1351.

Gaboriaud, C., Bissery, V., Benchetrit, T., & Mornon, J. P. (1987) *FEBS Lett. 244*, 149–155.

Gibson, Q. H., Swoboda, B. E. P., & Massey, V. (1964) *J. Biol. Chem. 239*, 3927–3934.

Heilmann, H. J., Mågert, H. J., & Gassen, H. G. (1988) *Eur. J. Biochem. 174*, 485–490.

Hiromi, K., Hamauzu, Z., Takahashi, K., & Ono, S. (1966) *J. Biochem. 59*, 411–418.

Hiromi, K., Tanaka, A., & Ohnishi, M. (1982) *Biochemistry 21*, 102–106.

Hiromi, K., Ohnishi, M., & Tanaka, A. (1983) *Mol. Cell. Biochem. 51*, 79–95.

Hones, J., Jany, K.-D., Pfleiderer, G., & Wagner, A. F. V. (1987) *FEBS Lett. 212*, 193–198.

Jany, K.-D., Ulmer, W., Fröschle, M., & Pfleiderer, G. (1984) *FEBS Lett. 165*, 6–10.

Johnson, L. N., Cheetham, J., McLaughlin, P. J., Acharya, K. R., Barfod, D., & Phillips, D. C. (1988) *Curr. Top. Microbiol. Immunol. 139*, 81–134.

Jörnvall, H., v. Bahr-Lindström, H., Jany, K.-D., Ulmer, W., & Fröschle, M. (1984) *FEBS Lett. 165*, 190–196.

Konstantinidis, A., & Sinnott, M. L. (1991) *Biochem. J. 279*, 587–593.

Laszló, E., Hollo, J., Hoschke, A., & Sárosi, G. (1978) *Carbohydr. Res. 61*, 387–394.

Makino, Y., Negoro, S. U., Urabe, I., & Okada, H. (1989) *J. Biol. Chem. 264*, 6381–6385.

Matsui, H., Blanchard, J. S., Brewer, C. F., & Hehre, E. J. (1989) *J. Biol. Chem. 264*, 8714–8716.

Matsuura, Y., Kusonoki, M., Harada, W., & Kakudo, M. (1984) *J. Biochem. 95*, 697–702.

Meagher, M. M., Nikolov, Z. L., & Reilly, P. J. (1989) *Biotechnol. Bioeng. 34*, 681–688.

Müller, D. (1928) *Biochem. Z. 199*, 136–170.

Nakano, K., & Fukui, T. (1986) *J. Biol. Chem. 261*, 8230–8236.

Palm, D., Goerl, R., & Burger, K. J. (1985) *Nature 313*, 500–502.

Pauly, H. E., & Pfleiderer, G. (1975) *Hoppe-Seyler's Z. Physiol. Chem. 356*, 1613–1623.

Pazur, J. H., & Kleppe, K. (1964) *Biochemistry 3*, 578–583.

Quiocho, F. A. (1989) *Pure Appl. Chem. 61*, 1293–1306.

Sierks, M. R., Ford, C., Reilly, P. J., & Svensson, B. (1989) *Protein Eng. 2*, 621–625.

Smith, L. D., Budgen, N., Bungard, S. J., Danson, M. J., & Hough, D. W. (1989) *Biochem. J. 261*, 973–977.

Street, I. P., Armstrong, C. R., & Withers, S. G. (1986) *Biochemistry 25*, 6021–6027.

Street, I. P., Rupitz, K., & Withers, S. G. (1989) *Biochemistry 28*, 1581–1587.

Strynadka, N. C. J., & James, M. N. G. (1991) *J. Mol. Biol. 220*, 401–424.

Svensson, B. (1988) *FEBS Lett. 230*, 72–76.

Svensson, B., & Sierks, M. R. (1992) *Carbohydr. Res. 227*, 29–44.

Svensson, B., Pedersen, T. G., Svendsen, I., Sakai, T., & Ottesen, M. (1982) *Carlsberg Res. Commun. 47*, 55–69.

Svensson, B., Larsen, K., Svendsen, I., & Boel, E. (1983) *Carlsberg Res. Commun. 48*, 497–502.

Svensson, B., Larsen, K., & Gunnarsson, A. (1986) *Eur. J. Biochem. 154*, 497–502.

Wilkinson, A. J., Fersht, A. R., Blow, D. M., & Winter, G. (1983) *Biochemistry 22*, 3581–3586.

Withers, S. G., & Rupitz, K. (1990) *Biochemistry 29*, 6405–6409.

Wolfenden, R., & Kati, W. M. (1991) *Acc. Chem. Res. 24*, 209–215.