Effects of Protein Glycosylation on Catalysis: Changes in Hydrogen Tunneling and Enthalpy of Activation in the Glucose Oxidase Reaction†

Amnon Kohen,‡ Thorlakur Jonsson,‡,§ and Judith P. Klinman*

Department of Chemistry, University of California, Berkeley, California 94720

Received October 3, 1996; Revised Manuscript Received December 18, 1996⊗

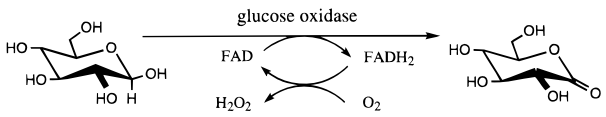
ABSTRACT: Three glycoforms of glucose oxidase, which vary in their degree of glycosylation and resulting molecular weight, have been characterized with regard to catalytic properties. Focusing on 2-deoxyglucose to probe the chemical step, we have now measured the temperature dependence of competitive H/T and D/T kinetic isotope effects and the enthalpy of activation using [1-^{2}H]-2-deoxyglucose. The D/T isotope effect on the Arrhenius preexponential factor (A_D/A_T) is 1.47 (±0.09), 1.30 (±0.10), and 0.89 (±0.04) for the 136, 155, and 205 kDa glycoforms, respectively. The value obtained for the 136 kDa glycoform is well above the range expected for semiclassical−classical (no tunneling) reactions (upper limit of 1.22). The abnormal A_D/A_T is rationalized by extensive tunneling. The enthalpies of activation are 8.1 (±0.4), 11.0 (±0.3), and 13.7 (±0.3) kcal/mol for the 136, 155, and 205 kDa glycoforms, respectively. Apparently, less glycosylation results in more tunneling and a lower enthalpy of activation. The crystal structure, kinetic analysis, and other studies suggest that the enzyme active site is not conformationally changed by the degree of glycosylation. Hence, the differences among the glycoforms, which indicate that changes in the enzyme polysaccharide envelope lead to a significant change in the nature of the hydrogen transfer step, suggest a dynamic transmission of protein surface effects to the active site.

The detailed origin of the rate acceleration of chemical reactions by enzymes is not yet fully understood, despite the immense amount of work that has been done in this area (Jencks, 1987; Fersht, 1985). In addition to the commonly accepted roles of transition-state stabilization/reactant destabilization and enzyme conformational changes, the contribution of protein dynamic effects and possibly tunneling to the rate enhancement may be of considerable importance (Mulholland & Karplus, 1996).

The contribution of quantum-mechanical tunneling to the hydrogen transfer step of enzymatic catalysis was first demonstrated for a dehydrogenase and a topa quinone-containing oxidase, and subsequently for a flavin-dependent oxidase [for a review, see Bahnson and Klinman (1995). More recently, the unusual properties of the lipoxygenase reaction (Glickman et al., 1994; Hwang & Grissom, 1994) have been ascribed to extensive tunneling (Jonsson et al., 1996). Hydrogen tunneling may be a general feature of enzymatic catalysis both for reaction of C−H bonds and for the more general transfer of protons during acid−base catalysis.

The interaction of soluble proteins with their solvent environment and the effect of this interaction on protein dynamics and activity have been discussed in some detail (Gregory, 1995). Many investigators have concluded that internal molecular dynamics are unlikely to make a contribution to catalysis (Kraut, 1988), while others believe that "it is generally accepted that the structural dynamics of proteins in solution play an important role in achieving and regulating their biochemical functions" (Gavish & Yedgar, 1995). A number of reports purport to link protein dynamics to enzyme activity [see Gregory (1995) and references cited therein], but none of these studies could distinguish effects on the chemical step(s) from effects on protein conformational changes and the binding and release of substrate/product.

In the present work, we have investigated the kinetic properties of three forms of glucose oxidase (GO)[1] that vary in their degree of glycosylation, with the goal of determining the contribution of H-tunneling to the C−H bond cleavage step. Glucose oxidase (EC 1.1.3.4) catalyzes the oxidation of glucose to gluconolactone and the subsequent reduction of oxygen to hydrogen peroxide according to a ping-pong steady-state kinetic mechanism (Pazur & Kleppe, 1964; Crueger & Crueger, 1990):



The enzyme contains one very tightly, but noncovalently, bound FAD cofactor per monomer and is a homodimer with a molecular mass of 130−320 kDa, depending on the extent of glycosylation. The native enzyme is glycosylated, with a carbohydrate mass percentage of 16−25% (Pazur & Kleppe, 1964; Swoboda & Massey, 1965; Kalisz et al., 1991). The enzyme has also been cloned (Kriechbaum et al., 1989; Frederick et al., 1990) and expressed in yeast, leading to a highly glycosylated form with a carbohydrate mass percentage approximating 60% (Frederick et al., 1990). Despite their very different degrees of glycosylation, it was

† This work was supported by a grant from the National Science Foundation.

* To whom correspondence should be addressed.

‡ These authors contributed equally to this work.

§ Current address: Decode Genetics, Lynghals 1, 110 Reykjavik, Iceland.

⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1997.

[1] Abbreviations: GO, glucose oxidase; KIE, kinetic isotope effect; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; kDa, kilodalton(s).

S0006-2960(96)02492-0 CCC: $14.00

found that the catalytic activity of the recombinant enzyme (Frederick et al., 1990) and the deglycosylated enzyme (Kalisz et al., 1991) with glucose is similar to that of the wild-type enzyme. The three-dimensional structure of a deglycosylated form of native enzyme is available as a frame of reference (Hecht et al., 1993).

The properties of glucose and 2-deoxyglucose oxidation by GO have been investigated using both steady-state and pre-steady-state kinetic methods (Bright & Gibson, 1967; Bright & Appleby, 1969). Although a large deuterium isotope effect was indicated from pre-steady-state measurements with glucose, the properties of the second-order rate constant for the steady-state oxidation of glucose implicated partial rate limitation from substrate binding. By contrast, the oxidation of 2-deoxyglucose has been concluded to be largely limited by the hydrogen transfer step (Bright & Appleby, 1969). The value of k_{cat} for oxidation of 2-deoxyglucose by GO is 50 s^{-1} at pH 5.6, 25 °C, in comparison to a value of 1200 s^{-1} for glucose (Kriechbaum et al., 1989). This led to the choice of 2-deoxyglucose for the isotope effect studies reported herein. As described below, we have prepared anomerically protonated, deuterated, and tritiated 2-deoxyglucose, and used these substrates as probes of hydrogen tunneling in the glucose oxidase reaction.

Our results indicate that the degree of hydrogen tunneling with glucose oxidase varies with the extent of protein glycosylation, such that tunneling is greatest with the lightest (least glycosylated) protein. These findings indicate a link between modification of the protein envelope and the amount of nuclear tunneling during catalysis at the active site. The origin of these differences may reside in different degrees of protein dynamic mobility among the three glycoforms of GO.

EXPERIMENTAL PROCEDURES

Materials

[1-^{3}H]-2-Deoxyglucose (10 Ci/mmol), 2-deoxyglucose (grade III), glucose oxidase (EC 1.1.3.4) from *Aspergillus niger* (VII-S, for synthesis only), and all salts and buffers (unless otherwise indicated) were from Sigma. [UL-^{14}C]-2-Deoxyglucose (255 mCi/mmol) was from American Radiolabeled Chemicals, Inc. Bis-tris propane was from Calbiochem. D_2O (99.9% and 99.996%) was from Cambridge Isotope Laboratories or Aldrich. Sodium amalgam (5%) was from Anachima. Glucose oxidase from *Aspergillus niger* (grade I, 211 units/mg), hexokinase, endoglycosidase-H, and α-mannosidase were from Boehringer Mannheim. The recombinant glucose oxidase from *Aspergillus niger* expressed in yeast (Frederick et al., 1990) was a generous gift of Dr. Steven Rosenberg from Chiron Co. (Emeryville, CA). The pH of all buffers was adjusted at the experimental temperature.

Synthesis of Anomerically Deuterated 2-Deoxyglucose. [1-^{2}H]-2-Deoxyglucose was synthesized from 2-deoxyglucose by oxidation to 2-deoxygluconolactone catalyzed by GO, followed by sodium amalgam reduction in D_2O yielding the deuterated product. 2-Deoxyglucose (1 g; 3.78 mmol) was dissolved in l mL of 0.2 M potassium phosphate buffer, pH 5.6. Then 155 mg of GO (25 000 units) was added, and the reaction was stirred gently under oxygen until all the reactant was converted to product (as detected by TLC). Twenty-five milliliters of acetonitrile was added, and the aqueous phase was reextracted twice with acetonitrile. The organic phases were pooled and dried under reduced pressure and then lyophilized twice from 50 mL of D_2O (99.9%) to exchange all exchangeable hydrogens. The produced lactone, 0.894 g (3.38 mmol; 89.5% yield from previous step), was dissolved in 130 mL of D_2O (99.996%) in a three-neck flask equipped with a mechanical stirrer and stirred under argon for 10 min. Reduction to [1-^{2}H]-2-deoxyglucose was performed by a modification of the procedure of Isbell et al. (1962). The flask was cooled to −2 °C in a ice−salt bath, and 10 g of $NaHC_2O_4$ (lyophilized twice from D_2O) and 23 g of sodium amalgam (5%) were successively added. The reaction mixture was stirred under argon, and the temperature was raised to 4 °C. After all the lactone was converted to sugar (∼1 h, by TLC detection), the pH was raised to 10 by addition of NaOD, and 1 L of methanol was then added. The mercury and the precipitated salts were filtered through a fine sinter-glass, and the volume was reduced to 5 mL; 500 mL of methanol was added, and more salts were filtered out. The product was passed through a mixed ion-exchanger (BioRad, TMD-8; H^+/HCO_3^- form) until the conductivity was similar to that of distilled water, lyophilized, and then crystallized twice from hot ethanol to yield 0.502 g of [1-^{2}H]-2-deoxyglucose (1.8 mmol, 50.2% overall yield). The product appeared to be quantitatively deuterated at the 1-position, as determined by loss of ^{1}H-NMR signals at 5.23 ppm (d) and 4.78 ppm (dd) and loss of the vicinal anomeric *J*-coupling to the 2-deoxy protons (J_{1-2ax} = 9.8Hz at δ = 1.37 ppm, J_{1-2ax} = 3.2 Hz at δ = 1.58, J_{1-2eq} < 1Hz at δ = 1.98 ppm, and J_{1-2eq} = 3.2 Hz at δ = 2.11 ppm as measured for the protonated sugar). Electrospray mass spectrometry (MNa^+ = 188.1) detected no ^{1}H contamination. Microanalysis found C 43.49% (calcd 43.68%) and H 7.54% (calcd 7.33%).

Synthesis of Anomerically Deuterated [UL-^{14}C]-2-Deoxyglucose. [UL-^{14}C, 1-^{2}H]-2-Deoxyglucose was synthesized from [UL-^{14}C]-2-deoxyglucose according to the same strategy described above. All the purification steps were performed by HPLC (Phenomenex C18-NH_2 column eluted with 87% acetonitrile in water); 65 μCi of [UL-^{14}C]-2-deoxyglucose (250 mCi/mmol) was "diluted" with unlabeled 2-deoxyglucose to a specific activity of 8.4 mCi/mmol and dissolved in 170 μL of 0.1 M potassium phosphate buffer, pH 7.2. Ten microliters of catalase (5000 units) and 8 mg of GO (1600 units) were added. The reaction was quenched after 14 min by adding 700 μL of acetonitrile, and the precipitated protein and salts were spun down (the pellet contained 9 μCi of ^{14}C). Unreacted sugar and the lactone were separated by HPLC (Altech NH_2 column) and pure lactone (∼30 μCi) fractions were pooled and lyophilized. Exchangeable hydrogens were replaced by deuterium by lyophilizing twice from D_2O (99.9%). The lactone was reduced in a similar apparatus to the one described above with 750 mg of $NaHC_2O_4$ (lyophilized twice from 99.9% D_2O) and 2.1 g of sodium amalgam (5%) in 10 mL of D_2O (99.996%). After 45 min, 50 mL of acetonitrile was added. The mercury and the precipitated salts were removed by filtration through a sinter-glass. The product was purified by HPLC, yielding 10.8 μCi of [UL-^{14}C, 1-^{2}H]-2-deoxyglucose (17% overall yield). The deuterium content of radiolabeled product was not directly determined. In subsequent experiments, no trend in D/T isotope effect values was

detected with fractional conversion of substrate. The latter has been seen previously with incompletely deuterated substrates (Cha et al., 1989).

Deglycosylation of Glucose Oxidase. Both wild-type (molecular mass 155 ± 10 kDa) and recombinant (molecular mass 205 ± 15 kDa) glucose oxidases were deglycosylated enzymatically to the same glycoform (molecular mass 136 ± 3 kDa). Deglycosylation was conducted under nondenaturating conditions using a modification of the procedure described by Kalisz et al. (1990). Since the recombinant enzyme deglycosylation was much faster, and the deglycosylated enzyme from both sources had the same MW and activity parameters (data not shown), the recombinant enzyme was used to produce the deglycosylated GO used below. In a typical experiment, 50 mg of GO was incubated for 24 h in 60 mL of 30 mM potassium phosphate buffer, pH 5.0, with 2.6 units of endoglycosidase H and 80 units of α-mannosidase. The reaction was followed by SDS−PAGE (Figure 1) until the GO showed a narrow (<3 kDa) band at 68 kDa. The reaction mixture was concentrated by ultrafiltration (Amicon, YM30 membrane). The buffer was replaced with 100 mM sodium acetate, pH 4.5, the mixture was then loaded on a cation-exchange column (Mono-S, Pharmacia), and the GO was eluted with 100 mM sodium acetate, pH 4.5. The GO was concentrated by ultrafiltration to 3.4 mL in 0.1 M potassium phosphate, pH 7.

The enzyme activity was measured by a continuous spectrophotometric assay (see Methods), the active site concentration was determined by the FAD absorption at 452 nm ($\epsilon = 12.83$ mM^{-1} cm^{-1}) as described by Frederick et al. (1990), and the protein concentration was measured by the Bradford assay (BioRad reagent) with bovine serum albumin as the standard or by its absorption at 280 nm using a published factor of 1.67 ODU/mg (Swoboda & Massey, 1965). The specific activity was 430 units/mg, and the overall yield of enzyme, based on active site measurement (452 nm), was about 40%.

Methods

Competitive Kinetic Isotope Effect (KIE) Experiments. In these experiments, a tritiated substrate was mixed with ^{14}C-labeled, protonated (for H/T KIE experiments) or deuterated (for D/T KIE experiments) substrate. The mixture was allowed to react in the presence of enzyme, under defined conditions of pH and temperature, and quenched at different fraction conversions. The quenched mixtures were analyzed by HPLC and liquid scintillation counting to determine the fraction conversion (f, determined from ^{14}C counting) and tritium to ^{14}C ratio in the products ($[^3H/^{14}C]_f$ and $[^3H/^{14}C]_\infty$ for the ratio at the time point and the infinity point, respectively). The L/T KIE's [also denoted by $^T(V/K)_L$ or k_L/k_T] were calculated by eq 1 (Melander & Saunders, 1987):

$$^T(V/K)_L = \frac{\ln (1 - f)}{\ln \{1 - f[(^3H/^{14}C)_f/(^3H/^{14}C)_\infty]\}} \quad (1)$$

These experiments were carried out in 10 mM buffer. The buffers used were sodium citrate (for pH 4.2), potassium phosphate (for pH 5.6 and 7.0), bis-tris propane (for pH 9.0), and borate (for pH 10). To determine which pH would be the most suitable for carrying out a detailed analysis of GO, H/T competitive KIE experiments were performed using the wild-type enzyme at 25 °C at a few different pH values. The measured KIE was observed to increase with pH, reaching a fairly constant level at pH 9 (suggesting that the hydrogen transfer step was closest to being rate-limiting at this pH). All further experiments were therefore carried out at pH 9.0. Prior to kinetic experiments, tritiated and ^{14}C-labeled substrates were copurified by the same HPLC procedure used in the preparation of labeled compounds (see Materials). Typically, each experiment contained 1.35 μCi of ^{3}H and 0.13 μCi of ^{14}C (about 0.2 mM and 0.6 mM total substrate concentration for H/T and D/T KIE experiments, respectively). The reaction mixture (1.6 mL) was preequilibrated in a Neslab waterbath (±0.1 °C) and three zero time points (t_0) of 150 μL each were removed. The reaction was initiated by addition of enzyme, and, in general, seven time points (t_f) were removed. After incubation overnight with more enzyme, an infinity time point (t_∞) was taken. All samples were quenched with 1 μL of 70% $HClO_4$. The sample's pH was raised to 8.5 by addition of 2.5 μL of 6 M NaOH, and all remaining substrate was converted to the 6-phospho derivative by addition of 4 mM $MgCl_2$, 0.5 mM ATP, 90 mM Tris-HCl, pH 8.5, and 30 units of hexokinase followed by a 1 h incubation.[2] The coupling reaction was quenched with 0.4 mM $HgCl_2$ and stored at −70 °C until HPLC analysis. Before analysis, each sample was thawed, and tetrabutylammonium hydrogen phosphate was added to a final concentration of 5 mM, after which precipitated salts were spun down. The HPLC analysis was performed with a C18 column (Phenomenex, UltraCarb) equilibrated with 5 mM tetrabutylammonium hydrogen phosphate, pH 6.8. After injection of the sample, the same eluent was used for 3.5 min followed by 12 min of 0−30% acetonitrile gradient. Tritiated water eluted at the dead-volume followed by 2-deoxygluconolactone, 2-deoxygluconic acid, and 6-phospho-2-deoxyglucose. Fractions were collected and analyzed using a liquid scintillation counter (Wallac). Since the substrate ^{3}H/^{14}C ratio at t_0 was equal to the ^{3}H/^{14}C ratio in the products at t_∞, an average of the three t_0 values was used as $(^3H/^{14}C)_\infty$ in eq 1.

Analysis of the temperature dependence of KIE's was done by fitting them to eq 2 as described previously [see Bahnson and Klinman (1995) and references cited therein]

$$k_L/k_T = A_L/A_T \exp[(E_T - E_L)/RT] \quad (2)$$

where k_L/k_T is L/T KIE, A_L/A_T is the KIE on preexponential factors, and $E_T - E_L$ is the isotope effect on the enthalpy of activation. The fitted parameters enable comparison of values for the A_L/A_T to theoretical limits from semiclassical models. The semiclassically calculated values range from 0.6 to 1.6 for A_H/A_T and from 0.9 to 1.2 for A_D/A_T (Schneider & Stern, 1972; Bell, 1980; Melander & Saunders, 1987). For proper error analysis, curve fitting was carried out as a least root mean square fit exponential regression of KIE vs $1/T$ (which resulted in larger errors than posttransformation linear regression). In order to ensure that the calculation of

[2] Derivatization of glucose to glucose 6-phosphate was found necessary for efficient HPLC separation of reactants and products. The quantitative conversion of unreacted substrate was achieved through the use of a large excess of hexokinase over initial GO and of ATP over 2-deoxyglucose. No regain of glucose oxidase activity could be detected (using an oxygen uptake assay) in acid-quenched samples that had undergone neutralization prior to the addition of hexokinase.

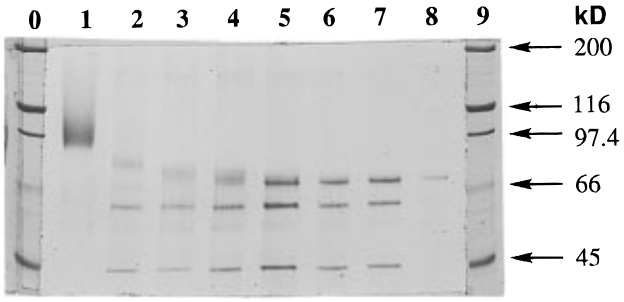


FIGURE 1: Analysis of the deglycosylation of recombinant GO by 7.5% acrylamide SDS−PAGE gel electrophoresis. Lanes 0 and 9 are the molecular mass standards. Lane 1 is the fully glycosylated enzyme. Lanes 2−7 are samples taken from the reaction mixture at 0.1, 2.5, 5, 12, 21, and 25 h (the upper band is the GO and the lower bands are the deglycosylating enzymes). Lane 8 is the deglycosylated enzyme after purification.

the errors in A_L/A_T was not algorithm-dependent, the output from several programs was compared (GraFit, Igor, and mainly KaleidaGraph). The value of the KIE at each temperature was an average of two or three independent experiments with at least six time points and three zero time points per experiment. The standard deviations for KIE's at each temperature were weighted directly (through the exponential regression) without any transformation.

Initial Velocity Measurements. Initial velocity measurements for GO activity (in the course of enzyme purification) were performed by a continuous spectrophotometric assay as described by Lockridge et al. (1972) and modified by Frederick et al. (1990) using a Hewlett-Packard 8452A diode array spectrophotometer equipped with a thermostated cell holder. This assay is based on coupling the GO reaction to that of the horseradish peroxidase reaction with H_2O_2 in the presence of Triton-stabilized *o*-dianisidine.

Initial velocity measurements used for kinetic experiments involved monitoring the consumption of oxygen using a Yellow Spring Instruments (1 mL chamber) with fast stirring. For each measurement, the reaction mixture was preequilibrated with the desired oxygen/nitrogen mixture. The oxygen concentration was determined from the known concentration of dissolved oxygen under conditions of air-saturated water at a given temperature. The reaction was initiated by injecting enzyme into the chamber.

K_M and k_{cat} and their standard errors were determined by nonlinear least root mean square fit to eq 3 (using KaleidaGraph):

$$v/[E] = k_{cat}[S]/(K_M + [S]) \quad (3)$$

where v is the initial velocity, [E] is the enzyme concentration, and [S] is the substrate concentration.

RESULTS

Comparative Characterization of the Glucose Oxidase Glycoforms. The deglycosylated enzyme shows a narrow (3 kDa) band on SDS−PAGE at 68 kDa, suggesting a high homogeneity (Figure 1). The apparent molecular mass of the deglycosylated monomer differs (by 1.5 kDa) from the calculated molecular mass of the glucose oxidase polypeptide chain plus FAD cofactor, which is in accordance with six *N*-acetylglucosamine and two mannose remaining (Hecht et al., 1993). This is in contrast to the wide bands for the glycosylated forms (20 kDa band at 155 kDa and 30 kDa band at 205 kDa for the wild-type and the recombinant glycoforms, respectively), demonstrating significant heterogeneity of their polysaccharide chains (Figure 1). The native gel (not shown) showed a similar behavior. The number of isoelectric forms decreased from about 5 (for the wild-type enzyme) or 4 (for the recombinant enzyme) to a doublet with a p*I* of 4.14 as previously reported by Schmid and co-workers (Kalisz et al., 1990).

Table 1: Kinetic Parameters with Glucose as the Variable Substrate for the Three GO Glycoforms[a]

glycoform (kDa)	K_M (mM)	k_{cat} (s^{-1})
205	28.3 (±0.7)	498 (±7)
155	27.9 (±0.8)	508 (±9)
136	28.5 (±0.8)	505 (±9)

[a] Measured with air saturation oxygen concentration (258 μM) in 0.2 M potassium phosphate at 25 °C by the continuous spectrophotometric assay as described under Methods.

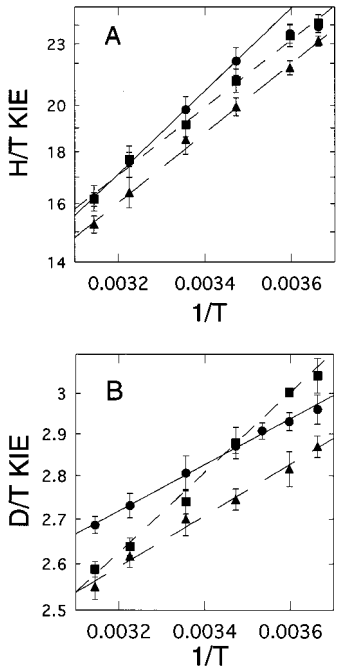


FIGURE 2: Temperature dependence of H/T KIE's (A) and D/T KIE's (B) for the GO glycoforms: 136 kDa (●), 155 kDa (▲), and 205 kDa (■). Data presented on a semilogarithm scale as KIE Arrhenius plots (see explanation and description under Methods). All the experiments were carried out in 10 mM bis-tris propane buffer, pH 9.0, as described under Methods.

Kinetic parameters for the glycoforms, using glucose as the variable substrate and air saturation of oxygen at 25 °C, are summarized in Table 1. As shown, kinetic parameters do not differ significantly among the three glycoforms. This result is in accordance with two previous reports that compared the wild-type (Kalisz et al., 1991) and recombinant forms (Frederick et al., 1990) of enzyme to their deglycosylated forms. This is the first comparative characterization of the 136, 155, and 205 kDa GO glycoforms.

Temperature Dependence of H/T and D/T Kinetic Isotope Effects. The H/T and D/T KIE's for the three GO glycoforms were measured with the substrate 2-deoxyglucose (as described under Methods). The temperature was varied from 0 to 45 °C, and the results are presented in Figure 2. Values for the KIE's in the limit of infinite temperature are listed in Table 2.

Table 2: Values for A_H/A_T and A_D/A_T[a]

glycoform (kDa)	A_H/A_T[b]	A_D/A_T[c]
136	0.84 (±0.34)	1.47 (±0.09)
155	1.28 (±0.18)	1.30 (±0.10)
205	1.46 (±0.20)	0.89 (±0.04)

[a] Nonlinear least root mean square fitted Arrhenius preexponential factors and their standard experimental error (calculated as described under Methods). [b] The semiclassically calculated values range from 0.6 to 1.6 for A_H/A_T. [c] The semiclassically calculated values range from 0.9 to 1.2 for A_D/A_T (Schneider & Stern, 1972; Bell, 1980; Melander & Saunders, 1987).

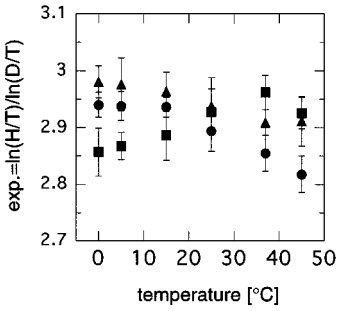


FIGURE 3: ln (H/T) to ln (D/T) ratio (the exponent of eq 5) as a function of temperature (in degrees centigrade). The exponents are the ratio between data from Figure 2A,B. Accordingly, the glycoforms are shown as 136 kDa (●), 155kDa (▲) and 205kDa (■).

From the data presented in Figure 2A for the H/T KIE, the differences among the three glycoforms appear to be small and close to experimental error. The magnitudes of KIE's on the Arrhenius preexponential factors (A_H/A_T) are all within the range for semiclassical calculated values (Table 2). By contrast, significant differences can be seen among the glycoforms for the D/T measurements (Figure 2B). The A_D/A_T for the heaviest GO glycoform (205 kDa) is at the lower semiclassical limit (0.9). The ratio for the wild-type enzyme (155 kDa) is slightly above the upper semiclassical limit (1.22), and the preexponential KIE ratio for the lightest, deglycosylated form (136 kDa) is significantly above this limit (Table 2). This latter observation is quite rare but has been predicted and observed for reactions characterized by substantial amounts of tunneling (Jonsson et al., 1996).

Kinetic Complexity. The different trends observed for A_H/A_T and A_D/A_T among the three glycoforms suggest that kinetic complexity contributes to the H/T KIE's. Comparison of the experimental magnitude of H/T to D/T KIE between 0 and 45 °C indicates the following relationship:

$$k_H/k_T < (k_D/k_T)^{3.26-3.34} \quad (4)$$

where k_H/k_T and k_D/k_T are H/T and D/T KIE, respectively. As previously discussed (Cha et al., 1989; Bahnson et al., 1993; Bahnson & Klinman, 1995, and references cited therein), the behavior of eq 4 differs from the semiclassical relationship (Swain et al., 1958; Saunders, 1985):

$$k_H/k_T = (k_D/k_T)^{3.26-3.34} \quad (5)$$

and suggests that some kinetic complexity "masks" the intrinsic H/T KIE.

The magnitudes of the exponential relationship between measured H/T and D/T KIE are plotted in Figure 3 as a function of temperature. The distribution of the exponent values is rather narrow (2.82−2.98), suggesting a small temperature dependence for the exponents. All the exponents are somewhat less than 3.0 and fit eq 4 above. The observed H/T isotope effect, $(k_H/k_T)_{obs,}$ may thus be smaller than the intrinsic effect, $(k_H/k_T)_{int}$, according to eq 6 (Northrop, l977):

$$(k_H/k_T)_{obs} = [(k_H/k_T)_{int} + C_H]/(1+C_H) \quad (6)$$

where C_H is the commitment to catalysis, defined as the ratio of rate constants for the chemical step versus dissociation of substrate or product from the enzyme. The value of C_H for protonated 2-deoxyglucose at 25 °C is estimated to be 0.5 from eq 6, using the observed D/T KIE to calculate the intrinsic H/T KIE from eq 5. This calculation assumes that the commitment for the D/T KIE is insignificant, consistent with the magnitude of the D/T KIE at 25 °C which is larger than predicted from semiclassical considerations (Melander & Saunders, 1987). For reactions with moderate commitments and large primary KIE's, the contribution of C_D to $(k_D/k_T)_{obs}$ becomes quite small [cf. Grant and Klinman (l989)]. The value for C_H could be greater than 0.5 if extensive tunneling is present for the protonated substrate (Cha et al., l989). In the case of such tunneling, deviations of A_H/A_T from classical Arrhenius behavior, as well as deviation of exponents from their semiclassical value, are expected to be masked by the kinetic complexity. This is almost certainly the case for the H/T KIE data reported herein.

The possibility that the high A_D/A_T preexponential factor for the 136 kDa glycoform is an artifact caused by a temperature-dependent commitment can be ruled out by following the trend in exponents at increased temperature. From Figure 3, it is clear that the commitment increases (exponent decreases) with increasing temperature for the two lighter glycoforms (136 and 155 kDa). That should cause a steeper Arrhenius slope and a lower observed KIE on the preexponential factor. In other words, the observed D/T KIE on the pre-exponential factor for these two glycoforms could be lower then the intrinsic one. Interestingly, the opposite trend in exponents occurs for the heavy (205 kDa) glycoform such that the observed preexponential factor D/T isotope effect may be larger than the intrinsic value. Overall, the contribution of a small, temperature-dependent commitment to the observed D/T isotope effects would only increase the differences seen among the three glycoforms.

From Figure 2A, it is noticeable that the plot for the H/T isotope effect with the lightest glycoform is curved at low temperatures (under 15 °C), indicative of a change in the rate-limiting step. Since the exponents for the 136 kDa glycoform decrease with increasing temperature, the chemical step appears to be more rate determining at low temperature. Using only the low-temperature region of the curve in Figure 2A, a KIE on the preexponential factor of 5.7 (±3.2) is calculated. This value is significantly higher than the value of 0.8 obtained with the full data set. Thus, the intrinsic value of A_H/A_T may be considerably in excess of 1.6 (the upper limit of the semiclassical range).

Enthalpy of Activation for Oxidation of [1-^{2}H]-2-Deoxyglucose. The enthalpy of activation for the oxidation of [1-^{2}H]-2-deoxyglucose was determined with the three glycoforms. As argued above, the rate-determining step with anomerically deuterated 2-deoxyglucose is fully (or close to fully) rate-limited by C−D bond cleavage, whereas C−H bond cleavage is only partially rate-determining. We

Table 3: K_M Values for O_2 and 2-Deoxyglucose with GO[a]

glycoform mass (kDa)	O_2 K_M (μM)	2-deoxyglucose K_M (mM)
	[25 °C]	
205	86 (±5)	29 (±2)
155	87 (±6)	31 (±3)
136	93 (±4)	30 (±2)
	[45 °C]	
205	280 (±20)	60 (±4)
155	280 (±10)	60 (±5)
136	270 (±30)	60 (±7)

[a] O_2-saturated concentration at 25 °C and 45 °C was 1150 and 920 μM, respectively. 2-Deoxyglucose concentration was 500 mM.

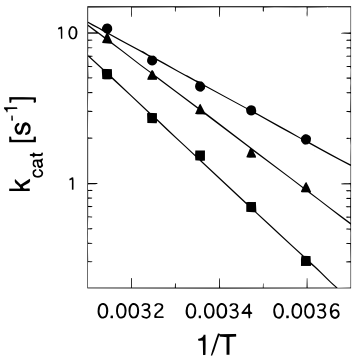


FIGURE 4: Arrhenius plot of [1-^{2}H]-2-deoxyglucose oxidation rates (k_{cat}) with the three GO glycoforms: 136 kDa (●), 155 kDa (▲), and 205 kDa (■). All experiments were carried out in 10 mM bis-tris propane buffer, pH 9.0, with 0.5 M substrate and under pure oxygen as described under Methods.

therefore synthesized [1-^{2}H]-2-deoxyglucose on a gram scale (see Materials). Initial velocities for its oxidation with the three GO glycoforms were determined at different temperatures by following the rate of oxygen consumption using an oxygen electrode (see Methods).

An unambiguous determination of the enthalpy of activation on the C−D bond cleavage step requires that measurements of k_{cat} be under full substrate saturation over the experimental temperature range. Values of K_M for 2-deoxyglucose and oxygen were therefore determined at 25 and 45 °C and are summarized in Table 3. The K_M values for 2-deoxyglucose were found to be 30−60 mM, such that saturation with this substrate could be ensured by maintaining its concentration at 0.5 M (there was no indication for any substrate inhibition). In the case of O_2, the K_M is seen to rise to 276 μM at 45 °C. Since saturation with pure O_2 is 0.9 mM at this temperature, it was not possible to fully saturate enzyme with oxygen at elevated temperatures (under atmospheric pressure). A simple Michaelis factor was therefore applied to correct the k_{cat} values at increasing temperature:

$$k_{cat} = V_{max(obs)}(K_M + [S])/[E][S] \quad (7)$$

where $V_{max(obs)}$ is the observed V_{max}, [S] is the oxygen concentration, and [E] is the enzyme concentration. This follows from the ping-pong steady-state kinetic mechanism of GO (Swoboda & Massey, 1965; Bright & Gibson, 1967). The overall correction to the Arrhenius slope was less than a factor of 1.14 for all glycoforms, and the trend among them was not affected. From Figure 4, it is visibly apparent that the Arrhenius slopes (and subsequently the enthalpies of activation) increase with the size of the polysaccharide

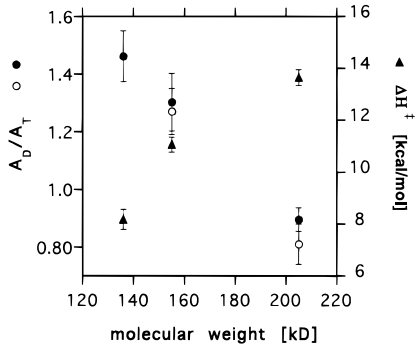


FIGURE 5: Relationship between the KIE on the Arrhenius preexponential factors A_D/A_T (● and ○), and enthalpies of activation for [1-^{2}H]-2-deoxyglucose (▲). All the experiments were under the same experimental conditions (see Methods). The A_D/A_T semiclassical calculated range is 0.9−1.22 (see text). For the 155 and 205 kDa glycoforms, two independent measurements of A_D/A_T are shown [○ are from Jonsson (l994)]. The earlier measurements of A_D/A_T (○) are within experimental error of more recent measurements of A_D/A_T (●), although the magnitude of individual isotope effects was smaller by 20−25%.

envelope on the surface of the GO glycoforms. The enthalpies of activation are plotted in Figure 5, together with the observed values for A_D/A_T. There is a clear trend in these data, whereby the A_D/A_T takes on increasingly larger values as the enthalpy of activation gets smaller. These trends are ascribed to different degrees of tunneling for the three glycoforms of GO, as discussed below.

DISCUSSION

Contribution of Hydrogen Tunneling to Glucose Oxidase Catalysis. The isotope effects on the Arrhenius prefactors (A_H/A_T and A_D/A_T) for the deglycosylated (136 kDa) glycoform of glucose oxidase appear to be significantly larger than unity. The experimental value of A_D/A_T of 1.46 is essentially free of complications due to multiple rate-determining steps and is clearly larger than the upper limit of 1.2 possible without tunneling (Bell, 1980). In a recent study, it has been proposed that hydrogen tunneling in enzyme reactions can lead to several types of temperature dependencies for rates and isotope effects. A diagram, based on earlier experimental and computational results of others (Bromberg et al., 1970; Stern & Weston, 1974; Glickman et al., 1994; Hwang & Grissom, 1994), was proposed to encompass the range of biological observations (Jonsson et al., 1996). Reproduced in Figure 6, this diagram shows ln (rate) as a function of inverse temperature for two arbitrary isotopes, L_1 and L_2 (any combination of H, D, and T), where 1 represents the lighter isotope and 2 the heavier one. Curvature is a consequence of increasing hydrogen tunneling, which in this simplified picture can lead to a temperature independence of both rates and isotope effects.

In reality, due to various experimental complications, measurement of rates and isotope effects is usually limited to a fairly narrow temperature range. In the case of enzymatic reactions under physiological conditions, the typical experimental range is 0−50 °C. In such a narrow temperature range, curvature due to tunneling is unlikely to be observed. Apparent isotope effects on Arrhenius prefactors, extrapolated from the accessible temperature range, give information about the position of the experimental system on the diagram of Figure 6. Classical behavior

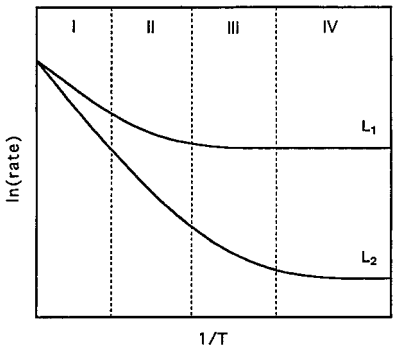


FIGURE 6: Schematic representation of models suggesting various A_1/A_2 values associated with different degrees of tunneling. In this diagram, systems are shown that include no tunneling (I), $A_1/A_2 = 1$; a moderate tunneling contribution (II), $A_1/A_2 < 1$; and more extensive tunneling (III), $A_1/A_2 \geq 1$; and (IV), $A_1/A_2 \gg 1$. The plot is an ordinary Arrhenius plot of ln (rate) versus reciprocal absolute temperature ($1/T$); experimentally, the temperature range is generally restricted to between 0 and 50 °C for enzyme reactions. The upper line represents the light isotope behavior (L_1), and the lower line represents the behavior of the heavy isotope (L_2).

(region I) leads to a linear behavior of ln (rate) or ln (isotope effect) vs inverse temperature, and values for A_1/A_2 close to unity. Tunneling of the lighter isotope to a greater extent than that of the heavier one is expected to lead to A_1/A_2 values less than unity (region II). This type of behavior has been observed in many small molecule reactions in solution [see Bell (l980) and Melander and Saunders (l987) for overviews] and also recently in the reactions catalyzed by bovine serum amine oxidase (Grant & Klinman, l989) and monoamine oxidase B (Jonsson et al., l994). More extensive tunneling (regions III and IV) is expected to give A_1/A_2 values that vary from less than unity to significantly larger than unity, depending on the curvature in the accessible temperature range. In an extreme case, rates and isotope effects are close to temperature-independent (i.e., the process is close to activationless), and the entire isotope effect can be expressed in the Arrhenius prefactor. Behavior of this type has recently been proposed for the soybean lipoxygenase reaction at room temperature (Jonsson et al., l996).

How do the glucose oxidase data fit into this description? Within the simplicity of Figure 6, the different nature of hydrogen transfer for the three GO glycoforms can be rationalized. According to this diagram, the heaviest glycoform (recombinant GO: 205 kDa) is represented by system III (or I, tending toward a system II behavior). The 155 kDa wild-type glycoform suits a system between III and IV, and the lightest glycoform (deglycosylated GO: 136 kDa) is represented by system IV. The above description predicts a correlation between the enthalpy of activation and the extent of tunneling. Using 2-deoxyglucose to study the C−D bond cleavage step directly, the enthalpy of activation is found to correlate with the degree of protein glycosylation (Figure 4). Most significantly, a decrease in protein glycosylation is found to lead to *both* an increase in A_D/A_T and a decrease in the enthalpy of activation. The smallest A_D/A_T value (approximately 0.9) is accompanied by the largest activation enthalpy (13.6 kcal/mol), while the largest A_D/A_T value (approximately 1.5) is accompanied by the smallest activation enthalpy (8.2 kcal/mol). It appears that decreased glycosylation leads to an increase in the degree of tunneling for the oxidation of 2-deoxyglucose by glucose oxidase.

Correlation between the Degree of Tunneling and Reaction Rate. It might be expected that an increased degree of tunneling would lead to an increased turnover rate. Inspection of Figure 4 shows that at 25 °C, the deglycosylated (136 kDa) variant of glucose oxidase has a k_{cat} roughly 3-fold higher than the highly glycosylated recombinant form (205 kDa). While this is not a large difference in turnover rates, it can be understood in the context of Figure 6. The experimental values for A_1/A_2 represent tangents to the curves of Figure 6. In regions where ln (k) vs $1/T$ is close to linear, parameters that perturb k_{cat} may lead to imperceptible changes in A_1/A_2. By contrast, when ln (k) vs $1/T$ begins to curve appreciably (region III), relatively small changes in k_{cat} may lead to significant differences in A_1/A_2 which could then vary from inverse to normal [cf. Jonsson et al. (1996)]. We believe this latter behavior pertains to GO.

In addition to the studies on 2-deoxyglucose oxidation, catalytic parameters have been determined for the three glycoforms of glucose oxidase using the natural substrate glucose under optimal conditions (pH 5.6) (Table 1). As discussed earlier, 2-deoxyglucose was chosen as substrate for isotope effect studies, as there was evidence that the oxidation of glucose was controlled (at least in part) by steps other than hydrogen transfer. The rate parameters measured for the oxidation of glucose (Table 1) are essentially identical for the three glycoforms. This is an intriguing result, suggesting that substrate binding and/or product release steps have not been affected by the degree of glycosylation of the enzyme. The different behavior of the substrates glucose and 2-deoxyglucose supports our interpretation that the effect of glycosylation is on the hydrogen transfer step.

Structural versus Dynamic Effects of Glycosylation. The above findings imply that glycoforms modify the nature of hydrogen transfer in the glucose oxidase catalyzed reaction. The polysaccharide envelope could cause two basic changes affect the hydrogen transfer, either changing the active site structure or modifying enzyme dynamics that alter the tunneling probability. Unfortunately, an X-ray structure is only available for the deglycosylated form of GO (Hecht et al., 1993), precluding a comparison of three-dimensional structures among the glycoforms. However, three approaches enable us to evaluate the effect of surface glycosylation on the active site conformation. First, the catalytic parameters for the three glycoforms of glucose oxidase using glucose as substrate (Table 1) show no significant difference, suggesting that substrate binding and/or product release steps have not been affected by the degree of glycosylation. Second, the crystal structure of the 95% deglycosylated wild-type GO (Hecht et al., 1993) indicates that all the glycosylation sites are on the surface of the protein, opposite to the dimer interface and at least 23 Å away from the active site. Third, numerous glycoenzymes have been examined by a variety of techniques (X-ray crystallography, NMR, CD, etc.). Most of the enzymes show a very small interaction of the polysaccharide with the surface of the protein and no conformational effect of polysaccharide on the interior regions (Varghese et al., 1995; Shaw et al., 1993; Puett, 1973; Williams et al., 1987; Wlodawer et al., 1988; Terashima et al., 1994; Rudd et al., 1994; Mer et al., 1996). On the other hand, for some enzymes, the glycosylation may play a role in the enzyme globular folding and may have a major effect on the protein structure, in the event of glycosylation-dependent misfolding (Grochulski et al., 1994; Hickman et

al., 1977; Riederer & Hinnen, 1991; Marquardt & Helenius, 1992; Wyss et al., 1995). The last examples are probably not relevant to GO since there is no evidence that glycosylation causes a major change in protein structure. The polysaccharide chains of GO most likely do not interact directly with the protein surface or the active site, undergoing their primary solvation by water.

An alternative explanation for the effect of glycosylation on GO catalysis is through changes in protein dynamics. Recently, several works have reported that glycosylation can modify the dynamic stability and functional activity of an enzyme (Mer et al., 1996; Rudd et al., 1994). In the latter studies, comparative X-ray crystallographic studies, CD and NMR techniques, and NH−ND exchange data were used to demonstrate that the three-dimensional structure of ribonuclease was unaffected by glycosylation whereas dynamic fluctuations had changed. In this study, enzyme activity was found to correlate with decreased glycosylation and protein rigidity. Due to the nature of the catalyzed reaction, however, it was not possible to distinguish which steps of the reaction (binding, chemical steps, etc.) were being affected by glycosylation. By contrast, the results on GO reported herein demonstrate an effect on the isotopically sensitive hydrogen transfer step. This implies that protein dynamics may be involved in the transition state of the bond cleavage step.

A Role for Protein Dynamics in Tunneling? Two questions, with many general applications, arise: First, what is the mechanism whereby glycosylation could modify protein dynamics; and second, how does such a change in protein dynamics affect tunneling in an enzymatic reaction?

A possible answer to the first question might lie in the relationship of surface changes to internal protein dynamics. Recently, Yedgar and co-workers provided direct evidence that viscosity decreases the specific volume and the adiabatic compressibility of the protein interior (Preiv et al., 1996). Based on the kinetics of tryptophan phosphorescence decay, Cioni and Strambini (1994) hypothesized that viscosity and hydrostatic pressure act similarly in decreasing internal free volume and increasing protein rigidity. Rudd et al. (1994) and Mer et al. (1996) described a similar phenomenon where glycosylation decreased dynamic fluctuations throughout the molecule. It is possible that protein glycosylation leads to a marked change in solvation at the protein surface that resembles the effects of viscosity, and possibly hydrostatic pressure.

As for the second question, many models connecting protein dynamics to its function have been described and reviewed [cf. Welch, et al. (1982), Welch (1986), Gregory (1995), Williams (1995), and Mulholiand and Karplus (1996) and references cited therein]. Several investigators have simulated hydrogen transfer processes incorporating both protein dynamics and quantum-mechanical tunneling (Sumi & Ulstrup, 1988; Borgis & Hynes, 1989; Bruno & Bialek, 1992; Bala et al., 1996). These models demonstrate that hydrogen tunneling in enzymatic systems may be mediated by thermal fluctuations, leading to a decrease in the tunneling distance and disruption of the tunneling coherence with a concomitant localization of the particle to the product side ("dissipative tunneling").

Experimentally, various techniques have been used to examine the role of protein dynamics in enzyme action. Examples of these techniques are fluorescence quenching (Matko et al., 1980), X-ray crystallography (Lumry, 1995), Rayleigh scattering of Mossbauer radiation (Goldanskii & Krupyanskii, 1995), volumetric measurements (Timasheff, 1993), ultrasonic absorption (Preiv et al., 1996), kinetic studies (Gavish & Werber, 1979), flash photolysis (Beece et al., 1980), fluorescence lifetime studies (Farnum et al., 1991), and NMR and site-directed mutagenesis (Li et al., 1992). Many of these studies conclude a link between protein dynamics and reaction rate, but none could distinguish effects on internal enzyme−substrate rearrangements or product release steps from effects on the chemical step. By using isotope effects, we have provided evidence that the hydrogen transfer step in an enzyme-catalyzed reaction is modified by remote changes in glycosylation on the surface of the protein. Thus, our results may provide the first instance of a connection between protein dynamics and an isolated chemical step of an enzyme reaction. Work is underway to test the validity of this suggestion by assessing the effect of protein glycosylation on GO mobility. Additionally, we plan to alter the surface properties of GO by chemical modification and to investigate the consequence of these changes to the hydrogen transfer step.

ACKNOWLEDGMENT

We thank Dr. Steven Rosenberg (Chiron Co., Emeryville, CA) for the generous gift of recombinant glucose oxidase.

REFERENCES

Bahnson, B. J., & Klinman, J. P. (1995) *Methods Enzymol.* 249, 373−397.
Bahnson, B. J., Park, D., Kim, K., Plapp, B. V., & Klinman, J. P. (1993) *Biochemistry* 32, 5503−5507.
Bala, P., Grochowski, P., Lesyng, B., & McCammon, J. A. (1996) *J. Phys. Chem.* 100, 2535−2545.
Beece, D., Eisenstein, L., Frauenfelder, H., Good, D., Marden, M. C., Reinisch, L., Reynolds, A. H., Sorensen, L. B., & Yue, K. T. (1980) *Biochemistry* 19, 5147−5157.
Bell, R. P. (1980) *The Tunneling Effect in Chemistry*, Chapman and Hall, London and New York.
Borgis, D., & Hines, J. T. (1989) in *The Enzyme Catalysis Process* (Cooper, A., Houben, J., & Chien, L., Eds.) pp 293−303, Plenum, New York.
Bright H. J., & Gibson, Q. H. (1967) *J. Biol. Chem.* 242, 994−1003.
Bright, H. J., & Appleby, M. (1969) *J. Biol. Chem.* 244, 3625−3634.
Bromberg, A., Muszkat, K. A., & Warshel, A. (1970) *J. Chem. Phys.* 52, 5952−5959.
Bruno, W. J., & Bialek, W. (1992) *Biophys. J.* 63, 689−699.
Cha, Y., Murray, C. J., & Klinman, J. P. (1989) *Science* 243, 1325−1330.
Cioni, P., & Strambini, G. B. (1994) *J. Mol. Biol.* 242, 291−301.
Crueger, A. & Crueger, W. (1990) in *Microbial Enzymes and Biotechnology* (Rogarty, W. M., & Kelly, C. T., Eds.) pp 177−227, Elsevier Applied Science, London.
Farnum, M. F., Magde, D., Howell, E. E., Hirai, J. T., Warren, M. S., Grimsley, J. K., & Kraut, J. (1991) *Biochemistry 30*, 11567−11579.
Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman, New York.
Frederick, K. R., Tung, J., Emerick, R. S., Masiarz, E. F., Chamberlain, S. H., Vasavada, A., Rosenberg, S., Chakraborty, S., Schopter, L. M., & Massey, V. (1990) *J. Biol. Chem. 265*, 3793−3802.
Gavish, B., & Werber, M. M. (1979) *Biochemistry 18*, 1269−1275.
Gavish, B., & Yedgar, S. (1995) in *Protein−Solvent Interactions* (Gregory, R. B., Ed.) pp 343−373, Marcel Dekker, New York.
Gibson, Q. H., Swoboda, B. E. P., & Massey, V. (1964) *J. Biol. Chem. 239*, 3927−3934.

Glickman, M. H., & Klinman, J. P. (1995) *Biochemistry 34*, 14077−14092.
Glickman, M. H., Wiseman, J. S., & Klinman, J. P. (1994) *J. Am. Chem. Soc. 116*, 793−794.
Goldanskii, V. I., & Krupyanskii, Y. F. (1995) in *Protein−Solvent Interactions* (Gregory, R. B., Ed.) pp 289−326, Marcel Dekker, New York.
Grant, K. L., & Klinman, J. P. (1989) *Biochemistry 28*, 6597−6605.
Gregory, R. B. (1995) *Protein−Solvent Interactions* (Gregory, R. B., Ed.) Marcel Dekker, New York.
Hecht, H. J., Kalisz, H. M., Hendle, J., Schmid, R. D., & Schomburg, D. (1993) *J. Mol. Biol. 229*, 153−172.
Hickman, S., Kulczycki, A., Lynch, R. G., Kornfeld, S. (1977) *J. Biol. Chem. 252*, 4402−4408.
Hwang, C. C., & Grissom, C. B. *J. Am. Chem. Soc. 116*, 795−796.
Isbell, H. S., Holt, N. B., and Frush, H. L. (1962) in Methods in Carohydrate Chemistry (Whistler, R. L., and Wolfarm, M. L. Ed.) Academic Press, New York, pp 276−280.
Jencks, W. P. (1987) *Catalysis in Chemistry and Enzymology*, Dover, New York.
Jonsson, T. (1994) Thesis, University of California at Berkeley, Berkeley, CA.
Jonsson, T., Glickman, M., Sun, S., and Klinman, J. P. (1996) *J. Am. Chem. Soc.* in press.
Kalisz, H. M., Hecht, H. J., Schomburg, D., & Schmid, R. D. (1990) *J. Mol. Biol. 213*, 207−209.
Kalisz, H. M., Hecht, H. J., Schomburg, D., and Schmid, R. D. (1991) *Biochim. Biophys. Acta 1080*, 138−142.
Kraut, J. (1988) *Science 242*, 533−242.
Kriechbaum, M., Heilmann, H. J., Wientjes, F. J., Hahn, M., Jany, K. D., Gassen, H. G., Sharif, F., & Alaeddinoglu, G. (1989) *FEBS Lett.* 255, 63−66.
Li, L., Falzone, C. J., Wright, P. E., & Benkovic, S. J. (1992) *Biochemistry 31*, 7826−7833.
Lockridge, O., Massey, V., & Sullivan, P. A. (1972) *J. Biol. Chem. 247*, 8097−8106.
Lumry, R. (1995) in *Protein-Solvent Interactions* (Gregory, R. B., Ed.) pp 1−142, Marcel Dekker, New York.
Marquardt, T., & Helenius, A. (1992) *J. Cell Biol. 117*, 505−513.
Matko, J., Tron, L., Balazs, M., Hevessky, J., Somogyi, B., & Damajanovich, S. (1980) *Bichemistry 19*, 5782.
Melander, L., & Saunders, W. H. (1987) *Reaction Rates of Isotopic Molecules*, R. E. Krieger Publishing Co., Malabar, FL.
Mer, G., Hietter, H., & Lefevre, J. (1996) *Nat. Struct. Biol. 3*, 45−53.
Mulholland, A. J., & Karplus, M. (1996) *Biochem. Soc. Trans. 24*, 247−254.
Northrop, D. B. (1977) in *Isotope effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) p 122, University Park Press, Baltimore, MD.
Pazur, J. H., & Kleppe, K. (1964) *Biochemistry 3*, 578−583.
Priev, A., Almagor, A., Yedgar, S., & Gavish, B. (1996) *Biochemistry 35*, 2061−2066.
Puett, D. (1973) *J. Biol. Chem. 218*, 3566−3572.
Riederer, M. A., & Hinnen, A. (1991) *J. Bacteriol. 173*, 3539−3546.
Rudd, P. M., Joao, H. C., Coghill, E., Fiten, P., Saunders, M. R., Opdenakker, G., & Dwek, R. A. (1994) *Biochemistry 33*, 17−22.
Saunders, W. H. (1985) *J. Am. Chem. Soc. 107*, 164−169.
Schneider, M. E., & Stern, M. J. (1972) *J. Am. Chem. Soc. 94*, 1517−1522.
Shaw, A. L., Rothnagel, R., Chen, D., Ramig, R. F., Chiu, W., & Prasad, B. V. V. (1993) *Cell 74*, 693−701.
Stern, M. J., & Weston, R. E. (1974) *J. Chem. Phys. 60*, 2803−2807, 2808−2814, 2814−2821.
Sumi, H., & Ulstrup, J. (1988) *Biochim. Biophys. Acta 955*, 26−42.
Swain, C. G., Stivers, E. C., Reuwer, J. F., & Schaad, L. J. (1958) *J. Am. Chem. Soc. 80*, 5885−5893.
Swoboda, B. E. P., & Massey, V. (1965) *J. Biol. Chem. 240*, 2209−2215.
Timasheff, N. S. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 67−97.
Varghese, J. N., Epa, V. C., & Colman, P. M. (1995) *Protein Sci. 4*, 1081−1087.
Welch, G. R. (1986) *The Fluctuating Enzyme* (Welch, G. R., Ed.) J. Wiley and Sons, New York.
Welch, G. R., Somogyi, B., & Damjanovich, S. (1982) *Prog. Biophys. Mol. Biol. 39*, 109−146.
Williams, R. J. P. (1995) *Eur. J. Biochem. 234*, 363−381.
Williams, R. L., Green, S. M., & McPherson, A. (1987) *J. Biol. Chem. 263*, 16020−16031.
Wlodawer, A., Svensson, A. L., Sjolin, L., & Gilliland, G. L. (1988) *Biochemistry 27*, 2705.
Wyss, D. F., Choi, J. S., Li, J., Knoppers, M. H., Willis, K. J., Arulanandam, A. R., & Smolyar, A. (1995) *Science 269*, 1273−1278.

BI962492R