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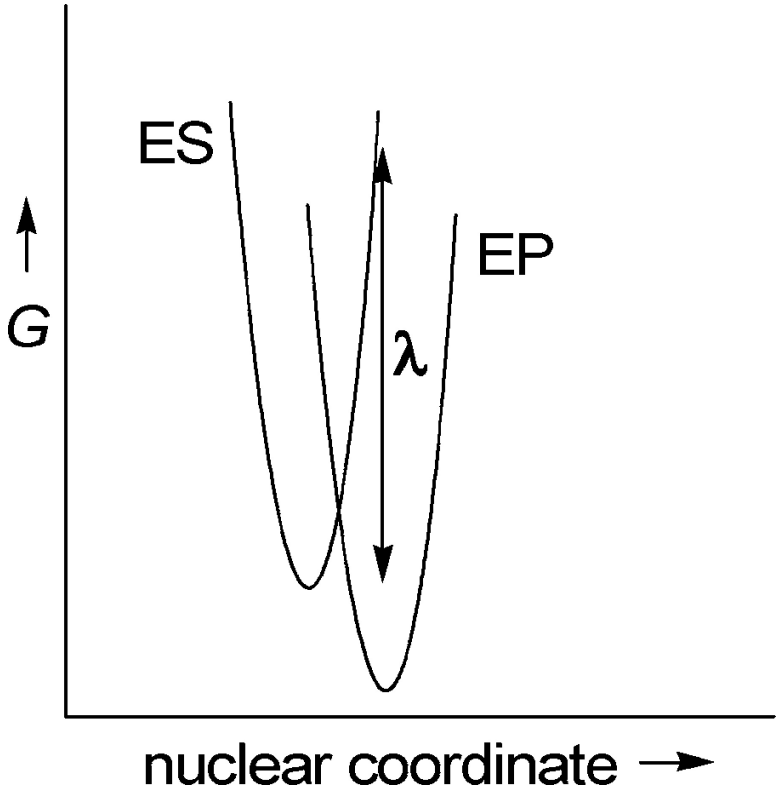

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Determination of a Large Reorganization Energy Barrier for Hydride Abstraction by Glucose Oxidase

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How protein environments facilitate the cleavage of strong chemical bonds is a fundamental problem in enzyme catalysis with implications ranging from biotechnology to drug design.[1] Computational studies have suggested roles for protein motion and optimized electrostatics in modulating the barriers to these reactions.[2] Yet the experimental data needed to test such predictions are rarely available. Described here is a case that is an exception, where the mechanism of C−H oxidation is defined and the intrinsic barrier analyzed within the context of Marcus theory.[3]

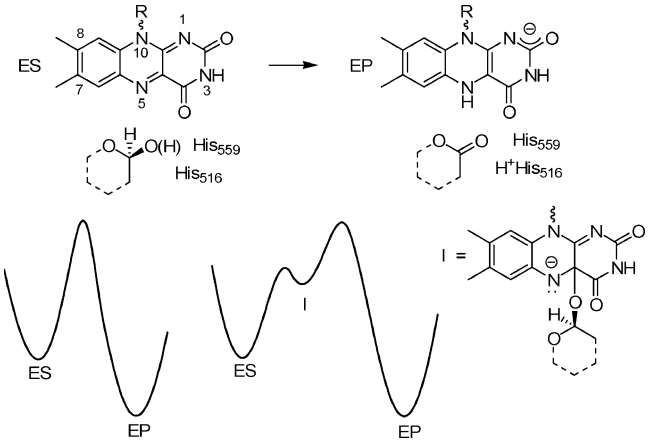
Glucose oxidase (GO) uses a noncovalently bound flavin (FAD) to oxidize sugars to lactones.[4] The reduced cofactor ($FADH^-$) is reoxidized by O_2 in a ping-pong kinetic mechanism.[5] Two descriptions of the sugar oxidation step have persisted over the years (Scheme 1). In one, FAD abstracts H^- from the bound substrate in a single step. In the alternative, two steps are involved with nucleophilic attack upon the isoalloxazine ring preceding intramolecular H^+ transfer. Both mechanisms allow for the rate-limiting cleavage of the anomeric C−H of the substrate as indicated by kinetic isotope effects.[6] Further, these isotope effect studies have provided evidence that protein motions impact the probability of hydrogen tunneling. It has been proposed that such quantum effects may be linked to catalytic rate accelerations in a number of C−H oxidizing enzymes including glucose oxidase.[7]

In previous studies, it was shown that reconstitution of the GO apoprotein with the chemically modified cofactors **7Me 8Cl**, **7H 8Cl**, and **7,8-Cl** (for numbering, see Scheme 1) affords active enzymes with turnover rates that are accelerated relative to that of the native GO, which contains **7,8-Me**.[8] The 5-deazaflavin (**5-Deaza**) and 8-OH 5-deazaflavin cofactors (**5-Deaza 8OH**), in which N(5) is replaced by a CH, have also been incorporated into apoGO.[9] All of the proteins react rapidly with D-glucose or 2-deoxy-D-glucose at pH 5, evidenced by bleaching of the absorbance (400−450 nm) due to the oxidized cofactor. No reaction occurs upon treating the unbound flavins with excess sugar under the same conditions.

Redox potentials ($E_{1/2}$ vs NHE) determined for the free cofactors correspond to $2e^-$ processes.[10] A small effect due to the protein environment is indicated at pH 7, where $E_{1/2} = -0.208$ V for free **7,8-Me** and $E_{1/2} = -0.184$ V for native GO.[11] Since $2e^- + 1H^+$ is thermodynamically equivalent to H^-, the driving force for H^- abstraction can be estimated using a thermochemical cycle as previously described by Parker.[12]

The driving force for the oxidation of 2-deoxyglucose recast in terms of the relative affinities for H^- and H^+ ($\equiv 2e^- + 2H^+$) is $\Delta G^\circ_{redox} = -F(E^\circ_{ox} - E^\circ_{red})$, where F is Faraday's constant (23.06 kcal mol^{-1}/V) and the E° are the $E_{1/2}$ for the oxidant and reductant extrapolated to pH 0. The $E_{1/2}$ (pH 7) $= -0.469$ V was determined for 2-deoxygluconolactone/2-deoxyglucose by cyclic voltammetry.[13] This value is only 0.034 V more positive than that reported for glucose/gluconolactone at the same pH.[14] ΔG°_{redox} reflects the reversible conversion of the unbound reactants and products. For **5-Deaza** and **7,8-Me**, the values are close to the ΔG°_{eq} extracted from equilibrium measurements upon adding sugar to the enzyme-bound cofactors.[13] Comparison of ΔG°_{redox} and ΔG°_{eq} suggests a 2.0 kcal mol^{-1} greater affinity of the enzyme for the substrate over the product. This small difference is included in the ΔG°_{eq} for the reversible conversion of ES to EP in Table 1.

Scheme 1. Reaction Coordinate Diagrams for C−H Oxidation by GO



Stopped-flow experiments were performed under N_2 with 2-deoxyglucose (pH 5, acetate buffer, $\mu = 0.1$ M).[15] Data were fitted by a single-exponential decay to obtain rate constants (k_1 and k_2) at saturating (≥ 0.5 M) and presaturating (< 0.05 M) substrate concentrations. No evidence for intermediates was obtained by global analysis of the data collected between 350 and 600 nm.

Analysis of the single-turnover reaction kinetics together with the thermodynamics provides insight to the mechanism of sugar oxidation. The similar increasing slopes observed when k_1 and k_2 are plotted versus $-\Delta G^\circ_{eq}$ (Figure 1) indicate the following: (i) substrate binding, which is reflected in k_2 but not in k_1, has a negligible impact on the driving force dependence of the reaction barrier, (ii) the rate-limiting steps at high and low substrate concentrations are the same, and (iii) the redox mechanism is most likely H^- abstraction. Decrease of k_1 in response to increasing $-\Delta G^\circ_{eq}$ would be expected for a two-step mechanism in which H^+ transfer within a negatively charged intermediate (**I**) is rate-limiting (cf. Scheme 1). **I** should be stabilized by electron-withdrawing groups, making the product-forming step less thermodynamically favorable and consequently reducing k_1. In further support of H^- abstraction, electrochemical and kinetic data for **8-OH 5-Deaza**[16] agree with the trend defined by the enzymes containing flavin cofactors. Its nicotinamide-like structure makes the deazaflavin unsusceptible to nucleophilic attack, thus precluding a two-step mechanism.

Marcus theory[3] is a useful starting point for discussions of enzyme catalysis. The formalism in eq 1 expresses the free energy activation barrier ($\Delta G^\ddagger$) for a unimolecular reaction in intrinsic (kinetic) and thermodynamic terms, reorganization energy (λ) and

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Table 1. Kinetic and Thermodynamic Parameters for Oxidation of 2-Deoxyglucose by GO at pH 5 and 25 °C unless Noted.[a] Errors are $\pm 2\sigma$

cofactor	$E_{1/2}$ at pH 7 (V)[b]	$\Delta G°_{redox}$ (kcal mol^{-1})[c]	$\Delta G°_{eq}$ (kcal mol^{-1})[c]	$k_1 \times 10^{-2}$ (s^{-1})	$k_2 \times 10^{-3}$ (M^{-1} s^{-1})
7,8-Cl	−0.126	−7.9	(−5.9)	5.25(0.29)	4.03(0.16)
7-Me 8-Cl	−0.137	−7.6	(−5.6)	3.38(0.16)	2.68(0.37)
7-H 8-Cl	−0.144	−7.5	(−5.5)	2.41(0.10)	2.67(0.12)
7,8-Me	−0.208	−6.0	−4.0	0.51(0.06)	0.689(0.254)
5-Deaza	−0.320	−3.4	−1.1		n.d.
8-OH 5-Deaza	−0.350[d]	−2.7	(−0.7)		0.0247[d]

[a] [GO] = 5−20 μM, [2-deoxyglucose] = 0.001−0.5 M. [b] From ref 10 unless noted. Values at pH 0 were calculated from the Nernst equation assuming a change of 60 mV per pH unit. [c] Calculated as described in the text. Values in parentheses were estimated by adding 2.0 kcal mol^{-1} to $\Delta G°_{redox}$. [d] From ref 16.

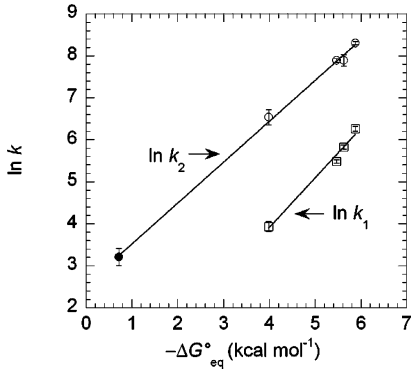

Figure 1. Rate constants as a function of the driving force for H$^-$ abstraction. Data for **8-OH 5-Deaza** (closed circle) are from ref 16.

$\Delta G°$, respectively. In Scheme 1, $\Delta G°$ is the energy gap between ES and EP, and λ is the energy required to change the equilibrium nuclear configuration of ES to that of EP without H$^-$ transfer taking place. Other terms include Boltzmann's (k_B) and Planck's (h) constants, temperature (T) and the reaction probability (κ), which is close to unity for an adiabatic reaction.

$$\Delta G^{\ddagger} = -k_B T \ln\left[\frac{k_1}{(\kappa k_B T/h)}\right] = \frac{(\Delta G° + \lambda)^2}{4\lambda} \qquad (1)$$

Theoretical calculations have indicated that H$^-$ abstraction often occurs in the near-adiabatic regime,[2] where environmental reorganization is strongly coupled to the charge transfer. The extent of the coupling is related to the H$^-$ donor−acceptor distance. In GO, 3.3 Å has been estimated from C(1) of the sugar to N(5) of the FAD.[4] We have used eq 1 with $\kappa = 1$ to estimate $\lambda = 68 \pm 1$ kcal mol^{-1} by fitting ln k_1 as a function of $-\Delta G°_{eq}$. Similar enthalpy and entropy contributions to $\Delta G^{\ddagger}$ and, therefore, to λ are indicated by temperature studies of **7,8-Me**, which reveal $\Delta H^{\ddagger} = 8.6 \pm 0.3$ kcal mol^{-1} and $-T\Delta S^{\ddagger} = 6.3 \pm 2.4$ kcal mol^{-1}.[13]

Two ways by which an enzyme can catalytically lower the barrier to a redox reaction include increasing the favorability of $\Delta G°$ and minimizing λ relative to the reaction in solution. For **7,8-Me** and **5-Deaza**, the $\Delta G°$ from equilibrium measurements is close to the $\Delta G°$ estimated from $E_{1/2}$ of the unbound reactants. Therefore, binding to the protein does not appear to greatly perturb the hydride affinities of these cofactors. Extending the result to the other cofactors, that is, assuming the thermodynamics are altered slightly and in the same way, implies that the catalytic rates in GO do not result from the enhancement of $\Delta G°$. The possibility of catalysis due to optimized protein electrostatics, such that λ is minimized, has been predicted by Warshel.[17] The effect has been simulated for H$^-$ transfer in the NAD$^+$-dependent lactate dehydrogenase and an outer-sphere reorganization of $\lambda_o = 53$ kcal mol^{-1} calculated. In the present study, λ is ∼15 kcal mol^{-1} larger but includes the energy associated with distorting bond lengths and angles within the reactants. Interestingly, λ in GO is only 10 kcal mol^{-1} less than reported for related solution phase H$^-$ abstraction reactions;[18] this translates into a barrier lowering of ca. 2.5 kcal mol^{-1} and a rate acceleration of $<10^2$ s^{-1} (cf. eq 1).

In summary, studies of glucose oxidase containing cofactor analogues reveal a correlation of rate to driving force that is consistent with sugar oxidation by H$^-$ abstraction. This result resolves a mechanistic uncertainty of many years and provides a basis for computational studies. Analysis of the electrochemical and equilibrium data for two of the enzymes studied suggests that the reaction thermodynamics is not significantly altered by the surrounding protein. Application of Marcus theory reveals a large reorganization energy barrier comparable to related reactions in solution. Implications for catalytic rate acceleration and its relationship to hydrogen tunneling in glucose oxidase will be addressed in a forthcoming paper.[19]

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Supporting Information Available: Experimental descriptions and kinetics plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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