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# Binding of protons and zinc ions to transition states for tautomerization of α-heterocyclic ketones: implications for enzymatic reactions

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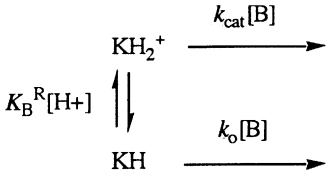
ABSTRACT: The description of catalysis in terms of binding of a catalyst to the transition state propoposed by Kurz is applied to tautomerization of the α-heterocyclic ketones phenacylpyridine, phenacylpyrazine, phenacylphenanthroline and phenylacetylpyridine catalysed by protons and zinc ions. Binding constants for protonated and zinc-coordinated transition states, $K_B^{\neq}$ are reported and Brønsted coefficients are calculated from comparison of $K_B^{\neq}$ with binding constants for the keto reactant and enolate anion intermediate. The formal equivalence of the binding formalism to a conventional Brønsted analysis is emphasized, and the results are compared with those from a 'generalised' Brønsted plot of rate constants against equilibrium constants for reactions of uncomplexed, protonated and zinc ion-coordinated ketones. This plot confirms that intrinsic reactivities of metal-coordinated and protonated substrates are similar even where differences exist between substrates. Application of a comparable Kurz–Brønsted treatment to enzymatic reactions depends in principle upon (a) dissecting binding contributions to catalysis from approximation of covalently reacting groups and (b) separating binding at the reaction site of the substrate, to which Kurz's treatment applies, from 'remote' binding, which, to a first approximation, is unchanged between Michaelis complex and transition state. The Brønsted relationship highlights stabilization of reactive intermediates as a thermodynamic driving force for binding catalysis at the reaction site. A formal expression which describes this stabilization, and also accommodates stabilization by remote binding of the substrate and intermediate by the enzyme, is proposed. Its relationship to the usual expression for application of the Kurz approach to enzyme catalysis, $(k_{cat}/k_0)/K_m = K_B^{\neq}$, is discussed and the usefulness of the Brønsted and Marcus relationships for interpreting $K_{B^{\neq}}$ is emphasized. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: proton binding; zinc ion binding; transition states; α-heterocyclic ketones; tautomerization; enzymatic reactions

## INTRODUCTION

Lewis acid catalysis by metal ions provides alternative pathways for specific acid-catalysed reactions at mild pH.[1] Experimental comparisons of catalysis by metal ions and protons have therefore been of interest,[2] and in this paper we report a study of keto–enol and imine–enamine tautomerization of α-heterocyclic ketones catalysed by zinc ions and protons. The comparison makes use of the approach advocated by Kurz[3–5] in which catalytic efficiency is expressed in terms of the strength of binding of the catalyst to reactants, transition state and products, and which has previously been applied to competing pathways of acid catalysis for the same reactions.[6] The study offers an opportunity for examining the scope and limitations of the binding formalism for interpreting chemical and enzymatic catalysis more widely.

A reaction scheme comparing acid-catalysed and uncatalysed pathways for the base-promoted C—H bond-breaking accompanying enolization of a ketone is shown in Scheme 1. The rate constants $k_{cat}$ and $k_0$ refer to reactions of protonated and unprotonated substrates ($KH_2^+$ and KH) with a base B, and $K_B^R$ is the equilibrium constant for binding the catalyst to the reactant. The use of a binding rather than dissociation constant ($K_a = 1/K_B$) is optional,[4–6] but normal for metal ion catalysis.[1] The




**Scheme 1**

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ratio of rates of catalysed and uncatalysed reactions $v/v_0$ is given by the equation

$$v/v_o = K_B{}^R(k_{cat}/k_o)[H^+] \quad (1)$$

and characteristically depends on the concentration and binding constant of the catalyst and the ratio of rate constants for bound and unbound substrate, $k_{cat}/k_0$.

Interpretations of catalysis normally separate binding of the catalyst ($K_B{}^R$) from its activating effect ($k_{cat}/k_0$)[4,6,7] (although this is not always possible in practice). For acid catalysis, Stewart and Srinivasan[7] have called $k_{cat}/k_0$ a 'proton activating factor.' More generally, $k_{cat}/k_0$ represents a substitutent effect upon the reactivity of the bound substrate, which may be related to an equilibrium effect by invoking the Brønsted or Marcus relationship, as in the equation[6]

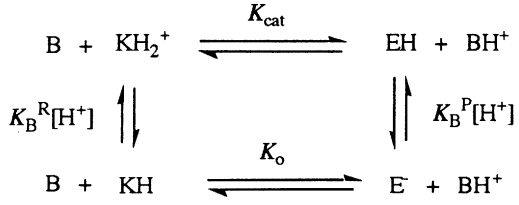
$$k_{cat}/k_o = (K_{cat}/K_o)^\alpha \quad (2)$$

where $K_{cat}$ and $K_0$ are equilibrium constants for formation of enol and enolate anion from the protonated ketone and ketone, respectively, and $\alpha$ is the Brønsted exponent. The 'equilibrium activating factor' $K_{cat}/K_0$ represents the thermodynamic driving force for the reaction arising from binding of the catalyst.[6]

The thermodynamic driving force may be related to the strength of binding of the catalyst in reactants and products by the equation

$$K_{cat}/K_o = K_B{}^P/K_B{}^R \quad (3)$$

This relationship is based on the thermodynamic cycle of Scheme 2, in which the equilibria for catalysed and uncatalysed reactions are coupled to equilibria for binding of the proton catalyst to the reactant and product of the uncatalysed reaction. The equilibrium constant $K_B{}^P$ is the binding constant of the proton to the product (for enolization, the enolate anion, $E^-$).




**Scheme 2**

Combining Eqns (2) and (3) to give

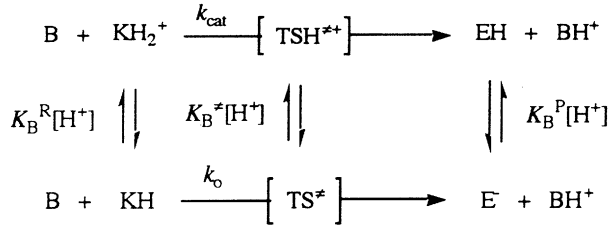
$$k_{cat}/k_o = (K_B{}^P/K_B{}^R)^\alpha \quad (4)$$

shows that the kinetic activation ($k_{cat}/k_0$) reflects stronger binding of the catalyst to the product than the reactant of the uncatalysed reaction. In so far as the 'product' refers to that from the rate-determining and not final step of the reaction, it is commonly a reactive intermediate (in our example the enolate anion). The Brønsted coefficient $\alpha$ in Eqns (2) and (4) is normally $<1$ and reflects attenuation of the effect of stabilizing the intermediate (relative to reactant) at the transition state.

The relationship between the stability of the transition state and of an intermediate provides both a qualitative and a quantitative framework for interpreting activation by catalysis. As discussed below, it remains appropriate even where 'imbalance' exists between factors influencing reaction rates and equilibria, as indeed is likely for the reactions considered here, which involve a rate–equilibrium relationship for carbon acids rather than the oxygen or nitrogen acids of a normal Brønsted relationship.

Kurz[3] has shown that stabilization of the transition state may also be expressed by an apparent binding constant $K_B{}^{\neq}$. Such a constant is defined by writing the rate constants $k_{cat}$ and $k_0$ as products of a frequency factor *(kT/h)* and equilibrium constant for formation of the transition states for uncatalysed and catalysed reactions, $TS^{\neq}$ and $TSH^{+\neq}$. Analogy with Scheme 2 then yields a relationship between $K_B{}^{\neq}$ and $k_{cat}/k_0$ based on the pseudo-thermodynamic cycle embracing reactants and transition states of Scheme 3, as shown in the equation

$$k_{cat}/k_o = K_B{}^{\neq}/K_B{}^R \quad (5)$$




**Scheme 3**

An important implication of Eqn (5) is that catalysis can now be considered equivalently in terms of rate and equilibrium constants for catalysed and uncatalysed reactions on the one hand and binding constants for association of the catalyst with reactant, product and transition state on the other. This equivalence derives from the cycles of Schemes 2 and 3, and is confirmed by comparing the Brønsted relationship between rate and equilibrium constants of Eqn (2) with the corresponding expression based on binding constants from combining Eqns (4) and (5) as

$$K_B{}^{\neq}/K_B{}^R = (K_B{}^P/K_B{}^R)^\alpha \quad (6)$$

However, the most widely used expression from Kurz's treatment comes from rearranging Eqn (5) to

$$k_{cat}K_B{}^R/k_o = K_B{}^{\neq} \quad (7)$$

This shows that the effectiveness of a catalyst depends only upon its binding at the transition state. The equation has the advantages (a) that it describes catalysis under the normal conditions that substrate and catalyst are uncomplexed and (b) does not require experimental separation

of the binding constant ($K_B^R$) and rate constant $k_{cat}$ (or activation factor $k_{cat}/k_0$). Moreover, the binding formalism now allows cancellation of $K_B^R$ between the pre-equilibrium binding of the catalyst and $k_{cat}/k_0$. Hence Eqn (6) may be rearranged to

$$K_B^{\neq} = (K_B^P)^{\alpha}(K_B^R)^{1-\alpha} \quad (8)$$

which reveals $K_B^{\neq}$ as a weighted mean of reactant and product binding constants.

The main significance of Eqn (8) is that it provides an interpretation of $K_B^{\neq}$. However, making use of this interpretation again normally requires the separation of $k_{cat}$ and $K_B^R$. Even in qualitative applications a notional assessment of the relative magnitudes of $k_{cat}$ and $K_B^R$ is required. The implications of this for the use of Eqn (7) to describe enzyme catalysis are discussed in this paper.

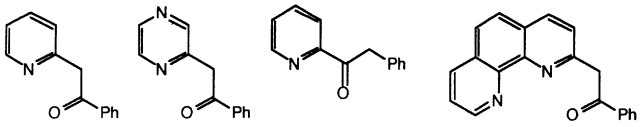
Applications of Kurz's approach to catalysis have been reviewed recently by Tee,[4] who pointed out that the method has been applied less to chemical than to enzymatic reactions.[8–11] One reason for this is certainly the non-covalent and 'environmental' character of stabilization by an enzyme. By contrast, chemical stabilization normally implies a structural modification of a substrate (and intermediate) in which protonation is naturally included. Such stabilization normally cannot be described as 'binding,' and discussions of chemical catalysis in terms of binding have been confined largely to non-covalent catalysts such as cyclodextrins[4,13] and to metal ions.[1,2,12]

One limitation to chemical applications of the binding formalism has been a lack of equilibrium data. As already stressed, equilibrium measurements permit the use of the Brønsted relationship to interpret binding at the transition state. A good example of the use of such data is provided by Cacciapaglia and Mandolini's comparison of binding of metal ions to reactants, transition state and products in a study of metal ion-catalysed cyclization of polyethers.[12] Where comparable equilibrium data are lacking, the usefulness of Eqn (7) is diminished.

The comparison of catalysis by protons and metal ions described in this paper seemed appropriate for a further study of 'binding' catalysis, both because metal ions are naturally considered from this viewpoint and because equilibrium and kinetic data are available. The availability of equilibrium data distinguishes this work from most previous investigations of metal ion catalysis, including other studies of the enolization of heterocyclic ketones.[1,2,14] However, a model for the present investigation is provided by Leussing and co-workers' measurements of enolization of pyruvate and oxaloacetate ions, in which he showed that catalysis by Mg, Mn, Zn and Cu ions may be described by a single rate–equilibrium relationship correlating proton-catalysed, metal ion-catalysed and uncatalysed reactions.[15] Our data are analysed similarly and also by calculation of binding constants for transition states.

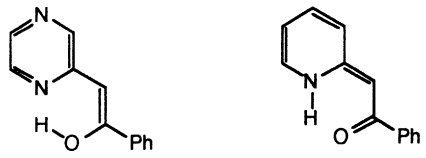
## RESULTS

The substrates investigated in this work comprised the heterocyclic ketones **1**–**4**. Rate and equilibrium constants for uncatalysed, proton-catalysed and zinc-catalysed tautomerizations were measured and the results plotted as a rate–equilibrium relationship, together with results of Chiang *et al.*[16] for uncatalysed and acid-catalysed enolization of actetophenone, to which the Kurz–Brønsted methodology has previously been applied by Tee.[4]



**1** **2** **3** **4**

The ketones **1**–**4** have p$K_a$s less than 14 and these p$K_a$s were measured spectrophotometrically using dilute solutions of aqueous sodium hydroxide. Ionization constants ($K_a$) for protonation of the heterocyclic nitrogen atoms were measured in solutions of HCl or an acidic buffer and converted to binding constants, $K_B = 1/K_a$. The unstable tautomers, which were enols for the ketones **2** and **3** (e.g. **5**) and enaminones for **1** and **4** (e.g. **6**), were generated in aqueous solutions by quenching solutions of their enolate anions into strong acid or acidic buffers in a Durrum 110 stopped flow spectrometer. Relaxation of these tautomers to their stable keto forms was monitored spectrophotometrically, and ionization constants were obtained either from a pH–rate profile for the ketonization reaction or by combining an equilibrium constant for ionization of the ketone with the appropriate keto–enol or keto–enaminone tautomeric constant.[17,18]

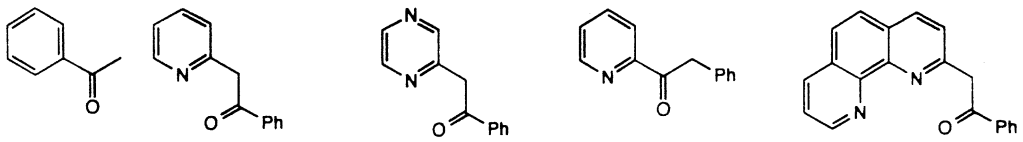


**5** **6**

Tautomeric constants were derived by combining rate constants for relaxation of the unstable tautomer with rate constants for enolization (or formation of the enaminone) of the ketone measured by the halogen trapping method.[19] A complication in obtaining appropriate binding constants for reactions of enols of ketones **2** and **3** was that acid-catalysed enolization occurs with protonation on the nitrogen atom of the heterocyclic ring rather than on the oxygen atom of the carbonyl group.[6,17] The required binding constants for the enolate anions were not the thermodynamically favoured values for protonation at oxygen therefore, but values for the unfavourable protonation on nitrogen. These had to be estimated from the p$K_a$ of the corresponding *N*-methyl

**Table 1.** Rate constants, equilibrium constants and p$K_a$s for binding of catalysts to reactants, products and transition states for the tautomerization of α-heterocyclic ketones in aqueous solution at 25 °C



| Base[a] | Catalyst | $AcO^-$ | $H_2O$ | $H_2O$ | $AcO^-$ | $AcO^-$ | $AcO^-$ | $H_2O$ |
|---|---|---|---|---|---|---|---|---|
| $k_H/k_{Zn}$[c] | | — | $1.6 \times 10^3$ | (132)[b] | (165)[b] | 2.5 | 0.4 | 4.9 |
| $\log k_H$[c] | $H^+$ | −2.54 | 4.03 | 0.33 | 3.45 | 2.02 | 7.29 | 4.40 |
| $\log k_{Zn}$[c] | $Zn^{2+}$ | | 0.83 | −1.79 | 1.22 | 1.62 | 7.70 | 3.71 |
| $\log k_{cat}$ | $H^+$ | 1.46 | −1.0 | −0.07 | 3.04 | −0.28 | 2.57 | −0.32 |
| | $Zn^{2+}$ | — | −0.82 | −1.79 | 1.22 | −0.07 | 4.20 | 0.21 |
| $\log k_0$ | | −5.87 | — | — | −1.79 | −2.69 | — | — |
| $pK_{cat}$[d] | $H^+$ | −0.82 | 6.08 | 4.8 | 0.02 | 2.12 | −0.46 | 4.32 |
| | $Zn^{2+}$ | — | 7.05 | (5.9)[b] | (1.1)[b] | 2.49 | −1.98 | 2.80 |
| $pK_0$ | | 13.52 | 13.27 | 11.90 | 7.12 | 7.22 | 8.27 | 13.05 |
| $\log K_B^R$ | $H^+$ | −4.0 | 5.03 | 0.4 | 0.4 | 2.30 | 4.72 | 4.72 |
| | $Zn^{2+}$ | — | 1.65 | (0.0)[b] | (0.0)[b] | 1.67 | 3.50 | 3.50 |
| $\log K_B^P$ | $H^+$ | 10.34 | 12.22 | 7.50 | 7.50 | 7.40 | 13.45[e] | 13.45[e] |
| | $Zn^{2+}$ | — | 7.87 | 6.05 | 6.05 | 6.40 | 13.75[e] | 13.75[e] |
| $pK^{\neq}$[f] | $H^+$ | 3.60 | — | — | 5.23 | 4.71 | — | — |
| | $Zn^{2+}$ | — | — | — | (3.01)[b] | 4.29 | — | — |
| α[g] | $H^+$ | 0.51 | — | — | 0.68 | 0.47 | — | — |
| | $Zn^{2+}$ | — | — | — | (0.50)[b] | 0.55 | — | — |

[a] $pK_a$ of acetic acid = 4.78.
[b] Values in parentheses are estimated.
[c] $k_H$ and $k_{Zn}$ are $k_{cat}K_B^R$ for $H^+$ and $Zn^{2+}$ catalysis, respectively; they are second-order rate constants (l $mol^{-1}$ $s^{-1}$) for water acting as a base and third-order (l$^2$ $mol^{-2}$ $s^{-1}$) for acetate as base.
[d] $pK_{cat} = -\log K_{cat} = pK^P - pK^R + pK_0$.
[e] *E*-isomer.
[f] Based on Eqn (7).
[g] Based on Eqn (12).

zwitterion for the phenylacetylpyridine,[17] and as previously described for phenacylpyrazine.[6] Details of these measurements have been reported elsewhere.[6,17,18]

Binding constants for zinc ions to enolate anions were obtained from spectrophotometric measurements of equilibrium constants *K* for the proton and metal ion concentration-dependent formation of the metal bound enolate ions ($EZn^+$) from the ketone in lutidine buffers based on the equations

$$KH + Zn^{2+} \overset{K}{\rightleftharpoons} EZn^+ + H^+ \qquad (9)$$

$$K = \frac{[EZn^+][H^+]}{[KH][Zn^{++}]} \qquad (10)$$

Combination of *K* with $K_a^{EH}$, the ionization constant of the enol, gave the appropriate binding constant. Equilibrium constants for binding of zinc ions to the keto tautomers were measured from kinetic saturation of the enolization reaction observed with increasing concentration of metal ion, as described by Cox[2a] and de Maria *et al.*[2b] For phenacylpyrazine this constant was not directly measured and had to be estimated.

Rate constants for metal-catalysed ketonization and enolization were obtained as indicated above from relaxation of the unstable tautomers and halogenation of the ketones, respectively.[18,19] Third-order rate constants for reaction with buffer base and zinc ions were obtained from the dependence of first-order rate constants on the concentration of these species. Where the base was water the corresponding constants for catalysis by metal ions were second order.

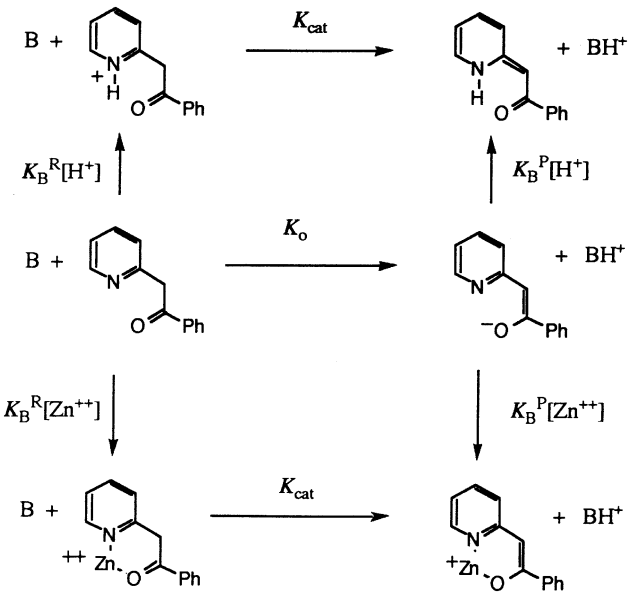
Rate constants for uncatalysed, acid-catalysed and zinc-ion catalysed enolization (or enaminone formation) are shown in Table 1, together with values of $pK_a$ ($\log K_B$) for dissociation (or binding) of the protonated and metal bound ketones and enolate ions, and rate and equilibrium constants for reaction of complexed ($\log k_{cat}$ and $\log K_{cat}$) and uncomplexed ($\log k_0$ and $\log K_0$) ketones to form enols or zinc-coordinated or free enolate anions. Where rate constants for enolization were not measured directly, they were obtained by combining corresponding rate constants for ketonization and the tautomeric constants. Table 1 also includes rate and equilibrium constants for acid-catalysed and uncatalysed enolization of acetophenone.[16]

The applicability of the Brønsted relationship to the

measurements in Table 1 was evaluated by plotting rate constants against equilibrium constants based on the log–log relationship

$$\log k = \alpha \log K + \text{constant} \qquad (11)$$

The rate constants ($K_{cat}$ and $K_0$) correspond to proton transfer from complexed or uncomplexed ketones ($KHZn^+$, $KH_2^+$ or KH) to the bases indicated in Table 1 (water or acetate) and the equilibrium constants ($K_{cat}$ and $K_0$) refer to the same reactions, i.e. they correspond (in their logarithmic forms) to the difference in p$K_a$s of the keto reactant and conjugate acid of the proton-abstracting base. These equilibrium constants are summarized for $H^+$- and $Zn^{2+}$-catalysed tautomerization of phenacylpyridine in Scheme 4, in which the normal double arrows for equilibria are replaced by single arrows to indicate the direction of reaction to which the constants refer.




**Scheme 4**

Equation (11) represents a 'generalised' Brønsted relationship in which the substrate (carbon base) and oxygen and nitrogen bases are varied. It is similar to that used by Leussing and co-workers[15] to compare activation of enolization of oxaloacetate and pyruvate anions by binding of $H^+$, $Mg^{2+}$, $Mn^{2+}$, $Zn^{2+}$ and $Cu^{2+}$ ions. The equation is based on the Brønsted relationship of Eqn (2) with values of $k_{cat}$ and $k_0$ and $K_{cat}$ and $K_0$ in this equation corresponding to $k$ and $K$, respectively, in Eqn (11). Although $K_{cat}$ and $K_0$ are not binding constants, replacement of $K_{cat}/K_0$ by $K_B^P/K_B^R$ in Eqn (2) [as in Eqn (3)] gives the alternative (and equivalent) relationship in terms of binding constants. Thus $K = K_{cat}$ in Eqn (11) would be replaced by $(K_B^P/K_B^R)K_0$.

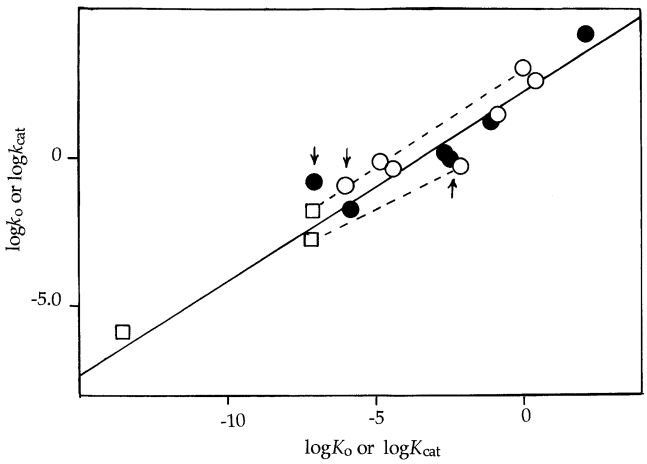



**Figure 1.** Extended Brønsted plot of logarithms of rate constants against logarithms of equlilibrium constants for deprotonation of zinc-coordinated (●), protonated (○) and free (□) α-heterocyclic ketones by water or acetate bases in aqueous solution at 25 °C.

## DISCUSSION

### Brønsted relationship

The plot of log $k$ versus log $K$ embracing unprotonated, protonated and metal-coordinated ketones is shown in Fig. 1. As expected of a relationship in which the nature of the carbon base is varied, some dispersion from a strictly linear correlation is observed. However, departures from linearity are not pronounced and the correlation upholds Leussing and co-workers' conclusion that enolizations of pyruvate or oxaloacetate ions show no discernible difference in intrinsic barrier between acid- and metal-catalysed reactions.[15] Leussing and co-workers' own measurements (which have acetate as base) also fall close to the correlation line. The slope of the line has a normal value, $\alpha = 0.63$.

Variations in intrinsic barrier lead to departures from a strict rate–equilibrium relationship.[20,21] Usually they reflect a difference in expression of polar, resonance and solvent effects upon a reaction rate and equilibrium, resulting from an imbalance in the progress of bond-making, bond-breaking and charge development at the transition state.[22] Although no consistent difference between protons and metal ions is evident in Fig. 1, variations in intrinsic barrier reflecting differences between substrates are apparent, most notably between measurements for phenacylpyridine (**1**), for which points for $H^+$ and $Zn^{2+}$ lie above the line (arrowed), and for phenylacetylpyridine (**3**), for which they lie below (also arrowed). This difference almost certainly reflects a difference between coordination of protons and metal ions to a pyridyl nitrogen atom which, for the phenacylpyridine, is directly conjugated with developing negative charge at the reacting carbon atom but, for

phenylacetylpyridine, mainly affects delocalization of charge to the carbonyl group.

Unlike Leussing and co-workers, we have not attempted to fit data to the Marcus equation. This is in recognition of the variation in intrinsic barrier noted above, and of the suggestion by Amyes and Richard[23] that curvature of Marcus correlations for carbon bases may be obscured by compensating changes in thermodynamic driving force and intrinsic barrier.

Also included in Table 1 are values of binding constants $\log K_B^{\neq}$ (equal to $pK_a^{\neq}$) for protonated and zinc-coordinated transition states. These correspond to ratios of rate constants of catalysed and uncatalysed reactions at concentrations not leading to complexation [Eqn (7)]. Their number is limited by lack of access to measurements for the uncatalysed reactions. However, ratios of $K_B^{\neq}$ values for catalysis by $H^+$ and $Zn^{2+}$ can be evaluated because $k_0$ cancels between the two reactions. These values are listed as $k_H/k_{Zn}$ in the first row of Table 1 (with $k_H$ and $k_{Zn}$ corresponding to $k_{cat}\,K_B^R$), and it is noticeable that $k_H/k_{Zn}$ shows a markedly higher value (1600 compared with 2.5) for phenacyl- than phenylacetylpyridine, even though the activating effect of bound protons and zinc ions is similar ($k_{cat}^H/k_{cat}^{Zn} \approx 0.7$). The difference reflects a difference in binding constants of the two ions and, in particular, that phenacylpyridine is more basic than phenylacetylpyridine whereas the ease of binding of zinc ions to the two substrates is almost the same.

Inspection of the values of $\log K_B^{\neq}$ in Table 1 shows that they lie between those of $\log K_B$ for the appropriate keto reactant and enolate anion product. This implies that the Brønsted coefficients are between 0 and 1. Application of the Brønsted relationship in the logarithmic form of Eqn (8) allows evaluation of the coefficients from the equation

$$\alpha = (pK_a^{\neq} - pK_a^R)/(pK_a^P - pK_a^R) \qquad (12)$$

which takes advantage of the identity of values of $\log K_B$ and $pK_a$. These values are also listed in Table 1 and correspond to the slopes of lines connecting points in the figure for the appropriate uncatalysed (□) and catalysed (○ or ●) reactions. Two of these are shown as dashed lines for acid catalysis of enolization of phenacylpyrazine (above the line) and of phenylacetylpyridine (below the line).

However, for the purpose of the present paper, the main function of Fig. 1 and Table 1 is to compare Kurz's approach to 'binding catalysis' with the more conventional rate–equilibrium correlation at the level of the Brønsted–Marcus relationship. It can be seen that the two approaches are equivalent in principle, but differ in emphasis in practice. Thus Kurz highlights values of $\alpha$ for individual substrates and the conventional method an averaged value for several substrates. Moreover, the conventional treatment focuses on activation of the substrate ($k_{cat}/k_0$) and treats the catalyst as a substitutent that can modify the rate and equilibrium constant of a reaction. Kurz's treatment emphasizes the low concentration limit of catalysis ($K_B^R k_{cat}/k_0$), where the binding formalism allows cancellation of contributions from $K_B^R$ to binding and activation steps, and places less emphasis on the catalyst as a substituent. However, there is no fundamental difference in the approaches and usage reflects preferences of different authors and of different fields of application. For both methods, it needs to be noted that the Brønsted relationship is applied to carbon substrates, which often show large reactivity differences and may imply variations of Brønsted coefficient and intrinsic barrier in addition to rate, equilibrium and binding constants.

In summary, the main feature of the binding approach is to express the thermodynamic driving force for the reaction as a difference in binding constants of the catalyst to reactant and product (or intermediate) and its kinetic effect as the corresponding difference between reactant and transition state. This provides a satisfactory treatment of the catalysis by protons and metal ions, and the remainder of the discussion will attempt to assess its application to enzyme catalysis, for which the binding approach has been widely used. The discussion will focus on use of the Brønsted relationship to relate kinetic and equilibrium effects of binding because this provides an interpretation of binding behaviour. In particular it will aim to develop a formal expression analogous to that of Eqn (8) for chemical catalysis. Although a quantitative analysis cannot be expected, the usefulnes of Eqn (8) in providing a conceptual framework for interpreting binding at the transition state strongly suggests its extension to enzymatic processes.

## Scope and limitations of 'binding' catalysis

When we attempt to extend the scope of Kurz's method, several limitations appear. The first comes from the distinction between 'binding' catalysis and 'covalent' catalysis. This is apparent from the present study, which shows that the method applies when the catalyst is bound to the substrate prior to the rate-determining step of the reaction. This is true of proton and metal ion catalysts, but inspection of Schemes 1–3 reminds us that the tautomerism is also subject to base catalysis. This has not been considered hitherto because the base participates in both catalysed and uncatalysed reactions. However, if an attempt is made to describe catalysis by the base within the same formalism as for protons and metal ions it becomes apparent that an appropriate uncatalysed reaction cannot be identified. This remains true even if both steps of the tautomeric reaction are included in the catalytic cycle. As noted by Tee,[4] this reflects the fact that catalyst and substrate undergo a covalent reaction in

which an atom or group is transferred between catalyst and substrate in the rate-determining step.

The distinction between covalent catalysis and binding catalysis in enzyme reactions has long been recognized.[8,24] However, the term covalent has often been used to indicate a reaction between enzyme and substrate at any stage of the catalytic cycle. At the risk of ambiguity, we restrict its use to the rate-determining step or (since more than one step may require catalysis) when the covalent step is under discussion. Thus general acid or general base catalysis is included in this definition but covalent participation of a catalyst prior to the rate-determining step is not. Conversion of a carbonyl group to an iminium ion or the covalent binding of most coenzymes, for example, fall within the scope of 'binding' catalysis, provided that the binding step is rapid.

Covalent and binding catalysis commonly exist side by side in both chemical and enzymatic reactions. To apply the Kurz–Brønsted treatment the two need to be separated. One means of doing this is illustrated by the tautomerization reactions just described. Provided that the covalent catalyst participates in both catalysed and uncatalysed reactions different substrates (and catalysts) may be compared within the binding formalism. A similar comparison may be made for enzymatic reactions when different substrates are compared for the same enzyme. However, because the covalent steps should remain the same this normally implies that structural changes made in the substrate should be remote from its reaction site.[8,25]

Sensitivity to structural changes remote from the site of a covalent reaction represents an important difference between enzymatic and chemical reactions. However, it introduces a second limitation to the Kurz method. A simplified view of 'remote' binding by an enzyme, which nevertheless is a convenient starting point for discussion, is that it is responsible for sequestration and molecular recognition of the substrate, but has no influence upon reaction of the enzyme–substrate complex, other than approximation of the covalently reacting groups.

An important implication of this view is that there is *no* influence of remote binding by an enzyme upon *activation* of the substrate. Since without activation there is no catalysis, in this approximation not only covalent catalysis but the most characteristic feature of binding by an enzyme fall outside the scope of Kurz's analysis.

In practice, Kurz's aproach is particularly useful in distinguishing *remote* binding of a substrate from binding at its *reaction site*. This distinction is similar to that between a substitutent and reacting functional group in a chemical reaction. Like substituents, remotely bound parts of the substrate undergo no change in the course of a reaction, whereas the reaction site (or a functional group) changes profoundly, usually as a result of changes in covalent bonding and the separation or combination of ionic charges.[25] It follows that binding at the reaction site also changes between the reactant and transition state, and that this is reflected in activation of the bound substrate.

Where a *change* in binding occurs, Kurz's treatment is applicable. Binding of the catalyst may then be represented as stabilizing the transition state of the catalysed reaction. Moreover, application of the Brønsted relationship, and analogy with the proton and metal ion catalysis described above, lead to interpretation of this binding in terms of thermodynamic driving force arising from stabilization of a structurally related *reactive intermediate*.[26]

Recognition of the need to stabilize reactive intermediates leads to a more systematic consideration of binding catalysis in enzymatic reactions. Although not all enzymatic (or chemical) reactions proceed through reactive intermediates, there is little indication that such intermediates are less important in enzymology than chemistry. Sometimes formation of an intermediate is mediated by a coenzyme, but commonly carbocations,[27] anionic tetrahedral intermediates,[28] enolate anions[29,30] and carboxyl-substituted carbanions[31] formed in enzymatic reactions depend for their stabilization solely on their protein environment.

Stabilization of an intermediate by an enzyme is more easily interpreted than stabilization of a transition state because again there is a separation of binding contributions from covalent catalysis. The stabilization corresponds to the free energy of transfer of the intermediate from the solvent used in an (implied) uncatalysed reaction, usually water, to the environment of the enzyme. If there are no covalent interactions between the enzyme and intermediate, a simple analogy exists between stabilisation by 'binding' of the enzyme and by solvation in a chemical reaction.

At first sight, the capacity of a protein to stabilize high-energy and usually charged intermediates by non-covalent interactions appears to be limited. To stabilize an anion, for example, the enzyme may marshal amidic hydrogen bonds, electrostatic interactions and, in metalloenzymes, coordination of a metal ion. These interactions are normally strengthened by being intramolecular in character, but the apparent magnitude of the stabilization has remained a puzzle to enzymologists. Special effects have been invoked, such as unusually strong hydrogen bonds,[29] but there is little evidence that non-covalent interactions in proteins differ substantially from those of simple molecules.[26,30]

Recent discussions of enzyme catalysis have emphasized 'remote' binding by the enzyme as a further source of stabilization of reactive intermediates. Before considering this, however, it is appropriate to recognize the complexity of distinguishing remote binding from binding at the reaction site (in the reactant, transition state and intermediate) and from the influence of covalent catalysis. The situation is more complicated than for

the proton and metal ion-catalysed tautomerization reactions discussed above, for which only binding at the reaction site was considered. The added complexity reinforces the suggestion above that it would be desirable to develop a more quantitative formulation of the catalysis which, even if not applicable numerically, would differentiate these contributions. The importance of stabilizing a transition state (or intermediate) by binding at the reaction site suggests, as a first step, that the Kurz–Brønsted formalism should be applied to this element of the catalysis and the result extended to 'remote' binding and covalent catalysis.

## A reference reaction for enzyme catalysis

A normal requirement for applying the Kurz–Brønsted formalism is to identify an uncatalysed reaction. This raises again the separation of binding catalysis from covalent catalysis. As we have seen, covalent catalysis is formally incompatible with an uncatalysed reaction. However, as discussed by Schowen,[32] considerable freedom exists in the choice of a more arbirtrary but nevertheless convenient reference reaction. One such choice is suggested by reactions in which the covalent catalysis is *intramolecular*. The rate of an intramolecular reaction is commonly compared with that of a corresponding intermolecular (usually bimolecular) process. The ratio of rate constants for the two processes then represents the entropic advantage of approximating the covalently reacting groups (modified by strain in the reactants or transition state).[33] The fact that the intermolecular reaction is itself subject to catalysis does not disqualify it as a reference.

Reactions of enzyme–substrate complexes are usually considered to be intramolecular. A favoured 'reference' therefore has been an intermolecular reaction in which the covalent functional groups of the enzyme are present in separate molecules with unchanged chemical reactivity. Measurement or estimation of a rate constant for such a reaction may not be possible, but this presents no serious difficulty if numerical comparisons are not required.

Sometimes an 'uncatalysed' reaction has been identified with that occurring in aqueous solution in the absence of enzyme. Such a reaction may be more accessible to experimental measurement, but usually the mechanism differs from that of the enzyme, making a comparison difficult to interpret.

## Brønsted–Kurz equation for enzyme catalysis

If the rate constant $k_0$ for the uncatalysed reaction is replaced by that for a reference reaction, Kurz's Eqn (7), expressing the ratio of rates of catalysed and uncatalysed reactions in terms of a binding constant for the transition state, may be rewritten for an enzymatic reaction in the form

$$(k_{cat}/k_o)/K_m = K_B^{\neq} \qquad (13)$$

Where $k_{cat}$ and $k_0$ are now unimolecular and multimolecular rate constants for reactions of the bound and unbound substrate, respectively, and $K_m$ is the Michaelis constant for forming the enzyme–substrate complex. Because the Michaelis constant is defined as a dissociation rather than binding constant, $K_B^R$ in Eqn (7) is replaced by $1/K_m$ in Eqn (13).

As it stands, Eqn (13) fails to separate the contributions to $(k_{cat}/k_0)/K_m$ that we wish to distinguish. However, it can be made a starting point for achieving such a separation if it can be modified (a) to differentiate remote binding from binding at the reaction site and (b) to dissect the latter into contributions from reactant and product binding by use of the Brønsted relationship.

The modification involves several steps. First, we restrict application of Eqn (13) (initially) to binding at the reaction site. Then, the Brønsted relationship is used to transform the binding constant of the transition state $K_B^{\neq}$ into binding contributions from reactant and product, $K_B^R(K_B^P/K_B^R)^{\alpha}$, as in Eqn (8). For an enzyme reaction $K_B^R$ represents the contribution specifically from binding at the reaction site to the overall binding of the Michaelis complex, while $K_B^P$ normally refers to binding of a reactive intermediate. Next, the contribution of remote binding is assigned by retaining our preliminary assumption that this is unchanged in the course of the reaction and denoting it $K_{rem}$. Finally, since our choice of reference reaction implies that the contribution of covalent catalysis corresponds to approximating the groups undergoing covalency change at the transition state, this contribution is designated $C_{ap}$. These contributions are combined in the equation

$$(k_{cat}/k_o)/K_m = \underbrace{K_{rem}K_B^R}_{1/K_m}\ \underbrace{(K_B^P/K_B^R)^{\alpha}C_{ap}}_{k_{cat}/k_o} \qquad (14)$$

Equation (14) summarizes the different elements contributing to enzymatic catalysis. It separates remote binding of the substrate $K_{rem}$ from binding at the reaction site $K_B^R$ in a manner which recalls the 'split site' model considered by Menger.[34] The Michaelis constant is expressed in terms of a product of contributions of $K_{rem}$ and $K_B^R$ for binding the reactant, i.e. $K_m = 1/K_B^R K_{rem}$. In addition, the activating effect of binding at the reaction site is expressed as a ratio of binding constants in the product (reactive intermediate) and reactant $(K_B^P/K_B^R)^{\alpha}$. This is analogous to the situation for metal ion and proton catalysis in Eqn (4) and represents the kinetic effect of stabilizing an intermediate relative to reactants by binding the catalyst. The Brønsted coefficient $\alpha$ reflects the extent of this stabilization available at the transition state. Although it is sometimes said that maximum binding of the enzyme occurs at the transition state, Eqn

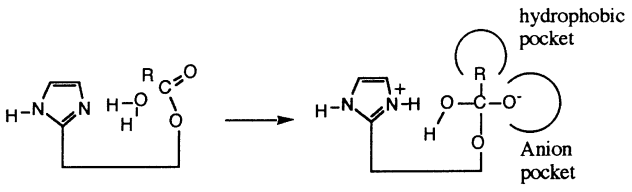
 

(14) implies that this is more likely to be true of a reactive intermediate.

## Changes in remote binding

Equation (14) can now be examined to identify the effect on reactivity of remote binding by the enzyme to the substrate. A first effect arises from the contribution of $K_B^R$ to both the Michaelis constant $K_m$ and activation of the bound substrate [$k_{cat}/k_0 = (K_B^P/K_B^R)^{\alpha} C_{ap}$]. The latter contribution implies that reactivity is increased by *un*favourable binding of the substrate, corresponding to destabilization of the reacting functional group in the enzyme–substrate (reactant) complex. Although a destabilizing contribution is normally unfavourable to $K_m$ its effect can be compensated by favourable binding at 'remote' sites on the enzyme through the dependence of $K_m$ on both $K_B^R$ and $K_{rem}$. Thus stabilization of the intermediate by the enzyme, represented by $K_B^P$, may be complemented by destabilization of the reacting functional group of the substrate in the Michaelis complex represented by $K_B^R$, without a deleterious effect on $K_m$.[24,25,34] Unfavourable binding of a (normally uncharged) reactant functional group is understandable if the binding site is adapted to accommodating a (normally charged) reactive intermediate.

Further stabilization of the intermediate comes from relaxing the assumption that remote binding is unchanged between the Michaelis complex and the reactive intermediate. This takes account of experimental evidence that remote binding as well as binding at the reaction site may be more favourable for the intermediate than the substrate, even though the 'remote' structure of the substrate is unchanged. A familiar and much discussed example of this occurs in hydrolysis of the covalently bound acyl enzyme of chymotrypsin (shown schematically below):[8]




The rate of this hydrolysis is strongly dependent on the (remote) structure of the acyl group RCO. As the nature of the acyl group should have little influence on the stability or 'chemical' reactivity of the acyl enzyme, it follows that formation of a tetrahedral intermediate accommodates the bulky structures characteristic of natural substrates in their optimum binding sites. This is not altogether surprising if it is recognized that the reaction involves separation of ionic charges and conversion of a trigonal to tetrahedral carbon atom, both of which must entail considerable electronic and geometric reorganisation at the reaction site.[8] Similar behaviour has been recorded for other enzymes.[35]

A change in remote binding between the Michaelis complex and reactive intermediate can be accommodated in Eqn (14) by adding the term $K_{rem}^{\neq}/K_{rem}$ as in the equation

$$(k_{cat}/k_o)/K_m = \underbrace{K_{rem}K_B^R}_{1/K_m}\ \underbrace{(K_{rem}^{\neq}/K_{rem})(K_B^P/K_B^R)^{\alpha}C_{ap}}_{k_{cat}/k_o} \qquad (15)$$

In principle, the use of this notation implies application of the Kurz formalism to remote binding of the substrate. However, $K_{rem}^{\neq}$ must take account not only of formation of a reactive intermediate but also juxtaposition of substrate and enzyme to allow approximation of the covalently reacting catalytic groups, i.e. to accommodate also the contribution of the term $C_{ap}$ to the transition state. There will also be a strong interaction between changes in remote binding and binding at the reaction site[24,25,34] (which are already subject to the characteristic interaction associated with multiple binding[36]). Dissection of $K_{rem}^{\neq}$ into contributions from reactant and product binding therefore does not seem appropriate.

While considering changes in remote binding, it is pertinent to enquire whether or not such changes occur in a pre-equilibrium step before the onset of changes in covalent bonding or are concerted with them. Analogy with solvation in a chemical reaction suggests that small structural changes, such as relaxation along preformed hydrogen bonds, may be concerted with covalent bond making, but that more radical changes, including making and breaking of hydrogen bonds, should involve pre-equilibria. Intuitively it seems likely that stabilization of charges developed at the reaction site occurs mainly in a concerted manner but that changes in remote binding take place in pre-equilibria. This may be an oversimplification, but it is not the intention here to discuss enzyme catalysis further than necessary to illustrate the scope and limitations of the Kurz–Brønsted analysis.

## Stabilization of transition states

In conclusion, we may recognize that the concept of stabilizing a transition state by binding an enzyme as expressed in the simplified form of Eqn (13) has already been widely applied to enzymatic reactions. Since this equation also provided the starting point for developing Eqns (14) and (15), it seems appropriate briefly to summarize its influence and to comment further on the relationship between the three equations.

An important influence of Eqn (13) in enzymatic catalysis has been in distinguishing binding of the transition state from binding of the substrate in the Michaelis complex. In this respect it has rationalized the design of transition state analogues for use as enzyme

inhibitors which bind to an enzyme more strongly than its natural substrates.[9,37] Although most inhibitors may be regarded as analogues of reactive intermediates, structures of transition states are also modeled on intermediates. In a further application of the equation, Critchlow and Dunford[5] have related p$K_a$s of transition states to the pH of onset of an acid-catalysed reaction in a pH profile and discussed the mechanistic implications of this. In non-enzymatic applications, Tee[4] has stressed the usefulness of comparisons between different reactions based on numerical evaluation of $K_B^{\neq}$ [as $(k_{cat}/k_0)K_B^{R}$].

An attractive feature of Eqn (13) is its conciseness. One way of regarding its relationship to Eqns (14) and (15) therefore is as a shorthand for these more cumbersome expressions. For example, although Eqn (13) is formally restricted to 'binding catalysis,' the separate contribution of approximating reacting groups is well recognised and readily identified explicitly by replacing $K_B^{\neq}$ in Eqn (13) by $K_B^{\neq} C_{ap}$.

Of course, the 'shorthand' nature of Eqn (13) implies that it should be expanded if separate contributions to catalysis are to be identified. The simplest expansion separates contributions to binding and activation ($K_m$ and $k_{cat}/k_0$), by multiplying and dividing $K_B^{\neq}$ (or $K_B^{\neq} C_{ap}$) by $K_m$ as in

$$(k_{cat}/k_o)/K_m = (K_m K_B^{\neq})C_{ap}/K_m \qquad (16)$$

Although $K_m$ then awkwardly appears in numerator and denominator [*cf.* also $K_{rem}$ in Eqn (15)], this is appropriate to considering its dual role in binding and activation. Indeed, in practice Eqn (13) is often represented by free energy diagrams in which the Michaelis complex and separate binding and activation steps are explicitly included, while avoiding the inelegance of Eqn (16). However, the viewpoint emphasized in this paper is that Eqns (14) and (15) provide an interpretation of Eqn (13), and that the relationship between them is comparable to that between Eqns (7) and (8) for chemical catalysis. The advantage of Eqns (14) and (15) then lies in dissecting and exposing for analysis factors affecting catalysis implicit in $K_B{\neq}$. In summary, these include binding at the reaction site, remote binding, approximating covalently reacting or binding groups and sequestering substrate and catalyst. The equations imply identification of a reference reaction and show the effect of stabilizing a reactive intermediate or destabilizing a reactant. All of these factors have been recognized and extensively discussed by enzymologists. It seems appropriate that they should also be expressed formally in a suitable elaboration of Eqn (13).

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