Exceptional active site H-bonding in enzymes? Significance of the 'oxyanion hole' in the serine proteases from a model study

William L. Mock * and Dave C. Y. Chua
Department of Chemistry, University of Illinois at Chicago, Chicago, IL 60607-7061, USA

For a series of secondary (*N*-butyl) and corresponding tertiary (*N*,*N*-tetramethylene) 5-substituted salicylamides, phenolic pK_a values have been measured in order to assess the energetic dependence of intramolecular carboxamide-NH hydrogen bonding upon the basicity of an acceptor oxyanion. The results are summarized in a Brønsted-type α coefficient of 0.12 (slope for ΔpK_a, tertiary minus secondary, *versus* phenolic pK_a of tertiary amide). Relative acidities of the same salicylamides in dimethylacetamide solution indicate that offsetting differences in anion hydration are not hidden in this coefficient. It is concluded that rather less transition-state stabilization from hydrogen bonding may be available within the active site of serine proteases than has previously been inferred from directed point mutations involving the enzymes.

Introduction

Serine proteases are a ubiquitous class of polypeptide-cleaving enzymes. They are found in digestive juices (mammalian and bacterial), in the blood-clotting cascade, and in physiological hormone processing, for example. The chemical mechanism of these enzymes is thought to be understood in outline, but an explanation for the rapidity of amide bond scission brought about by the catalytic proteins ($\geqslant 10^8$-fold hydrolytic acceleration) remains under debate. Appropriately plain model studies of such complex systems can have the virtue of isolating individual components of reactivity. By means of a surrogate we have investigated, with a view to evaluating its significance, one aspect of the enzymic active site that has been hypothesized to be important in peptide cleavage, namely, the so-called 'oxyanion hole'.

Background

The serine proteases, as well as papain and a number of mechanistically related lipases, are known to function by an acyl-enzyme mechanism. The hydroxymethyl side-chain of an active site serine residue, assisted by coupled histidine and aspartate residues (general base catalysis), displaces the amino group from the scissile amide of substrate, resulting in a transient ester species involving the enzyme and the acyl portion of the substrate. That entity (detectable in special circumstances [1]) is subsequently cleaved by solvent water, releasing a carboxylate and regenerating the enzyme for another catalytic cycle. The usual addition–elimination mode of acyl transfer, involving a metastable tetrahedral adduct, is necessarily involved in each of these steps. The latter intermediates must be stabilized by the enzyme, for their formation and decomposition represent the activation barriers which the enzyme is able to level in performing catalysis. Crystallographic investigations have suggested one way in which the enzyme may accomplish this.[2] It appears that the *anionic oxygen* resulting from substrate-amide carbonyl addition [(Ser)O–C(R,NHR)–O^-] can accept *hydrogen bonds* from the NH portion of enzymic carboxamides (protein backbone linkages or asparagine side-chain), and that these interactions are responsible for its stabilization. Because these groups reside in a depression on the enzyme surface, that region of the active site has come to be known as the 'oxyanion hole'. In the case of the bacterial protease subtilisin, an estimate of its significance is available. Replacement with other amino acids by point mutation of an asparagine residue (Asn 155), which supplies a $-CH_2CONH_2$ side-chain donating one of a pair of such H-bonds within the active site, has suggested that moiety provides a rate factor of *ca.* 330 in the relevant specificity constant k_{cat}/K_m (predominantly in k_{cat}).† However, this should be regarded as an upper limit to the H-bonding contribution, since induced dislocations in the active site of an enzyme can have multiple adverse consequences (perturbed hydration, conformational distortion, *etc.*). For example, the carbamoyl functionality of asparagine possesses a sizable group dipole moment, oriented generally orthogonal to the pertinent H-bond in the case of Asn 155 (*cf.*, major resonance contributor $H_2{}^+N{=}CR{-}O^-$). Perturbation of the overall active site polarization by Asn side-chain replacement could have deleterious kinetic consequences apart from diminished H-bonding within the transition state.

Until now there has been a shortage of autonomous data indicating just how much stabilization that NH hydrogen bonds directed toward the reacting species might actually be capable of providing to the developing anionic intermediate during hydrolysis, yet recent surveys have accorded to this phenomenon a pervasive importance throughout enzymology.[4] This article seeks to estimate independently the kinetic contribution that such H-bonding interactions could make to catalysis in a serine protease.

Premise

The central idea is that, in order to be productive, an H-bond to an oxyanion such as described [(adduct):(Ser)O–C(R,NHR)–O^- ··· HNCO:(enzyme)] must be considerably stronger than

† Subtilisin Asn 155 mutants, $(k_{cat}/K_m)_{rel}$: N155G, 0.0058, 0.0047, 0.0035; N155L, 0.004; N155T, 0.0003; N155Q, 0.006; N155D, 0.002; N155H, 0.029; comparable papain Gln 19 mutant Q19A, 0.005; Q19S, 0.0005; mean value = 0.003 (log weighted).[3]

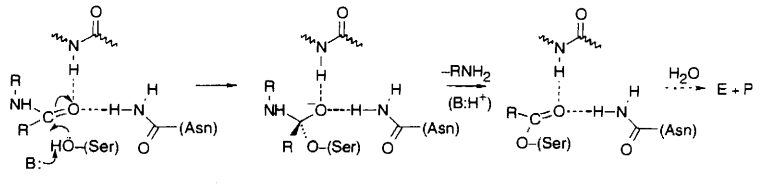
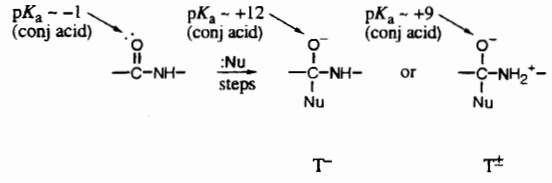


Table 1 5-Substituted salicylamide phenolic pK_a values, relative pK_{DMA} values, and FTIR C=O frequencies

5-Substituent	pK_a **1**[a]	pK_a **2**[a]	rel. pK_{DMA} **1**[b]	rel. pK_{DMA} **2**[b]	$\nu_{C=O}$ **1**/cm^{-1}[c]	$\nu_{C=O}$ **2**/cm^{-1}[c]
NO_2	5.10	5.93	−4.8	−2.3	1649	1632
SO_2Me	5.66	6.76	−2.9	1.6	1647	1628
SOMe	6.31	7.37	−1.3	3.9	1644	1626
CO_2Me	6.35	7.27	−0.7	2.4	1649	1630
Br	7.26	8.45	1.1	—	1644	1624
Cl	7.28	8.46	1.1	—	1646	1626
SMe	7.88	—	2.1	—	1647	1628
H	8.03	9.28	—	—	1644	1628
OMe	8.31	9.75	4.2	—	1649	1632
Me	8.48	9.73	—	—	1647	1632

[a] Maximal standard error ±0.03. [b] Relative acid dissociation in *N,N*-dimethylacetamide solution, estimated error ±0.2; zero corresponds to a phenol that spontaneously ionizes to an extent of 50% in dimethylacetamide. [c] $CHCl_3$ solution.

that involving the precursor amide carbonyl [(non-covalently bound substrate):NH–C=O · · · HNCO:(enzyme)]. Only in that instance can enzymic binding to the transition state at this locus be tighter than was bonding to its antecedent substrate, which constitutes the foundational principle of enzymic catalysis (Pauling).[5] The oxygen atom of the substrate carbonyl undergoes an increase in basicity of 10–13 pK units in the course of traversal of the reaction coordinate for tetrahedral adduct formation (*i.e.*, a protonated carboxamide has pK_a of *ca.* −1, whereas the conjugate acid of the oxyanion intermediate has an estimated intrinsic pK_a of 9 to 12).[6] The question then becomes, how does H-bond strength involving an amide NH donor vary energetically as a function of the *basicity* of an oxyanion acceptor over this pK_a range?[7] Any H-bonds to T^- or $T^\pm$ should be very much stronger than to the precursor substrate carbonyl, if this is to provide a significant contribution to catalysis.



Results

Method

The H-bonding factor can be estimated by a systematic model study of the pK_a values of suitably substituted *salicylamides*. An applicable series consisting of pairs of phenolic amides **1** and **2**, differing in the substituent X, has been examined (X = H, OMe, Cl, NO_2, *etc.*). In alkaline aqueous solution the secondary amides unquestionably H-bond intramolecularly to the phenolate, thereby stabilizing the anion (as shown on the right for **1**). That interaction is manifested by a decrease in the phenolic pK_a values relative to the tertiary amides **2**, which lack an available NH. On the other hand, strength of internal H-bonding within the conjugate acid forms of **1** and **2** (on the left) ought to be the same, and ought not to depend upon the *para* substituent X, since phenol acidity is primarily a delocalization phenomenon of the anion. This finds confirmation in the IR spectra for **1** and **2**, which show an absence of systematic variation with substituent X for the H-bonded amide carbonyl stretching frequency, and a consistent difference between the measured values of $\nu_{C=O}$ for **1** and **2** (*cf.*, Table 1).

The central idea exploited in this work is that any phenolic pK_a perturbation caused by H-bonding within the anion of **1** necessarily has a direct connection to the strength of that interaction.[8] Thermodynamics enforces the relationship: if the free energy difference in H-bonding as regards any pair of non-ionized structures on the left (**1** *versus* **2**) is negligible, then the corresponding ΔG for the same pair on the right (after deprotonation) must equal the disparity for energy of ionization, as reflected in pK_a values [$\Delta G = 2.3RT\Delta(pK_a)$]. What one ultimately desires to know is the *differential* of the H-bonding interaction energy with respect to pK_a of the phenolic acceptor oxyanion, since that relates to the potential stabilization available to a comparably basic tetrahedral reaction intermediate as it forms at an enzyme active site under the influence of NH-donor amides.

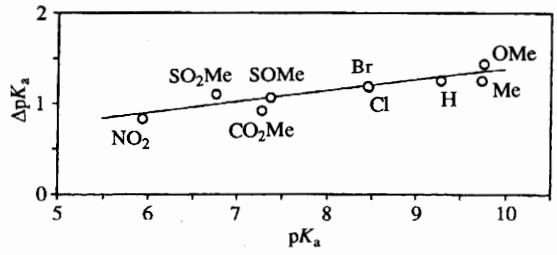
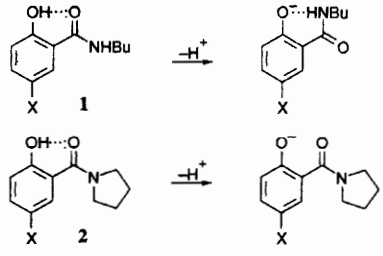


Fig. 1 Plot of ΔpK_a (pK_a of **2** minus pK_a of **1**) *versus* pK_a of **2**. Least-squares regression line has a slope of 0.12 (±0.02), intercept 0.14 (±0.18).



Coefficient

The pK_a value of each phenol was ascertained by spectrophotometric (UV–VIS) titration in dilute buffered aqueous media, as recorded in Table 1. It may be seen that those salicylamides that can intramolecularly H-bond as the anion (**1**) are consistently more acidic than their counterparts which may not (**2**), but that the difference depends upon the substituent X. Fig. 1 presents a plot of ΔpK_a values (**2** minus **1**) *versus* the corresponding pK_a values of **2**, which yields a straight line having a slope designated α (a Brønsted-type coefficient). The latter parameter has a value of 0.12 (±0.02). As we subsequently argue, it contains the desired estimate of the significance of an 'oxyanion hole', since relevant pK_a-determining factors other than amide H-bonding (ArO^- · · · HNCO) ought to cancel in the subtractive comparisons of **1** and **2**. Also germane is the observation that the abscissa intercept occurs at a pK_a value of approximately −1, in general correspondence with that for the

solvent conjugate acid, as expected. This indicates that a linear extrapolation of our data to more acidic regions is justified.

Solvation

A concern in evaluating the influence of intramolecular H-bonding in the salicylamides is that their phenolates also engage in intermolecular H-bonding with solvent H_2O, and that could provide a levelling effect if it were not proportional across each series. In order to get a handle on this aspect, relative acidities for **1** and **2** were obtained in *N,N*-dimethylacetamide, a representative dipolar aprotic solvent containing a tertiary amide linkage. Such measurement becomes feasible since the present series of salicylamides constitutes a set of overlapping indicators. By appropriate adjustment of the acidity of a solution containing a suitable pair of such chromophores, it is possible to secure simultaneous partial ionization of each, and thus to measure directly a ratio of acid dissociation constants. In practice this entailed a spectrophotometric titration of the mixture with an appropriate strong proton donor (trifluoroacetic acid) or base (potassium *tert*-butoxide) in dimethylacetamide. It proved feasible to establish relays across most of the series, and so to obtain a set of relative pK_{DMA} values, as also recorded in Table 1. For the salicylbutylamides a good linear correlation exists between these pK_{DMA} values and the corresponding aqueous pK_a values (slope of 2.6 ± 0.1, plot not shown), validating the procedure.

In order to evaluate the significance of hydration, a comparison similar to that in Fig. 1 may be made. In Fig. 2 the difference between pK_{DMA} and pK_a values for the various representatives of **1** is plotted against the pK_{DMA} values for **1**. The resulting straight line has a slope α' of 0.62 (± 0.02). This is a measure of the relative efficacy of H_2O in solvating the phenolates, in conjunction with the influence of the internal amide-NH. The parameter includes an H-bonding contribution from that solvent (which is absent in dimethylacetamide), but also other aspects unique to H_2O as well, so that the intermolecular H-bonding cannot be factored out. The inset to Fig. 2 contains a similar analysis for **2**. For the tertiary salicylamides only four points were accessible in dimethylacetamide, but they give a reasonably straight line with a slope α'' of 0.76 (± 0.04), when the difference (pK_{DMA} minus pK_a) is similarly plotted against pK_{DMA} for the representatives of **2**. The pertinent observation is that the discrepancy between α'' and α' amounts to 0.14, practically the same value as previously observed for α itself in Fig. 1. The simplest explanation is that phenolate stabilization from hydration is the same for both **1** and **2**, but that **1**-anion enjoys an additional increment energy-wise from intramolecular H-bonding involving the secondary salicylamide-NH, which is of the same magnitude in dimethylacetamide as in water. That would ensue if the internal NH were contributing in each instance the greatest stabilization of which it was capable. Although multiple factors govern the extent of acid dissociation and several of these are appraised subsequently, the difference-plot method here employed performs the purpose of cancelling many systematic deviations, so that the influence of the chief structural variation (H-bond in **1**-anion) may be examined.

Discussion

Theory

The importance of the Brønsted-type coefficient to the question of transient oxyanion stabilization may be appreciated by examining limiting cases. For example, were the slope in Fig. 1 found to be $\alpha = 1$, then with regard to the bolstering of an oxyanion that would say that an NH hydrogen bond was energetically just as beneficial as complete proton transfer to RO^- by an external acid; such a result of course constitutes an unlikely eventuality. On the other hand, a slope of $\alpha = 0$ would indicate that the H-bonding stabilization is a constant factor which does not depend at all upon the basicity of acceptor RO^-. This would imply that the oxyanion hole within the serine proteases is incapable of contributing enthalpically to the enzyme kinetics, since bonding to the more basic metastable intermediate *must* be stronger than is bonding to its precursor substrate, in order for the catalytically all-important stabilization of transition state to occur. Consequently, an intermediate result was anticipated ($0 < \alpha < 1$).

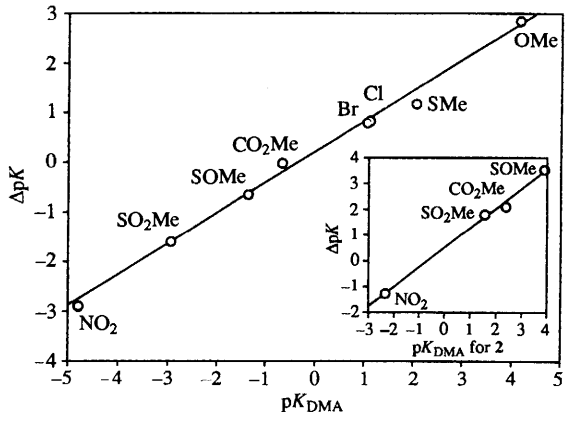


Fig. 2 Plot of ΔpK (pK_{DMA} of **1** plus 7 minus pK_a of **1**) *versus* pK_{DMA} of **1**. Least-squares regression line has a slope of 0.62 (± 0.02), intercept 0.17 (± 0.06). Inset: Similar plot for **2**, slope of 0.76 (± 0.04), intercept 0.5 (± 0.01). An ordinate offset of 7 units has been introduced in each case to rescale the aqueous pK_a values to the same basis as the pK_{DMA} values (zero corresponding to neutrality).

Magnitude

The relatively small value of $\alpha = 0.12$ that was actually observed in Fig. 1 may be roughly converted into an enzyme kinetic factor as follows. Multiplication of the experimentally derived coefficient (α) by the *ca.* 12 p*K* units of basicity change, which a substrate carboxamide oxygen apparently undergoes during formation of a tetrahedral intermediate (T^-) at the protease active site, gives an estimate of 30-fold for the kinetic contribution from each of the H-bonds within the first catalytic step of peptide cleavage in the case of the serine proteases; *i.e.*, antilog(12 × 0.12). Because there appear to be two such interactions within the oxyanion hole, an overall rate acceleration from this source might reasonably be the square of this value. The foregoing computation nominally frames reactivity as a fraction of the corresponding specific-acid catalysed transformation, which normally would be observed only at very low pH. The latter constitutes the apposite default mechanism for purposes of evaluating the significance of H-bonding (upon extrapolation to neutral pH where the serine proteases operate), but a potential stabilization energy of *ca.* 2 kcal mol^{-1} per NH ought to be the maximum available regardless. More particularly, this 'upper-limit' calculation would hold only for a very late transition state for tetrahedral adduct formation, in which negative charge is nearly fully developed on the incipient oxyanion. An earlier transition state would necessarily yield a lesser effect. Of course comparison may also be drawn to general acid catalysis, for which a Brønsted coefficient of 0.12 would likewise indicate relative inefficacy.‡

‡ Stahl and Jencks[7] give a similar α value for intermolecular H-bonding between substituted phenolates and ethylenediamine dication. Abraham[9] has provided a collection of solute hydrogen bond acidity and basicity parameters, which do not encompass species of the basicity of aryloxides, however.

Conclusions

It would appear that the point mutation results with subtilisin lead to an over-estimate the significance of the H-bonding factor by an order of magnitude (*ca.* 330-fold *versus* ≤30-fold). That is perhaps not surprising, for as indicated previously mutational disruption of an active site can have multiple adverse consequences. It might be argued that the enzymic H-bonds are exceptional in some respect, and that they are more efficacious biochemically than this surrogate system would seem to indicate.[4] Although the $ArO^- \cdots HN$ hydrogen bond might not achieve collinearity in the case of **1**-anion, crystallographic evidence suggests no great disadvantages to non-linear H-bonds.[10] It has been proposed theoretically that H-bonds in six-membered rings have *ca.* 60% of the strength of that for a linear counterpart.[11] Should that be the case, the discrepancy with the point mutation results may be reconciled, provided that complete negative charge becomes developed on oxygen within the transition state for the catalytic reaction. That seems unlikely, however, inasmuch as the fully anionic tetrahedral adduct has been shown crystallographically to accumulate in one case as a discrete intermediate,[2b] and consequently a fractionally charged transition state is indicated. With regard to feasibility of the hyper-stabilization that has been hypothesized in these cases (*i.e.*, especially strong, 'low-barrier' H-bonds[4a]), the O ··· N separation for a strain-free, fully coplanar structure for **1**-anion calculates to ≤2.5 Å, providing an optimum interatomic distance between those H-bond constituents. If there were any benefit to be derived, it should cost only a fraction of a kcal mol^{-1} to bend the NH bond into near colinearity with phenolate oxygen in the model. The short gap between O and N should allow a maximized interaction without requiring major heavy-atom bond deformations. Any necessary geometric adjustment could well be a constant factor across the series of substrates, also tending to cancel in such manner as not to perturb systematically the value of α. It might have been imagined that serine proteases gain extra transition-state stabilization by recourse to the tactic of enhancing the H-bond strength in reacting intermediates through a conversion of unrealized substrate binding energy from adjoining sites, specifically so as to compress the O ··· N interatomic distance to an ideal span. However, that explanation fails, since the resulting H-bond length would merely be equivalent to that already present in this model, which shows no such effect.§

No chemical reason exists for regarding the salicylamide-NH as a significantly different donor than a peptide-NH. One might seek a distinction in the H-bonding capabilities of the geometrically distinguishable *syn*- and *anti*-NH orientations with respect to the amide C=O. The anion of **1** employs the *anti*-NH, whereas Asn 155 in subtilisin makes use of a *syn*-NH. However, the peptide-backbone NH moieties, as also employed by subtilisin and exclusively in most other serine proteases, have the *anti* orientation found in **1**. A countervailing argument might be tendered that because of geometrical constraints, the **1**-anion models sustain concurrent H_2O or counterion association, which putatively is more efficacious and consequently levels the influence of the intramolecular H-bonding by amide. However, the pK_{DMA} comparisons appear to exclude a specific hydration explanation (*cf.*, preceding analysis of Fig. 2). Substrate concentrations were deliberately kept low (*ca.* 0.1 mmol dm^{-3}, UV assay) so as to exclude cation chelation and to minimize other forms of ion pairing. Dependence of p*K* on substituent is actually stronger in dimethylacetamide, where counterion electrostatic 'levelling' ought to be greatest. For significant levelling to occur, these factors would have to be more beneficial than intramolecular H-bonding, which raises a question of why the enzymes under considerationg use amide-NH in preference.¶

In this vein, a tempting hypothesis has been that in the biochemical system the substrate carbonyl is held out-of-range for hydrogen bonding with respect to at least one of the enzymic carboxamides within an initial E·S complex, so that an additional energetic advantage accrues when geometric adjustment in the course of reaction subsequently renders H-bonding feasible within the tetrahedral adduct. This stratagem represents a variation of the bound-substrate destabilization mechanism of enzymic reactant activation, which may be conceptually distinguished from transition state stabilization. As in all other such schemes, no beneficial effect is provided in the enzymic specificity constant k_{cat}/K_m by such action.[5] The latter parameter is the only proper index of kinetic acceleration, since it correlates the ground state energy of the substrate in solution with that of the transition state for the first committed step involving the enzyme. The substrate binding step does not enter in this second-order rate constant. In the posed counter-example wherein H-bonding is initially occluded upon substrate docking, an accompanying loss of ground state interactions upon formation of the E·S complex, specifically those involving water engaged in solvation of the substrate carbonyl (as well as the enzymic amide), would offset the purported kinetic gain as regards substrate destabilization. So long as mutational effects in proteases are judged by the pertinent k_{cat}/K_m comparison, substrate destabilization can be of no consequence. Of course the potential entropy-negating role of the enzymic carboxamides in properly orienting the substrate for nucleophilic addition ought not to be ignored. However, that would most certainly involve H-bonding within the productive E·S complex; *i.e.*, there must be an entropy–enthalpy compensation in this regard, and the net consequences, if any, would manifest exclusively within the first-order saturation rate constant k_{cat}, which admits only ambiguous interpretation.

It is instructive to compare this transient oxyanion buttressing by means of H-bonding, as found in the serine proteases, with Lewis-acid stabilization of the same intermediate oxyanion by a zinc dication, as occurs within the metallo-proteases.[12] A recent systematic examination of substrate-analogue phenolate binding to carboxypeptidase A has

§ It has been specified[4] that exceptional 'low-barrier' or 'short, strong' H-bonds should occur only when pK_a values of the donor and acceptor atoms match. This appears not to obtain in the case of the salicylamides, although bending of the NH so as to linearize the H-bond in **1**-anion creates H–N–C angle strain, which should have an effect of intramolecularly acidifying the NH group. Regardless, Fig. 1 shows no sign of curvature at the upper end, which would have to be extreme if the line were to extrapolate to an H-bond energy of 10–20 kcal mol^{-1} for an oxyanion of $pK_a \approx 15$ (approximate acidity of amide NH) as has been suggested. Since the probable basicity of an oxyanion within a carboxamide acyl-transfer transition state is less than that,[6] and nearer the range of the phenolates examined, the lower value of our empirically estimated H-bond stabilization energies seems more relevant to the enzymic situation here considered in any event. Also, the potential for existence of any especial stabilization in heteronuclear (O ··· HN) cases has been questioned.[11]

¶ The collateral observation that a plot of the pK_a values of **1** *versus* those of corresponding phenols, absent of any *ortho* substituent, yields a (linear) slope of 1.05 (±0.03) indicates that H-bonding by solvent to ArO^- is only slightly inferior to that by intramolecular amide NH; partial exclusion of either form of stabilization by shielding on one side of the phenoxide yields a slope of 1.2 (pK_a values of **2** *versus* same reference phenols); see also Stahl and Jencks.[7] These coefficients also indicate that resonance delocalization of anionic charge into the carboxamide substituent is not capable of masking the H-bonding effect here considered. The pK_a values of **2** are generally within a one unit of the corresponding reference phenols, and the subtractive comparison of **1** and **2** should result in cancellation of any such contribution as regards α.

provided a corresponding Brønsted-type coefficient for that family of enzymes, which is useful for judging the potency of the active site metal ion contained therein.[13] In that study K_i values were ascertained for a series of inhibitors presenting a phenolic oxyanion to the enzymic zinc when bound at the active site. The variation in affinity of *para*-substituted phenolate residues for Zn^{2+} (as abstracted from pK_i values), when plotted against proton affinities for the same oxyanions (pK_a values, as in the present study), yielded a coefficient of $\alpha = 0.76$. This indicates that an enzymic metal ion is considerably more efficacious in the role of stabilizing a tetrahedral-adduct oxyanion and its associated transition states (a phenomenon labelled as 'fluxionate Lewis acidity').[13] While a pair of H-bonds in subtilisin might together provide a kinetic contribution of perhaps a thousand-fold to peptide hydrolysis, a single zinc ion in carboxypeptidase can yield as much as a million-fold more than that (assuming the same *ca.* 12 pH-unit increment in oxygen basicity on the part of the incipient oxyanion upon traversal of the reaction coordinate to the tetrahedral-adduct transition state). This perhaps explains why the serine proteases need an internal nucleophile (Ser hydroxymethyl) and precisely positioned general-base catalysis (His imidazole coupled with Asp carboxylate), whereas the metalloproteases are able to exploit a direct hydration mechanism of peptide scission with a probable lesser demand upon a general base. With the *endo*metalloprotease thermolysin it is likely the diad His 231/Asp 226 that, in analogy to the serine proteases, fulfils the role of H^+-abstractor from the nucleophile (H_2O).[14] In the case of carboxypeptidase A it appears to be the substrate carboxylate which similarly helps to induce peptide scission by transient acceptance of a proton.[15] The catalytic general base is *not* provided by electrostatically augmenting residues Glu 143 or Glu 270 within these metalloproteases (as has commonly been assumed), according to our studies with those enzymes.[12-15]

While exceptionally strong H-bonds were not detected in this study, the anion-stabilizing capacity of dimethylacetamide as a solvent should not go unremarked. According to the data recorded in Table 1, phenols having an aqueous pK_a value of < 7 also spontaneously ionize to an extent of greater than 50% in dimethylacetamide solution. It appears that the dipolar environment provided by that solvent is capable of providing considerable support for the phenolates, comparable to that available in H_2O, but in the absence of solvent-donated H-bonds. Molecules of $AcNMe_2$ and H_2O have similar basicity, so that the spontaneous ionization of phenols in dimethylacetamide, comparable to that in water, cannot be attributed to a superior proton-accepting capability of the organic solvent. This is relevant to the enzyme active site deliberation. Dipoles similar to that in dimethylacetamide are abundantly available in the enzyme peptidic backbone and side-chains, as has been long recognized. It would seem probable that cumulative and geometrically enforced orientation of such moieties about an active site could provide a more potent anion-stabilizing factor, irrespective of pK_a, than is the case for the special-but-limited electrostatic interaction known as the hydrogen bond.[16] In summary, H-bonding within the 'oxyanion hole' of the serine proteases may be critical to the catalytic mechanism, but it does not appear to be capable of accounting energetically for more than a small portion of the abatement in intrinsic activation barrier for hydrolytic peptide cleavage that distinguishes the enzymic process. Of course that transition state stabilization does occur in some fashion enzymically, and the oxyanion hole notion can be modified to include the longer-range dipolar electrostatic factors that we have suggested. However, at least with regard to heteronuclear cases ($O^- \cdots HN$) such as here considered, any hypothesis of active-site 'low-barrier' transient hydrogen bonds as a universal component of enzymic catalysis[4] would seem to need more substantiation before being adopted as a legitimate major explanation for kinetic acceleration within active sites.

Experimental

Preparation of 5-substituted salicylamides

Substrates of type **1** and **2** were obtained by heating the methyl esters of the commercially available or otherwise previously known substituted salicylic acids with either *n*-butylamine or pyrrolidine at 80 °C for several hours, followed by recrystallization of the residue (or chromatographic purification in the case of oils). In the case of 2,4-$(CH_3O_2C)_2C_6H_3OH$ it was shown by IR spectroscopy that the *ortho* rather than the *para* ester had reacted (the H-bonded $-CO_2-$ absorption was that replaced). Sulfoxides were secured conventionally by oxidation of the methylthio-substituted amides. Structures were confirmed by NMR as well as by IR (Table 1).

pK_a measurements

UV–VIS spectra of buffered (*ca.* 0.025 mol dm^{-3}) solutions of the phenolic amides (*ca.* 0.1 mmol dm^{-3}) were serially obtained at 25 °C over the pH region of phenolic ionization [buffers: citrate, phosphate, tris(hydroxymethyl)aminomethane, bis-(hydroxymethyl)aminoethane, aminoethanol]. In each case the phenolate absorption as a function of pH was directly fitted to the pertinent sigmoidal curve by the least-squares method, yielding the pK_a value analytically (Table 1). A tight isosbestic was invariably noted between phenol and phenolate absorptions. Since the objective was direct pairwise comparison of **1** with **2** under equivalent conditions, calibrated pH meter readings at 25 °C were used directly in specifying pK_a values (*i.e.*, Table 1 presents practical constants). Metal ions (sodium) in the buffers were kept at concentrations below that required for chelation of salicylamide, excluding pK_a perturbations from this source.

pK_{DMA} measurements

Pairs of salicylamides were dissolved in *N*,*N*-dimethylacetamide (*ca.* 0.1 mmol dm^{-3}) and the acidity was incrementally adjusted by addition of concentrated (*ca.* 0.1 mmol dm^{-3}) dimethylacetamide solutions of trifluoroacetic acid or potassium *tert*-butoxide as needed, with UV–VIS spectral determination after each addition of several μl, yielding sigmoidal titration curves. Generally, the ionization of each species was followed at a previously determined isosbestic of the other. A mixture was then selected in which each component was partially ionized, and relative acid dissociation constants were obtained from the relationship $K_a/K_a' = [A][AH']/[A'][AH]$, with concentrations estimated from absorption measurements. Also useful in establishing relays were the following phenols (relative pK_{DMA}): 3,5-Cl_2-2-OH-C_6H_2CONH-C_4H_9 (−3.2), 3,5-Cl_2-2-OH-$C_6H_2CON(CH_2)_4$ (0.7), 5-NO_2-2-OH-$C_6H_3CO_2CH_3$ (−1.6), 2,4-$(CH_3O_2C)_2C_6H_3OH$ (2.2). For extremely acidic solutions it was necessary to estimate an acidity function from the titration curve for 5-NO_2-2-OH-$C_6H_3CONHC_4H_9$, in order to obtain internally consistent relative pK_{DMA} values. On the alkaline limb, relative pK_{DMA} values of > 4 could not be obtained without addition of excessive amounts of potassium *tert*-butoxide. Generally, tolerances mentioned in this article are standard errors from least-squares analysis; for the pK_{DMA} values error limits have been estimated from congruities within cyclic relays.

Acknowledgements

This work was supported by the US National Institutes of Health. D. C. Y. C. was the recipient of an Undergraduate Research Fellowship from the Baxter Healthcare Corporation.

References

1 (*a*) A. J. White, K. Drabble, S. Ward and C. W. Wharton, *Biochem. J.*, 1992, **287**, 317; (*b*) P. J. Tonge and P. R. Carey, *Biochemistry*, 1992, **31**, 9122.

2 (*a*) D. M. Blow, *Acc. Chem. Res.*, 1976, **9**, 145; (*b*) R. M. Sweet, H. T. Wright, J. Janin, C. H. Chothia and D. M. Blow, *Biochemistry*, 1974, **13**, 4212; (*c*) M. D. Finucane and J. P. G. Malthouse, *Biochem. J.*, 1992, **286**, 889; (*d*) A. Warshel, G. Naray-Szabo, F. Sussman and J.-K. Hwang, *Biochemistry*, 1989, **28**, 3629.

3 (*a*) P. Bryan, M. W. Pantoliano, S. G. Quill, H.-Y. Hsiao and T. Poulos, *Proc. Natl. Acad. Sci. USA*, 1986, **83**, 3743; (*b*) J. A. Wells, B. C. Cunningham, T. P. Graycar and D. A. Estell, *Phil. Trans. R. Soc. London*, 1986, **A317**, 415; (*c*) P. Carter and J. A. Wells, *Proteins: Struct., Funct., Genet.*, 1990, **7**, 335; (*d*) P. Carter, L. Abrahmsén and J. A. Wells, *Biochemistry*, 1991, **30**, 6142; (*e*) R. Ménard, J. Carrière, P. Laflamme, C. Plouffe, H. E. Khouri, T. Vernet, D. C. Tessier, D. Y. Thomas and A. C. Storer, *Biochemistry*, 1991, **30**, 8924.

4 (*a*) J. A. Gerlt and P. G. Gassman, *Biochemistry*, 1993, **32**, 11943; J. A. Gerlt and P. G. Gassman, *J. Am. Chem. Soc.*, 1993, **115**, 11552; (*b*) W. W. Cleland and M. M. Kreevoy, *Science*, 1994, **264**, 1887.

5 (*a*) L. Pauling, *Am. Sci.*, 1948, **36**, 51; (*b*) W. P. Jencks, *Adv. Enzymol. Relat. Areas Mol. Biol.*, 1975, **43**, 219; (*c*) R. L. Schowen, in *Transition States of Biochemical Processes*, Plenum, New York, 1978, pp. 77–114.

6 (*a*) W. P. Jencks, *Chem. Rev.*, 1972, **72**, 705; (*b*) J. P. Fox and W. P. Jencks, *J. Am. Chem. Soc.*, 1974, **96**, 1436; (*c*) A. C. Satterthwait and W. P. Jencks, *J. Am. Chem. Soc.*, 1974, **96**, 7018 and 7031; (*d*) T.-C. Liang and R. H. Abeles, *Biochemistry*, 1987, **26**, 7603.

7 N. Stahl and W. P. Jencks, *J. Am. Chem. Soc.*, 1986, **108**, 4196.

8 I. D. Sadekov, V. I. Minkin and A. E. Lutskii, *Russ. Chem. Rev.*, 1970, **39**, 179.

9 M. H. Abraham, *Chem. Soc. Rev.*, 1993, **22**, 73.

10 R. Taylor and O. Kennard, *Acc. Chem. Res.*, 1984, **17**, 320.

11 P. Gilli, V. Bertolasi, V. Ferretti and G. Gilli, *J. Am. Chem. Soc.*, 1994, **116**, 909.

12 W. L. Mock and J. Z. Zhang, *J. Biol. Chem.*, 1991, **266**, 6393.

13 W. L. Mock, D. J. Freeman and M. Aksamawati, *Biochem. J.*, 1993, **289**, 185.

14 W. L. Mock and M. Aksamawati, *Biochem. J.*, 1994, **302**, 57.

15 W. L. Mock and X. Xu, *Bioorg. Chem.*, 1994, **22**, 373.

16 J. P. Guthrie and R. Kluger, *J. Am. Chem. Soc.*, 1993, **115**, 11569.

Paper 4/07900K
Received 29*th December* 1994
Accepted 18*th July* 1995