Fluxionate Lewis acidity of the Zn^{2+} ion in carboxypeptidase A

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Competitive inhibition constants K_i for a series of phenol-ring-substituted derivatives of α -(2-hydroxyphenyl)benzenepropanoic acid have been ascertained by observing their influence on the catalytic hydrolysis of a peptide substrate by the zinc enzyme carboxypeptidase A. The pH-dependence of K_i shows that binding is maximal between two pK_a values: one is that of the phenol group of the inhibitor, and the other uniformly has a value of 6, the pK_a of a Zn^{2+} -bound water molecule on the enzyme in the absence of substrate or inhibitor. This is

the dependence expected if phenolate binds to the Zn^{2+} displacing its bound $\text{H}_2\text{O}/\text{HO}^-$. A log-log plot of the dissociation constants for the productive forms of inhibitor plus enzyme versus the acid dissociation constants of the phenolic residues in the inhibitors yields a straight line with a slope of +0.76. This number indicates that the active-site metal ion has special capacity for dispersing negative charge, such as builds up on the oxygen atom of a carboxamide group undergoing nucleophilic addition.

INTRODUCTION

Although many details of the chemical mechanism by which the metalloenzyme carboxypeptidase A (CpA) cleaves the susceptible peptide bond within its substrates are as yet unclear, it seems certain that the active-site Zn^{2+} ion participates as a Lewis acid in some fashion, most probably in polarizing the carbonyl group of the scissile amide linkage [1]. The enzymic metal centre has received extensive magnetic and spectroscopic investigation (usually with other ions substituted for Zn^{2+}) [2], but until now the characterization of its Lewis acidity has been incomplete. By one measure it is anomalously potent in this regard: the water molecule which binds to the externally available ligation position of the active-site Zn^{2+} ion in the absence of a substrate has its acidity increased, lowering its pK_a to a value of approx. 6, as previously suggested [3,4] and here subsequently confirmed. This can be compared with (hexa)quo- Zn^{2+} ion in homogeneous solution, for which a pK_a of approx. 9 is found [5]. The diminished proton affinity of the conjugate base, hydroxo- Zn^{2+} species at the active site may be attributed in part to the reduced co-ordination number of the metal ion as observed crystallographically [6–8]. This restriction of the number of chelating (donor) ligands augments its uncompensated electron deficiency. The proton acidity of an externally co-ordinating indicator ligand (such as H_2O) provides one practical index of the effective Lewis acidity of the metal ion.

However, there is another dimension to the phenomenon of Lewis acidity. Of greater significance for chemical insight into the mechanism of CpA is the response of the active-site metal ion to varying basicity in an external ligand. The reason for this can be appreciated by a cursory consideration of the addition of a nucleophile to a carboxamide when this is assisted by an electrophilic catalyst. The basicity of the oxygen atom of the carbonyl group (expressed conventionally as the protonic pK_a of its conjugate acid; Figure 1) undergoes a substantial enhancement

as the reaction co-ordinate is traversed (nucleophile adds) [9–11]. Because of this, a Lewis acid such as a metal ion, held in pre-equilibrium co-ordination to the carbonyl oxygen atom, may speed up the reaction by binding more tightly to the tetrahedral adduct than to the reactant. This is the essence of Lewis acid catalysis. What one wishes to know in the case of the enzyme is just how much more firmly does the active-site metal ion co-ordinate to a strongly basic external ligand (T^+ or T^-) than it does to a weakly basic one ($-\text{CONH}-$). This information embodies an integral aspect of the enzyme mechanism; most efficient catalysis should result if the energetic response by the enzyme to changing basicity in the substrate were large. Characterization of the latter property of the CpA active site, which we call the fluxionate Lewis acidity, comprises the subject of the present paper.

MATERIALS AND METHODS

Materials

CpA was supplied by Sigma Chemical Co. (no. C 0386). The Allan form was chosen for its reported greater solubility. It was recrystallized by dialysis according to established procedures before use [12]. Cobalt CpA was prepared by repeated extraction

Figure 1 Illustration of substantial increase in carboxamide oxygen basicity on tetrahedral adduct formation

Abbreviations used: CpA, bovine carboxypeptidase A (EC 3.4.17.1); FaFF, *N*-[3-(2-furyl)acryloyl]-L-phenylalanyl-L-phenylalanine; I-XY, generic representation of following inhibitors: I-H, α -(2-hydroxyphenyl)benzenepropanoic acid; I-NO, α -(2-hydroxy-5-nitrophenyl)benzenepropanoic acid; I-CN, α -(5-cyano-2-hydroxyphenyl)benzenepropanoic acid; I-Cl, α -(5-chloro-2-hydroxyphenyl)benzenepropanoic acid; I-NAC, α -(5-acetamido-2-hydroxyphenyl)benzenepropanoic acid; I-NOCl, α -(3-chloro-2-hydroxy-5-nitrophenyl)benzenepropanoic acid; I-CNCl, α -(3-chloro-5-cyano-2-hydroxyphenyl)benzenepropanoic acid; I-ClCl, α -(3,5-dichloro-2-hydroxyphenyl)benzenepropanoic acid.

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Figure 2 Structure of kinetic assay substrate (FaFF) and preparation of inhibitors I-XY

See Table 1 for substituents X and Y in various I-XY. Reagents in the upper row are: For I-NO, $X^+ = \text{La}(\text{NO}_3)_3$, NaNO_3 , HCl ; $2\text{H} = \text{N}_2\text{H}_4$, H_2O_2 . For I-CN, $X^+ = \text{I}_2$ followed by CuCN ; $2\text{H} = \text{Na}(\text{Hg})$, NaOH . For I-Cl, $X^+ = \text{SO}_2\text{Cl}_2$; $2\text{H} = \text{Na}(\text{Hg})$, NaOH . For I-NOCl, $X^+ = \text{La}(\text{NO}_3)_3$, NaNO_3 , HCl ; $Y^+ = \text{KClO}_3$, HCl ; $2\text{H} = \text{N}_2\text{H}_4$, H_2O_2 . For I-CNCl, $X^+ = \text{I}_2$ followed by CuCN ; $Y^+ = \text{KClO}_3$, HCl ; $2\text{H} = \text{Na}(\text{Hg})$, NaOH . For I-Nac, $\text{H}_2(\text{Pt})$ (on I-NO) followed by Ac_2O .

of a suspension of the crystalline enzyme with aqueous 1,10-phenanthroline with subsequent reconstitution with cobaltous chloride, following established procedures [13]. Enzyme concentrations were estimated using $\epsilon_{278} = 6.42 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Buffers employed in this work for kinetic analysis were (0.05 M each) as follows: 2-amino-2-methyl-1,3-propanediol, pH 8.75–10.0; Tris, pH 7.25–8.75; Mes, pH 5.3–7.25. All enzyme work was done with solutions 1.0 M in NaCl. Solutions at a pH of 6.5 or lower were made 0.05–0.1 mM in additional Zn^{2+} as required. The assay substrate for kinetic work was *N*-[3-(2-furyl)acryloyl]-L-phenylalanyl-L-phenylalanine (FaFF, $K_m = 0.1 \text{ mM}$) [14] from Sigma. Preparation of new inhibitors is outlined in Figure 2, with experimental details in the Appendix. Known analogues I-H and I-ClCl were synthesized by similar methods [3,4].

Methods

The pH-dependence of K_i for inhibitors I-XY with CpA was determined at $25.0 \pm 0.1^\circ\text{C}$ in buffers previously listed with FaFF as assay substrate at an initial concentration (20 μM) of less than K_m , with spectrophotometric (328 nm, 2 cm path-length) analysis and the method of initial rates. Enzyme concentration was maintained well below inhibitor concentration in K_i determinations (100-fold). Values of K_i were obtained at each pH by a non-linear least-squares fit of data from perturbation of

$k_{\text{cat.}}/K_m$ to the appropriate inhibition equation. The observed inhibition is known to be of the competitive type, and is due to the L-configuration enantiomers of I-XY exclusively [3]; K_i values for the racemic materials employed in this work have been adjusted accordingly. All pH values are calibrated pH-meter readings uncorrected for ionic strength effects. Tolerances listed are standard errors from least-squares analysis.

RESULTS

An active-site probe

It has previously been shown that phenolic substrate analogues of type I-XY (Figure 2) are tightly bound competitive inhibitors for CpA [3]. They contain the carboxy and adjacent benzyl groups that are specifically recognized by the enzyme, and are typically possessed by rapidly hydrolysed peptides (e.g. an *N*-acylphenylalanine such as FaFF, the substrate used here for assay). Additionally, in I-XY a phenolic hydroxy group appears to occupy the position of the carbonyl oxygen within the scissile linkage of the substrate (--- in depiction of FaFF; Figure 2), and consequently is appropriately situated for interaction with the active-site metal ion.

The Brønsted acidity of a phenol may be easily modulated through a desirable range of $\text{p}K_a$ values by variation in ring substituents (X and Y in I-XY). We reasoned that the contemplated characterization of enzymic Lewis acidity might be secured by a comparison of the ligation strength of differently substituted phenolate residues towards the metal ion contained within the active site of CpA. In principle, the required affinity measurements should be obtainable by determination of competitive K_i values for the inhibition of peptidase activity by various representatives of I-XY, provided that the ring substituents themselves do not interact differently with the enzyme. In an attempt to accommodate the latter restriction, only uncharged and similarly sized phenol substituents have been employed, as listed in Table 1. Variation of phenolic $\text{p}K_a$ was achieved primarily by incorporation of electron-withdrawing *para* substituents (X); however, in order to extend the scale, an *ortho*-chlorine (Y) was added in a comparable number of cases. Inhibitor *para*-substituents X within complexes $\text{E} \cdot (\text{I-XY})$ appear to be freely exposed to solvent in models built from a crystal structure of the enzyme, although the phenolic hydroxy of the flexible side chain of residue Tyr-248 could potentially make contact with the bound inhibitor. With larger inhibitor substituents than used in this study, the side chain of Ile-247 might become involved, resulting in a perturbation of K_i .

Table 1 Inhibition constants and $\text{p}K_a$ values for various I-XY

Parameters for least-squares fit of inhibition data in Figure 3 to expression in Scheme 1 (with $\text{p}K_{\text{EH} \cdot \text{HOH}} = 6$ and $\text{p}K_{\text{EH}} = 9.2$, from $k_{\text{cat.}}/K_m$ versus pH profile).

Inhibitor:	(substituents)	$\text{p}K_{\text{A/OH}}^*$	$(K_i)_{\text{lim.}} (\mu\text{M})^\dagger$	$\text{p}K_{\text{EH} \cdot \text{I}}^\dagger$
I-H:	(X = H, Y = H)	10.76 ± 0.08	51.8 ± 0.6	9.56 ± 0.12
I-NO:	(X = NO_2 , Y = H)	7.55 ± 0.01	0.082 ± 0.005	9.18 ± 0.08
I-CN:	(X = CN, Y = H)	8.70 ± 0.02	0.17 ± 0.01	9.81 ± 0.41
I-Cl:	(X = Cl, Y = H)	10.18 ± 0.01	0.34 ± 0.02	9.47 ± 0.10
I-Nac:	(X = NHCOCH_3 , Y = H)	10.57 ± 0.08	19.4 ± 0.3	9.54 ± 0.07
I-NOCl:	(X = NO_2 , Y = Cl)	5.96 ± 0.05	0.22 ± 0.02	8.03 ± 0.19
I-CNCl:	(X = CN, Y = Cl)	7.14 ± 0.04	0.56 ± 0.03	8.19 ± 0.04
I-ClCl:	(X = Cl, Y = Cl)	8.85 ± 0.05	1.04 ± 0.05	9.71 ± 0.38

* From spectrophotometric determination in the same saline buffer as for kinetics.

† From curve-fitting using $K_{\text{EH} \cdot \text{HOH}}$, K_{EH} and $K_{\text{A/OH}}$ as non-adjustable parameters.

Figure 3 Plot showing pH-dependence of specificity constant k_{cat}/K_m (a) and of competitive inhibition constants K_i (b) for CpA in buffered saline solution

Presentation is in log–log form, i.e. $\log k$ and $-\log K_i$ (or $\text{p}K_i$) versus pH. For the kinetic data $\{[3]$, hydrolysis of substrate FaFF, (a), $\blacksquare$), the curve is a least-squares fit to the expression $(k_{\text{cat}}/K_m)_{\text{apparent}} = (k_{\text{cat}}/K_m)_{\text{lim}} / \{(1 + [H]/K_{a1})(1 + K_{a2}/[H])\}$, with $\text{p}K_{a1} = 6$ and $\text{p}K_{a2} = 9.2$, as denoted by breaks in the asymptotic dashed line (Dixon plot: slopes of +1, 0 and -1 , formally indicating kinetic order in hydroxide for acidic, neutral and alkaline solution). For the inhibition data (b, obtained with assay substrate FaFF and $[S] < K_m$): $\circ$, I-NO; $\diamond$, I-CN; $\triangle$, I-Cl; $\bullet$, I-NOCl; $\blacklozenge$, I-CNCl; $\blacktriangle$, I-ClCl. The curves are least-squares fits to the expression in Scheme 1, with derived parameters listed in Table 1 (asymptotic lines comparable with the upper plot have been omitted for clarity).

Measurement of binding

The determination of competitive K_i values involves measurement of the velocity of substrate hydrolysis (with $[S] < K_m$) in the presence of various concentrations of inhibitor, in our case in buffered solution using a continuous spectrophotometric analysis and the method of initial rates. The expected hyperbolic dependence of substrate cleavage rate on inhibitor concentration was found, and experimental K_i values were secured by non-linear least-squares curve-fitting to the appropriate equation

$$(k_{\text{cat}}/K_m)_{\text{apparent}} = k_{\text{cat}} / \{K_m(1 + [I]/K_i)\}$$

It may be noted that these inhibitors are quite potent; under favourable conditions, I-NO adheres to the active site with a K_i of approx. $0.1 \mu\text{M}$. In order to extract the desired information, it was necessary to establish the hydronium ion-dependence of competitive inhibition for each I-XY, by repetitive determination over a range of pH values. The ionization state of functional groups on the enzyme as well as within the inhibitor have a marked effect on the affinity between the two species, as may be seen in the logarithmic plots in Figure 3. The general pattern is for tightest inhibitor binding at intermediate pH, with diminished enzyme affinity at the pH extremes, as indicated by the fitted curves for I-XY. The results for I-H [3,4] and I-NAc are not shown; they are similar to that of I-Cl, but with rather higher K_i values overall. In order to appreciate the significance of the present results, as well as to understand CpA generally, it is important to comprehend the origin of this pH-dependence.

To begin, it is necessary to review the catalytic response to pH variation of the enzyme itself. CpA has a bell-shaped pH-dependence for the relevant specificity index k_{cat}/K_m (which corresponds to a second-order rate constant for peptide hydrolysis at very low substrate concentration). Characteristic behaviour is shown in logarithmic form in Figure 3(a). Any downward bend in the curve represents the $\text{p}K_a$ for an ionization of the free enzyme or substrate [15]. In the present instance, the pH profile reveals two essential ionizable active-site functional groups, having $\text{p}K_a$ values of approx. 6.0 and approx. 9.2 which

bracket the region of maximum enzyme activity. From this it may be inferred that an intermediate protonation state of the enzyme (EH rather than EH_2 or E) is exclusively productive catalytically. The identity of the enzymic residues giving rise to this pH profile was in dispute until recently. In fact, inhibitors of type I-XY were specifically introduced for the purpose of resolving the question [3]. Firstly, we note that the enzymic protonation manifested on the acidic limb of the profiles ($\text{p}K_a$ value of 6) affects catalysis and inhibition equivalently; all of the inhibitors show a fall-off in enzyme affinity in acidic solution, as does the processing of substrates by the enzyme, quite possibly for the same reason. On the alkaline limb, however, the apparent decrease in inhibitor binding correlates with the acidity of the phenol moiety within the inhibitor. For the latter species, $\text{p}K_a$ values for phenolic ionization have been independently determined in the same buffered saline solutions as used for kinetics, by quantitative u.v.–visible spectrophotometric measurements of the phenolate absorbance as a (sigmoidal) function of pH. The resulting values are listed as $\text{p}K_{\text{IArOH}}$ in Table 1. In the cases of I-NO, I-NOCl and I-CNCl (and probably I-CN and I-ClCl), the fall-off in enzyme affinity manifested on the alkaline limb of the $\log K_i$ versus pH profile is clearly attributable to the phenol group of the inhibitor, because the down-turn correlates explicitly with the acidity of that moiety. The least-squares-fitted lines drawn in Figure 3 employ the spectrophotometric $\text{p}K_a$ as a non-adjustable parameter causing the observed curvature. In the cases of I-H, I-Cl and I-NAc, the phenol residue is less acidic, with $\text{p}K_{\text{IArOH}}$ values higher than 10. In the latter instances, we believe that the observed inflection near pH 9 in the $\log K_i$ profile is due to an enzymic residue, specifically, the same group that causes the break in the k_{cat}/K_m profile (Figure 3a), and that a perturbation due to deprotonation of the inhibitor phenol group goes undetected only because enzyme instability prevents examination of the appropriate pH range.

Reverse protonation

Although it seems certain from the pH profiles that the ionization state of the phenol group in the inhibitors regulates binding to the enzyme (as it should if the phenolic moiety were interacting with the active-site metal ion), there is a peculiarity in the pH profiles that requires explanation. Enzyme affinity is tightest at pH values below the $\text{p}K_a$ of the phenol group, and deprotonation of the inhibitor seems to cause a monotonic decrease in binding (i.e. on the alkaline limb, the value of the slope $\Delta \text{p}K_i / \Delta \text{pH}$ approaches -1). It appears as if the undissociated phenol were being bound, and the phenolate were being rejected. However, it has been spectroscopically established to the contrary that I-XY does in fact bind to the enzyme exclusively as the phenolate anion. This follows from direct u.v.–visible examination of enzyme–inhibitor complexes in relatively concentrated solution. A stoichiometric adduct with the phenylazo derivative (I-XY, with $\text{X} = \text{N}=\text{N}-\text{Ph}$ and $\text{Y} = \text{H}$) displays, at all accessible pH values (5–10), an indicative long-wavelength visible absorbance of the anionic form of the chromophore (azophenolate) [3]. Moreover, cobalt CpA (the fully functional enzyme in which Co^{2+} has been substituted for the active-site Zn^{2+}) displays a perturbation of the characteristic visible absorption of the metal ion, consequent to forming an adduct with this type of inhibitor, as exemplified in Figure 4 for the complex with I-CNCl. The broad Co^{2+} absorption in the vicinity of 560 nm for the free enzyme is transformed into a sequence of enhanced peaks at 511, 589, 610 and 627 nm on addition of slight excess of inhibitor. Such a drastic change is best interpreted as phenolate ligation to the

Figure 4 Visible absorption spectra of CoCpA

A buffered saline solution of enzyme (1.1 mM, pH 6.7) was scanned in the absence of inhibitor (---) and in the presence of a slight molar excess of I-CNCl (—).

Co^{2+} ion, with the latter apparently decreasing its co-ordination number. The longer-wavelength triad is diagnostic for a tetrahedral ligand field for Co^{2+} . This feature is common to other $\text{E} \cdot (\text{I-XY})$ complexes. However, the absorption in Figure 4 at 511 nm for the enzyme complex of I-CNCl occurs instead at 492, and at 504 nm in the cases of I-H and I-ClCl; this latter transition would appear to be a charge-transfer band involving the phenolate and the metal ion [4]. Recently secured crystallographic evidence confirms ligation of the phenolate oxygen to the active-site Zn^{2+} ion (G. Shoham and H. M. Greenblatt, personal communication).

The explanation for this apparent contradiction between kinetic and spectroscopic evidence for involvement of the phenolate group lies in the concept of reverse protonation [16]. The key realization is that binding of inhibitor also depends absolutely on the enzymic residue with a pK_a value of approx. 6, which must be in its conjugate acid form to allow effective co-ordination of the inhibitor phenolate moiety to the enzyme. Consequently at intermediate pH values there is a compensation, such that maximally tight binding occurs midway between the pK_a values of inhibitor and enzyme. The only chemically plausible explanation is that the crystallographically observed water molecule which co-ordinates to the active-site metal ion in the absence of substrates or inhibitors has had its acidity increased by the Zn^{2+} , with a resultant pK_a value of 6. A phenolate residue can easily displace H_2O from the enzyme, but it competes with OH^- very much less readily. Below a pH of 6, all of the free enzyme in solution becomes susceptible to inhibitor, but the apparent value of K_i increases (weaker binding) because the already low concentration of the exclusively bound form of the inhibitor (phenolate) becomes progressively diminished in solution with decreasing pH. Conversely, when the phenolate species becomes predominant (above a pH corresponding to the pK_a of inhibitor), the apparent value of K_i also increases, due to yet further depletion of the more susceptible form of the enzyme. At intermediate pH values, these factors cancel one another, resulting in a level pH-dependence for K_i . As developed subsequently, the true affinity of the bound form of the inhibitor (ArO^-) for the more receptive form of the enzyme ($\text{Zn}^{2+} \cdot \text{HOH}$) is stronger than is suggested by the apparent limiting value of K_i , since the latter does not account for the fact that it is exclusively minor species that formally participate in the binding equilibrium at intermediate pH values.

This same pK_a value of 6 as seen in the enzyme kinetics has been widely (but incorrectly) attributed to the side chain carboxy

Scheme 1 Equilibria responsible for kinetic behaviour of CpA in the presence of I-XY

The corresponding expression explaining apparent inhibition constant as a function of pH is as follows:

$$(K_i)_{\text{apparent}} = (K_i)_{\text{lim.}} (1 + [\text{H}]/K_{\text{EH} \cdot \text{HOH}}) (1 + K_{\text{IArOH}}/[\text{H}]) (1 + K_{\text{EH} \cdot \text{I}}/[\text{H}]) / (1 + K_{\text{EH} \cdot \text{I}}/[\text{H}]).$$

group of enzymic residue Glu-270. This assignment is rendered untenable by the experimental observation that this ionization controls binding of I-XY absolutely. The pH profiles for K_i contain no detectable compensatory inflection corresponding to the same functional group within the enzyme-inhibitor complex. Either the acidic group has been displaced (as we propose), or at least it has assumed a pK_a value of greater than 10 in the complex (which is implausible for a carboxy group). Furthermore, the conclusive evidence for reverse protonation requires that the conjugate acid form of this functionality be the productive species in the binding of inhibitors, and most probably also for substrates (which commonly bind more weakly than I-XY). This observation would not be reconcilable with the widely assumed (but as yet unproven) general base role for Glu-270- CO_2^- in the catalytic cycle, were that group the moiety with pK_a of 6. We do not exclude the possibility that the ionization of $\text{Zn}^{2+} \cdot \text{HOH}$ is influenced by the presence of Glu-270 (possessed of a probable typical carboxy pK_a of approx. 4), but it is the second acid dissociation constant of the coupled system comprised of these residues that is detected kinetically. For more complete analysis see discussion elsewhere [3].

The overall situation is summarized in Scheme 1. The formal enzyme-inhibitor dissociation constant for the components existing in the complex is given by K_d , which is related to the experimental value of $(K_i)_{\text{lim.}}$ by the magnitudes of K_{IArOH} and $K_{\text{EH} \cdot \text{HOH}}$, acid dissociation constants of functional groups on inhibitor and enzyme respectively. It is the K_d values that contain the pertinent chemical affinity information for this study, as developed subsequently.

Fit to the pH profile

A set of equilibria accommodating the complete pH-dependence of the experimental inhibition constant K_i as regards binding of all I-XY to CpA is provided in Scheme 1, along with an appropriate equation expressing the overall observed value of K_i in terms of the individual dissociation constants. The curves presented in Figure 3 are least-squares fits to this expression. As previously indicated, the primary determinants of the shape of the curves are $\text{pK}_{\text{EH} \cdot \text{HOH}}$ and pK_{IArOH} . The magnitudes of these acid ionization constants are independently established (fixed). The value of $\text{pK}_{\text{EH} \cdot \text{HOH}}$ is in every case 6.0 (from the $k_{\text{cat.}}/K_m$ versus pH-dependence). Separate spectrophotometric determination provides a value of pK_{IArOH} for each inhibitor I-XY as given in Table 1 (first column of data). The values given for $(K_i)_{\text{lim.}}$ (second column) correspond to the least-squares limiting value for the competitive inhibition constant midway between

Figure 5 Pictorial summary of pK_a values for active-site functionality within CpA and its I-XY complexes

these two pK_a values in Figure 3. The extreme alkaline limb of the profiles shows an additional small perturbation, and this merits a brief comment. It has previously been suggested that the pK_a value of 9.2 seen in the $k_{\text{cat.}}/K_m$ versus pH profile (pK_{EH}) is due to one of the two enzymic imidazole ligands that (in conjunction with Glu-72) hold the Zn^{2+} ion at the active site. This attribution is compatible with crystallographic and spectral evidence, and has been supported by induced-shift p.m.r. data for the imidazole ring protons as a function of pH with cobalt CpA [4]. The tentative assignment for the pK_a of 9.2 is to His-196. The K_i versus pH profiles in Figure 3 suggest that dissociation of a proton from this site influences phenolate binding to the metal ion, but only marginally so (as might be expected for an indirectly coupled peripheral ionization). Curve-fitting indicates that the value for K_i is perturbed only slightly by this deprotonation (in contrast with the pK_a of 6 ionization, which controls binding absolutely). Should this be the case, it becomes a thermodynamic necessity that the pK_a of the responsible functional group is also perturbed by a comparable small amount in the enzyme-inhibitor complex. Our least-squares refinement of the K_i versus pH data yields in most cases an estimate of the value of $\text{pK}_{\text{EH}\cdot\text{I}}$ (probably the same ionization as pK_{EH} , but in the presence of saturating amounts of inhibitor), as is also listed in Table 1 (third column of data). It may be noted that for the complexes with weakly basic phenolates (which are less capable of satisfying the electron deficiency of the metal ion) the $\text{pK}_{\text{EH}\cdot\text{I}}$ value is approx. 8, whereas for the more strongly basic phenolates the same acid dissociation has a value of approx. 9.5. This is chemically reasonable for a coupled system of this nature. Confirmation of such an ionization was sought by examining the visible spectrum of the complex $\text{E}\cdot(\text{I-CNCl})$ (Figure 4) as a function of pH, but, not surprisingly, no significant perturbation was noted. Whatever may be the source of the extreme alkaline limb inflection in the pH profile, it has no effect on the main conclusions of this paper, which have to do with the relationship between pK_{IArOH} and $(K_i)_{\text{lim.}}$ (or K_d). The pertinent enzymic ionizations are summarized in Figure 5.

DISCUSSION

Role of Zn^{2+} in CpA

By now it is a commonplace realization that no single universal chemical explanation for enzymic efficacy exists, but rather that enzyme active sites promote reaction by marshalling a number of factors so as to stabilize transition states for the catalysed reaction. Hence, enzyme mechanistic rationalization has become a reductionist programme of attempting to quantify contributing components to an overall rate acceleration. Although there is still uncertainty about the sequence of catalysis steps in the case of CpA [17], the metal ion is clearly the locus of reaction. A valuable static picture of its environment is provided by X-ray-crystallographic and various spectroscopic investigations. However, these can be ambiguous as regards mechanism, and until now functional characterization of the role of the active-site Zn^{2+}

has been limited. The previously devised 'synergism test' provides reactivity evidence that the oxygen atom of the scissile carbonyl group co-ordinates to the active-site metal ion at a critical juncture in peptide hydrolysis [1]. We refer to the finding that the native enzyme (ZnCpA) is a relatively poor catalyst for the cleavage of a thiopeptide substrate analogue $\text{PhCO-NH-CH}_2\text{-CO-NH-CH}_2\text{-C(=S)-NH-CH(CH}_2\text{Ph)-CO}_2\text{H}$, but that the thioamide linkage is cleaved efficiently by cadmium carboxypeptidase A (CdCpA, the enzyme species in which the active-site Zn^{2+} has been replaced by Cd^{2+}). Furthermore, CdCpA is a less efficient enzyme than ZnCpA for normal carboxamide-containing peptide substrates (as measured by $k_{\text{cat.}}/K_m$). We explain this behaviour by polarizability considerations in the dative interaction between substrate and enzymic metal ion (hard-soft acid-base theory; 'soft' acid Cd^{2+} beneficially interacts with 'soft' base S, but 'hard' Zn^{2+} prefers 'hard' O in substrate carbonyl). However, this pattern only makes sense if the metal ion functions in Lewis acid fashion to polarize the substrate carbonyl in the course of peptide hydrolysis, as is also suggested by enzyme crystallographic investigations.

Quantification of Zn^{2+} acidity

With acknowledgement of its substrate-activating role, perception of how well the metal ion is able to disperse increasing negative charge during the course of the catalytic process (fluxionate Lewis acidity) bears importantly on the question of mechanism, as indicated in the Introduction. A useful approach to the problem of evaluating this particular dimension of Lewis acidity may be found in inorganic co-ordination chemistry. The idea is to compare the metal ion with a proton, as regards its ligation by a series of oxyanions. For example, the strength of complexation of simple phenolates with ferric ion in aqueous solution has been evaluated quantitatively with respect to the influence of aryl substituents on affinity for the metal ion. When appropriate pK_a values (defined as the negative logarithm of the dissociation constant for $\text{Fe}^{3+}\cdot\text{ArO}^-$) for a series of phenols are plotted against the corresponding phenolic pK_a values, a linear correlation emerges. The slope of the regression line in the case of ferric ion has a coefficient α of approx. 0.9 ($\alpha = \Delta\text{pK}_d/\Delta\text{pK}_a$) [18,19]. This is a linear free energy (Brønsted-type) relationship, which in the present instance is measuring the 'proton-like' character of Fe^{3+} . What it says is that ferric ion is able to disperse the charge from a phenolate anion nearly as well as does a proton. A similar determination has been reported for vanadic ion [20].

Relevant information for this type of comparison in the case of CpA is contained in our results. The pertinent numbers are not the experimental K_i values in Table 1, but rather the computed dissociation constants for the formally bound minor component species (Scheme 1, top). These may be derived from our data by the thermodynamically enforced relationship $K_d = (K_i)_{\text{lim.}} \cdot K_{\text{IArOH}}/K_{\text{EH}\cdot\text{HOH}}$. In dealing with our limited data set, we have to take cognizance of the fact that in half of the inhibitors that we used the presence of *ortho*-Cl substituents

Figure 6 Plot of pK_a versus $pK_{a, \text{ortho-H}}$ for various I-XY

$pK_d = p(K_f)_{\text{lim}} + pK_{a, \text{ArOH}} - pK_{\text{EH} \cdot \text{HOH}}$. The resulting straight line (slope = 0.76 ± 0.01) is for a simultaneous least-squares fit of pK_d for *ortho*-H-containing (○) as well as for *ortho*-Cl-containing (△) inhibitors to the same gradient but with an iteratively adjusted offset (-0.79 ± 0.02 pK unit) on the vertical axis for I-NOCl, I-CNCl and I-ClCl.

(which were incorporated to extend the range of phenol acidities) might be expected to introduce a systematic perturbation of K_d from solvation or enzymic interactions not present in the case of the other inhibitors. Preliminary data fitting suggested that the halo group does cause an approximately 6-fold decrease in enzyme affinity uniformly across the series. In the ultimate correlation this was treated as an adjustable parameter. As may be seen in Figure 6, a quite satisfactory linear relationship results when pK_d is plotted against $pK_{a, \text{ArOH}}$, under the singular constraint that the subsets with and without an *ortho*-Cl substituent adopt the same slope. The least-squares value found for the latter coefficient is $\alpha = 0.76 \pm 0.01$. This is a conservative data treatment; the value for α is greater without a segregation of the *ortho*-Cl derivatives. In fitting the data, I-H and I-NAC have been excluded; both fall significantly off the regression line, for reasons requiring further investigation.

What is the mechanistic significance of α ? For one thing, it allows a comparison of the metal ion at the active site of CpA with ordinary aquated Zn^{2+} in homogeneous solution. Although simple phenols co-ordinate rather too poorly to this ion for convenient measurement, appropriate data exist for chelation by substituted salicylaldehydes of aqueous Zn^{2+} ion [21,22]. The derived value for the coefficient α in the latter case is only 0.35. It appears from this comparison that the enzyme-bound metal ion is considerably more efficacious at dispersing anionic charge than is Zn^{2+} in solution. The enzymic Zn^{2+} ion responds much more positively to a change in basicity in the oxyanion occupying its exchangeable ligand position.

Catalytic consequences

Although a phenolate may seem structurally removed from a peptide linkage, an attempt can be made to relate this evidence to the mechanism of the enzyme. As indicated in the introduction, the oxyanion in the transient tetrahedral adduct from a carboxamide ($\text{T}^\pm$ or T^-) is many orders of magnitude more basic than is the amide itself. Consequently, as the tetrahedral adduct forms by nucleophilic addition, a continuous increase in pK_a for the carbonyl oxygen atom must characterize the reaction co-ordinate. In the latter stages of addition (encompassing the transition state), the developing pK_a value should be within the range for the phenolate oxyanions of this investigation. Therefore the static response of the enzyme to changes in basicity in the

phenolate groups may well parallel its dynamic interaction with the incipient tetrahedral adduct. For that reason the magnitude of the enzymic coefficient α bears directly on the mechanism of catalysis. Succinctly but somewhat anthropomorphically put, the greater the value of α , the more effectively in principle should the enzyme be able to 'pull' a bound substrate toward the tetrahedral intermediate.

A rough calculation of the catalytic significance of this 'fluxionate' factor may be developed. For the sake of simplified argument, we will postulate an intrinsic 10 pK-unit increase in the pK_a of the carbonyl oxygen of the amide moiety as it traverses the reaction co-ordinate for nucleophilic addition, as shown in Figure 1 [9–11]. (Although strictly speaking one should be concerned with the transition states for formation and decomposition of tetrahedral adducts, we shall couch our argument in terms of stabilization of the intermediate $\text{T}^\pm$.) The consequences of a pre-equilibrium co-ordination of the substrate carbonyl group to the active-site Zn^{2+} ion of CpA are easily estimated. Initial ligation of substrate to the active-site Zn^{2+} ion may give in neutral solution 76% of the catalytic benefit to be derived from specific acid catalysis by the hydronium ion (i.e. pre-equilibrium protonation of the carboxamide group). Since at a pH of about 7.5 (the optimum for CpA), less than 1 in 10^8 substrate molecules would be so activated spontaneously by protonation, the kinetic acceleration from this consideration alone may amount to more than 10^6 (compared with a factor of approx. 10^3 for Zn^{2+} ion in homogeneous solution, provided that the latter could be induced to co-ordinate to a carboxamide group). An analogy might also be invoked with respect to protonic general acid catalysis, for which a Brønsted coefficient of 0.76 would indicate substantial participation.

There has been considerable speculation about an 'entatic state' in CpA, in which it is hypothesized that the resting enzyme is energetically 'poised for catalysis' so as to engage the substrate productively by virtue of intrinsic destabilizing interactions in the co-ordination environment of the metal ion which are relaxed as the catalytic cycle proceeds [23]. To us, this concept seems to be merely a special formulation of the Pauling principle of catalysis (transition-state mimicry), cast in a rather mystical and mechanismless form [24]. However, it could be argued that we have in fact quantified the phenomenon, since our analysis addresses changes in energy of interaction as the bound substrate proceeds to tetrahedral adduct within the active site.

Although the foregoing calculation of kinetic contribution from Zn^{2+} as a specific acid catalyst has a back-of-an-envelope flavour and involves considerable extrapolation, it provides a useful insight into the mechanism of CpA. What really matters catalytically at the active site is the difference in binding energy involving the co-ordinating acidic species in response to the change of basicity occurring within the reacting substrate. The new term 'fluxionate' emphasizes this most relevant dynamic aspect of Lewis acidity. This concept is central to explaining what the Zn^{2+} does during the course of catalysis; a large value for α helps us to understand how the enzyme can promote tetrahedral adduct formation during amide cleavage. The observed hyperacidity of this metal ion is presumably a result of the evolutionary process, and probably should primarily be associated physically with a constricted co-ordination sphere provided by the enzymic environment. An unavoidable consequence of the metal ion's special Lewis acidity is an enhanced Brønsted acidity for the water molecule which co-ordinates to the active site in the absence of substrate or inhibitor ($pK_{\text{EH} \cdot \text{HOH}} = 6$), as shown conclusively here and previously [3]. This probably represents the natural check on evolutionary pressure to increase Lewis acidity of the metal ion. Because

hydroxide competes with substrate for the active-site Zn²⁺ ion even at neutral pH, it constitutes an unavoidable endogenous inhibitor. The best compromise between efficaciousness of metal ion and tolerable level of inhibition by hydroxide is probably represented by this p*K*_a of 6 which characterizes the enzymic kinetics (and by the associated α of 0.76).

This paper has focused entirely on a functional characterization of the active-site Zn²⁺ ion of CpA. Previous papers have addressed other aspects of the enzyme mechanism, such as identity and stereochemistry of the nucleophile adding to the enzyme-activated amide linkage [1,3,17,25]. Such matters are not considered here in detail. Briefly, the kinetics and pH-dependence of catalytic peptide hydrolysis can be adequately rationalized by a 'reverse protonation' mechanism for substrate activation analogous to that invoked to explain inhibition by I-XY. The nucleophile that attacks the Zn²⁺-activated carboxamide group is probably a solvent water molecule aided by one of the two carboxylate groups that are present at the active site when substrate is bound [17].

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REFERENCES

- Mock, W. L., Chen, J.-T. and Tsang, J. W. (1981) *Biochem. Biophys. Res. Commun.* **102**, 389–396
- Auld, D. S. and Vallee, B. L. (1987) in *Hydrolytic Enzymes* (Neuberger, A. and Brocklehurst, K., eds), pp. 201–254, Elsevier, Amsterdam
- Mock, W. L. and Tsay, J.-T. (1986) *Biochemistry* **25**, 2920–2927
- Mock, W. L. and Tsay, J.-T. (1988) *J. Biol. Chem.* **263**, 8635–8641
- Perrin, D. D. (1982) *Ionization Constants of Inorganic Acids and Bases in Aqueous Solution*, 2nd edn., pp. 136–138, Pergamon, Oxford
- Woolley, P. (1975) *Nature (London)* **258**, 677–682
- Kimura, E. and Koike, T. (1991) *Comments Inorg. Chem.* **11**, 285–301
- Rees, D. C., Lewis, M. and Lipscomb, W. N. (1983) *J. Mol. Biol.* **168**, 367–387
- Jencks, W. P. (1972) *Chem. Rev.* **72**, 705–718
- Fox, J. P. and Jencks, W. P. (1974) *J. Am. Chem. Soc.* **96**, 1436–1449
- Satterthwait, A. C. and Jencks, W. P. (1974) *J. Am. Chem. Soc.* **96**, 7018–7044
- Mock, W. L. and Chen, J.-T. (1980) *Arch. Biochem. Biophys.* **203**, 542–552
- Auld, D. S. and Holmquist, B. (1974) *Biochemistry* **13**, 4355–4361
- Riordan, J. F. and Holmquist, B. (1984) in *Methods of Enzymatic Analysis*, 3rd edn. (Bergmeyer, H. W., ed.), vol. 5, pp. 44–55, Verlag Chemie, Deerfield Beach, FL
- Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol.* **45**, 273–387
- Cleland, W. W. (1982) *Methods Enzymol.* **87**, 390–405
- Mock, W. L. and Zhang, J. Z. (1991) *J. Biol. Chem.* **266**, 6393–6400
- Jabalpurwala, K. E. and Milburn, R. M. (1966) *J. Am. Chem. Soc.* **88**, 3224–3227
- Ernst, Z. L. and Herring, F. G. (1965) *Trans. Faraday Soc.* **61**, 454–461
- Galeffi, B. and Tracey, A. S. (1988) *Can. J. Chem.* **66**, 2565–2569
- Postmus, C., Jr., Magnusson, L. B. and Craig, C. A. (1966) *Inorg. Chem.* **5**, 1154–1157
- Leussing, D. L. and Bai, K. S. (1968) *Anal. Chem.* **40**, 575–581
- Vallee, B. L. and Galdes, A. (1984) *Adv. Enzymol. Relat. Areas Mol. Biol.* **56**, 283–430
- Schowen, R. L. (1978) in *Transition States of Biochemical Processes* (Gandour, R. D. and Schowen, R. L., eds.), pp. 77–114, Plenum, New York
- Mock, W. L. and Tsay, J.-T. (1989) *J. Am. Chem. Soc.* **111**, 4467–4472

APPENDIX

Details of syntheses of various I-XY, summarized in Figure 2, are presented below. Because of favourable crystallinity, some intermediates were isolated in the form of γ -lactones linking the phenolic hydroxy and the carboxy groups [i.e. 2(3*H*)-benzofuranones].

Preparation of I-NO

To a solution of 70 mg of lanthanum trinitrate hexahydrate and 2.75 g (32 mmol) of sodium nitrate in 17 ml of 15% HCl at 0–5 °C, was added 4.88 g (32 mmol) of (2-hydroxyphenyl)acetic acid in 120 ml of diethyl ether. The mixture was stirred vigorously in a closed flask for 18 h, during which the temperature came to approx. 20 °C and the mixture turned red (signifying completion of reaction). Saturated NaCl solution was added and the ether phase was separated and washed twice with additional saturated aqueous NaCl. The ether was then extracted twice with 4 M NaOH, and the combined extracts were washed several times with chloroform. The alkaline solution was then acidified to a pH of approx. 3 with conc. HCl. The acidic solution was boiled with charcoal, filtered through Celite, and the filtrate was thoroughly extracted with ethyl acetate. The organic phase was rotary evaporated, and 100 ml of toluene was added to the residue. The resulting mixture was brought to a vigorous boil with stirring for 1–2 min (dissolves *ortho* isomer). The hot mixture was then rapidly suction filtered, and the collected brown powder was washed with cool toluene, to give 2.6 g (41 %) of (2-hydroxy-5-nitrophenyl)acetic acid, m.p. 151–154 °C; 400 MHz p.m.r. δ (p.p.m.) [(²H)acetone] 3.76 (2 H, s), 7.06 (1 H, d, *J* 8.9 Hz), 8.0 (1 H, dd, *J* 8.9, 2.8 Hz), 8.19 (1 H, d, *J* 2.8 Hz); i.r. (KBr) 1320 cm⁻¹ (–NO₂), 1715 (–CO₂H).

A mixture of 1.07 g (5.4 mmol) of this material, 0.3 g of anhydrous sodium acetate, 1.04 g (1 ml, 9.8 mmol) of benz-

aldehyde and 1.6 g (1.5 ml) of acetic anhydride was heated to 90 °C with stirring for 16 h. Then 10 ml of water was added, and heating and stirring was continued for 15 min. After cooling in an ice bath, the aqueous layer was decanted, leaving a solid residue containing the product. This was triturated for 20 min with 15 ml of hot methanol, and the resulting mixture was chilled in an ice bath. Product was collected; after slow evaporation of the filtrate, additional product was obtained on trituration of the residue, yielding a total of 0.7 g (48 %) of *Z*- and *E*-5-nitro-3-(phenylmethylene)-2(3*H*)-benzofuranone. The isomers were submitted to di-imide reduction without separation. This material was dissolved in 10 ml of tetrahydrofuran and 1.5 ml of 4 M NaOH. After 10 min the solution was acidified with conc. HCl to a pH of approx. 3, and the tetrahydrofuran was removed by rotary evaporation. The organic material was extracted into methylene chloride, and the solvent was then evaporated. The residue was dissolved in 13 ml of water containing 4.6 ml (0.14 mol) of 95 % hydrazine. To this solution was added 10.3 ml (0.1 mol) of 30 % H₂O₂ by motorized syringe at 25 °C over a period of 24 h with stirring. Then conc. HCl was added until a precipitate was obtained. The resulting mixture was vigorously stirred with ethyl acetate and then filtered. The organic phase was separated and evaporated, and the residue was taken up in 5 ml of acetic anhydride and heated at 110 °C for 1 h (to achieve lactonization, for convenience of purification). Excess acetic anhydride was removed by rotary evaporation, and the residue was taken up in benzene and passed through a silica column. Material recovered on evaporation was taken up in chloroform, diluted with petroleum ether and allowed to stand at –20 °C for 24 h. A precipitate was collected and recrystallized twice in the same way to give 162 mg (23 %) of 5-nitro-3-(phenylmethyl)-2(3*H*)-benzofuranone, m.p. 92–93 °C; 400 MHz p.m.r. δ (p.p.m.) [(²H)chloroform] 3.14 (1 H, dd, *J* 8.3, 13.8 Hz), 3.53 (1 H, dd, *J*

5.1, 13.8 Hz), 4.11 (1 H, dd, J 5.1, 8.3 Hz), 7.13 (3 H, m), 7.30 (3 H, m), 7.74 (1 H, d, J 2.3 Hz), 8.22 (1 H, dd, J 2.3, 8.8 Hz); i.r. (chloroform) 1345 cm^{-1} ($-\text{NO}_2$), 1830 (lactone) (Found: C, 66.5; H, 4.0; N, 5.1; $\text{C}_{15}\text{H}_{11}\text{NO}_4$ requires C, 66.9; H, 4.1; N, 5.2%). To prepare stock solutions of I-NO, this material was dissolved in 2 M NaOH and the solution was then neutralized.

Preparation of I-CN

To 10.0 g (65.8 mmol) of (2-hydroxyphenyl)acetic acid in 300 ml of 3 M NaOH at 70 °C was added 16.7 g (65.8 mmol) of iodine, and the mixture was stirred at this temperature for 2.5 h. The chilled reaction mixture was carefully neutralized and then slowly acidified by dropwise addition of HCl in an ice bath until precipitation of a solid was complete. After standing for 10 min in the cold, the precipitate was collected and washed with cold water. This material was suspended in 600 ml of 0.1 M HCl and brought to the boil for 10 min. Undissolved material (mostly diiodide) was collected and again extracted with HCl as before. The filtrates were extracted with ethyl acetate, and the combined extracts were rotary evaporated. The residue was dissolved in ethanol and filtered. Evaporation yielded 6.14 g (34%) of (2-hydroxy-5-iodophenyl)acetic acid, m.p. 157–160 °C; 200 MHz p.m.r. δ (p.p.m.) [(^2H)acetone] 3.61 (2 H, s), 6.72 (1 H, d, J 8.5 Hz), 7.41 (1 H, dd, J 8.5, 2.3 Hz), 7.54 (1 H, d, J 2.3 Hz); i.r. (KBr) 1700 cm^{-1} ($-\text{CO}_2\text{H}$).

A mixture of 12.02 g (43.2 mmol) of the previous material and 5.4 g (60.3 mmol) of cuprous cyanide in 20 ml of *N*-methyl-2-pyrrolidinone was stirred for 3.75 h at 140 °C. The resulting mixture was then added to a solution of 107 g (1.9 mol) of KCN in 300 ml of water, and the mixture was stirred for 20 min. It was then acidified to a pH of about 3 with conc. HCl (hazard: toxic HCN gas liberated). After addition of 150 ml of methylene chloride, the mixture was stirred for several minutes. After filtration, the phases were separated and the aqueous portion was again extracted with methylene chloride. The combined organic extracts were evaporated and the residue was dissolved in 1 M NaOH. After being washed with ethyl acetate, the aqueous portion was acidified to a pH of about 3, again extracted with ethyl acetate, and the latter extract was rotary evaporated. The solid residue was extracted with boiling water. On cooling, pale-yellow crystals formed, yielding 4.3 g (56%) of (5-cyano-2-hydroxyphenyl)acetic acid, m.p. 179–182 °C; 200 MHz p.m.r. δ (p.p.m.) [(^2H)acetone] 3.69 (2 H, s), 7.04 (1 H, d, J 8.4 Hz), 7.51 (1 H, dd, J 8.4, 2.1 Hz), 7.61 (1 H, d, J 2.1 Hz); i.r. (KBr) 1725 cm^{-1} ($-\text{CO}_2\text{H}$), 2230 ($-\text{CN}$).

A mixture of 0.60 g (3.4 mmol) of this material, 0.16 g of anhydrous sodium acetate, 0.63 g (0.6 ml, 5.9 mmol) of benzaldehyde, 0.76 g (0.7 ml) of acetic anhydride and 0.5 ml of *N*-methyl-2-pyrrolidinone was heated to 95 °C with stirring for 13 h. Then 15 ml of water was added, and after an additional 10 min of heating the mixture was cooled in an ice bath. After decantation of the liquid, the residual solid was boiled in methanol for 20 min. A yellow powder was collected, and the filtrate was concentrated to give more such material. A total of 0.553 g (66%) of 5-cyano-3-(phenylmethylene)-2(3*H*)-benzofuranone was obtained, and was reduced without further purification. A 0.336 g (1.4 mmol) portion of this material was dissolved in 20 ml of tetrahydrofuran and 10 ml of 1 M NaOH. With stirring, 2% sodium amalgam was added in portions at intervals of several minutes, with monitoring of the reaction by silica t.l.c. (ethanol eluent; reactant R_f 0.59, 0.71; product R_f 0.66). At completion, the solution was decanted from mercury and was acidified with HCl. After removal of the tetrahydrofuran

by rotary evaporation, the product was extracted into methylene chloride. Removal of solvent gave a residue which was boiled in benzene for 30 min. On cooling, the precipitate was collected; the filtrate deposited additional product on slow evaporation, yielding a total of 0.351 g (97%) of α -(5-cyano-2-hydroxyphenyl)benzenepropanoic acid (I-CN), m.p. 170–172 °C; 200 MHz p.m.r. δ (p.p.m.) [(^2H)chloroform] 3.10 (1 H, dd, J 7.8, 13.5 Hz), 3.39 (1 H, dd, J 7.8, 13.5 Hz), 4.05 (1 H, t, J 7.8 Hz), 6.91 (1 H, d, J 8.4 Hz), 7.09 (2 H, m), 7.22 (3 H, m), 7.30 (1 H, d, J 1.9 Hz), 7.42 (1 H, dd, J 1.9, 8.4 Hz); i.r. (KBr) 1700 cm^{-1} ($-\text{CO}_2\text{H}$), 2240 ($-\text{CN}$) (Found: C, 72.2; H, 5.2; N, 5.5; $\text{C}_{16}\text{H}_{13}\text{NO}_3$ requires C, 71.9; H, 4.9; N, 5.2%).

Preparation of I-Cl

To a stirred solution of 1.70 g (11.2 mmol) of (2-hydroxyphenyl)acetic acid in 10 ml of acetonitrile at 25 °C was added over a period of 15 min 1.63 g (1 ml, 12.1 mmol) of sulphuryl chloride in 5 ml of acetonitrile. After 1 h, 5 ml of water was added, and the acetonitrile was removed by rotary evaporation. The residue was dissolved in 8 ml of 4 M NaOH, and the solution was washed with ethyl acetate. After acidification with conc. HCl, the product was extracted into methylene chloride. The organic solution was concentrated to 10 ml and allowed to stand, whereon a precipitate slowly formed. Further concentration gave additional material. A total of 1.23 g (59%) of (5-chloro-2-hydroxyphenyl)acetic acid; 400 MHz p.m.r. δ (p.p.m.) [(^2H)acetone] 3.63 (2 H, s), 6.86 (1 H, d, J 9.0 Hz), 7.10 (1 H, dd, J 9.0, 3.0 Hz), 7.23 (1 H, d, J 3.0 Hz) was collected.

A mixture of 0.83 g (4.45 mmol) of this material, 0.30 g of anhydrous sodium acetate, 1.04 g (1.0 ml, 9.8 mmol) of benzaldehyde and 1.5 g (1.4 ml) of acetic anhydride was heated to 95 °C with stirring for 16 h. Then 10 ml of water was added, and, after an additional 10 min of heating, the mixture was cooled in an ice bath. After decantation of the liquid, 10 ml of methanol was added to the residue, and after boiling for 10 min the mixture was chilled. A precipitate was collected; the residue from evaporation of the filtrate was similarly treated to afford additional material. A total of 0.67 g (59%) of 5-chloro-3-(phenylmethylene)-2(3*H*)-benzofuranone was obtained, and was reduced without further purification. This material was submitted to sodium amalgam reduction in the manner of the preparation of I-CN, giving a quantitative yield of α -(5-chloro-2-hydroxyphenyl)benzenepropanoic acid (I-Cl); 400 MHz p.m.r. δ (p.p.m.) [(^2H)acetone] 3.04 (1 H, dd, J 6.7, 13.3 Hz), 3.33 (1 H, dd, J 8.3, 13.3 Hz), 4.34 (1 H, dd, J 6.7, 8.3 Hz), 6.89 (1 H, d, J 8.8 Hz), 7.08 (1 H, dd, J 2.5, 8.8 Hz), 7.18 (5 H, m), 7.27 (1 H, d, J 2.5 Hz). It was converted into the dicyclohexylamine salt for final purification, m.p. 188–189 °C (Found: C, 70.7; H, 8.0; N, 3.0; $\text{C}_{27}\text{H}_{36}\text{NO}_3\text{Cl}$ requires C, 70.8; H, 7.9; N, 3.1%).

Preparation of I-NOCl

To a mixture of 0.504 g (2.6 mmol) of (2-hydroxy-5-nitrophenyl)acetic acid, 2 ml of acetic acid and 4 ml of conc. HCl was added 0.212 g (1.7 mmol) of potassium chlorate. After being stirred at 25 °C for 2 h, the solution was cooled in an ice bath and the precipitate was collected and washed with cold water. Recrystallization from water gave 0.379 g (64%) of (3-chloro-2-hydroxy-5-nitrophenyl)acetic acid, m.p. 170–176 °C; 200 MHz p.m.r. δ (p.p.m.) [(^2H)acetone] 3.88 (2 H, d, J 1.2 Hz), 8.20 (1 H, d, J 2.2 Hz), 8.23 (1 H, dt, J 1.2, 2.2 Hz).

A mixture of 1.96 g (9.7 mmol) of this material, 0.47 g of anhydrous sodium acetate, 1.6 g (15.7 mmol) of benzaldehyde and 2.5 g (2.3 ml) of acetic anhydride was heated to 95 °C with

stirring for 18 h. Then 15 ml of water was added, and, after an additional 10 min of heating, the mixture was cooled in an ice bath. After decantation of the liquid, the residue was boiled in 20 ml of methanol for 30 min. After cooling and dilution with water, a precipitate was collected, giving 1.82 g (71 %) of 3-chloro-5-nitro-3-(phenylmethylene)-2(3*H*)-benzofuranone, which was submitted to di-imide reduction without further purification. A mixture of 146 mg of the previous material in 10 ml of tetrahydrofuran and 1 ml of 1 M NaOH was warmed to open the lactone. To the solution, 1 ml (30 mmol) of 95 % hydrazine was added, followed by slow addition over a period of 8 h of 3 ml (20 mmol) of 6 % H_2O_2 . The resulting solution was carefully acidified to a pH of about 3 with HCl, and then extracted with methylene chloride. Evaporation of the extract gave 84 mg (54 %) of α -(3-chloro-2-hydroxy-5-nitro-phenyl)benzenepropanoic acid (I-NOCl) as an amorphous solid; 400 MHz p.m.r. δ (p.p.m.) [(^2H)acetone] 3.16 (1 H, dd, J 7.5, 13.7 Hz), 3.42 (1 H, dd, J 7.9, 13.7 Hz), 4.45 (1 H, t, J 7.7 Hz), 7.2 (5 H, m), 8.12 (1 H, d, J 2.5 Hz), 8.18 (1 H, d, J 2.5 Hz). For purification it was converted into the dicyclohexylamine salt, m.p. 212 °C (dec); i.r. (KBr) 1335 cm^{-1} , 1575 ($-\text{NO}_2$), 1445, 1640 ($-\text{CO}_2^-$) (Found: C, 64.9; H, 7.1; N, 5.5; $\text{C}_{18}\text{H}_{11}\text{N}_2\text{O}_5\text{Cl}$ requires C, 64.5; H, 7.0; N, 5.6 %).

Preparation of I-CNCl

To a mixture of 1.74 g (9.8 mmol) of (5-cyano-2-hydroxyphenyl)acetic acid, 7.7 ml of acetic acid and 15.4 ml of conc. HCl was added 0.40 g (3.3 mmol) of potassium chlorate. After being stirred at 25 °C for 2 h, the solution was cooled to -20 °C for 18 h and the precipitate was collected and washed with cold water. Recrystallization from warm diethyl ether by dilution with petroleum ether gave 0.81 g (39 %) of (3-chloro-5-cyano-2-hydroxyphenyl)acetic acid, m.p. 172–175 °C; 200 MHz p.m.r. δ (p.p.m.) [(^2H)acetone] 3.78 (2 H, s), 7.64 (1 H, d, J 1.9 Hz), 7.78 (1 H, d, J 1.9 Hz); i.r. (chloroform) 1715 cm^{-1} ($-\text{CO}_2\text{H}$), 2230 ($-\text{CN}$).

A mixture of 1.40 g (6.6 mmol) of this material, 0.28 g of anhydrous sodium acetate, 1.04 g (1.0 ml, 9.8 mmol) of benz-

aldehyde and 1.6 g (1.5 ml) of acetic anhydride was heated to 92 °C with stirring for 16 h. Then 10 ml of water was added, and after an additional 10 min of heating the mixture was cooled in an ice bath and the precipitate was collected. After boiling in 10 ml of methanol for 20 min the solid was again collected and recrystallized from petroleum ether, giving 0.76 g of mixed isomers of 7-chloro-5-cyano-3-(phenylmethylene)-2(3*H*)-benzofuranone, which were reduced without further purification. This material was submitted to sodium amalgam reduction in the manner of the preparation of I-CN, giving quantitatively α -(3-chloro-5-cyano-2-hydroxyphenyl)benzenepropanoic acid (I-CNCl), m.p. 156–158 °C (dec); 200 MHz p.m.r. δ (p.p.m.) [(^2H)acetone] 3.14 (1 H, dd, J 8.1, 13.8 Hz), 3.43 (1 H, dd, J 7.5, 13.8 Hz), 4.40 (1 H, t, J 7.8 Hz), 7.2 (5 H, m), 7.56 (1 H, d, J 2.0 Hz), 7.73 (1 H, d, J 2.0 Hz); i.r. (KBr) 1705 cm^{-1} ($-\text{CO}_2\text{H}$), 2250 ($-\text{CN}$) (Found: C, 63.0, 63.1; H, 4.0, 4.1; N, 4.6, 4.6; $\text{C}_{16}\text{H}_{12}\text{NO}_3\text{Cl}$ requires C, 63.7; H, 4.0; N, 4.6; $\text{C}_{16}\text{H}_{12}\text{NO}_3\text{Cl} \cdot 0.2\text{H}_2\text{O}$ requires C, 62.9; H, 4.1; N, 4.6 %; note: these phenolic acids lactonize on heating, limiting intensive drying).

Preparation of I-Nac

A solution of 250 mg (0.93 mmol) of the lactone of I-NO in 15 ml of ethyl acetate together with 50 mg of Adams platinum oxide catalyst was submitted to hydrogenation at atmospheric pressure. After 4 h at 25 °C hydrogen uptake (91.5 ml) was complete and t.l.c. showed one product. An equal volume of acetic anhydride was then added and the solution was allowed to stand overnight. Evaporation and recrystallization of the residue from ethyl acetate and hexane gave 142 mg (54 %) of 5-acetamido-3-(phenylmethyl)-2(3*H*)-benzofuranone (lactone of I-Nac), m.p. 148–149 °C; 400 MHz p.m.r. δ (p.p.m.) [(^2H)chloroform] 2.13 (3 H, s), 3.10 (1 H, dd, J 8, 14 Hz), 3.42 (1 H, dd, J 5, 14 Hz), 4.00 (1 H, d, J 5, 8 Hz), 6.92 (1 H, d, J 7 Hz), 7.13 (1 H, d, J 7 Hz), 7.23 (6 H, m); i.r. (KBr) 1670 cm^{-1} (amide), 1790 (lactone) (Found: C, 72.0; H, 5.3; N, 4.9; $\text{C}_{17}\text{H}_{15}\text{NO}_3$ requires C, 72.6; H, 5.4; N, 5.0; $\text{C}_{17}\text{H}_{15}\text{NO}_3 \cdot 0.1\text{H}_2\text{O}$ requires C, 72.1; H, 5.4; N, 5.0 %). To prepare stock solutions of I-Nac, this material was dissolved in 2 M NaOH and the solution was then neutralized.