Reactive-Site Design in Folded-Polypeptide Catalysts—The Leaving Group pK_a of Reactive Esters Sets the Stage for Cooperativity in Nucleophilic and General-Acid Catalysis

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Abstract: The second-order rate constants for the hydrolysis of nitrophenyl esters catalysed by a number of folded designed polypeptides have been determined, and 1900-fold rate enhancements over those of the 4-methylimidazole-catalysed reactions have been observed. The rate enhancements are much larger than those expected from the pK_a depression of the nucleophilic His residues alone. Kinetic solvent isotope effects were observed at pH values lower than the pK_a values of the leaving groups and suggests that general-acid catalysis contributes in the pH range where the leaving group is predominantly protonated. In contrast, no isotope effects were observed at pH values above the pK_a of the leaving group. A Hammett ρ value of 1.4 has been determined for the peptide-catalysed hydrolysis reaction by variation of the substituents of the leaving phenol. The corresponding values for the imidazole-catalysed reaction is 0.8 and for phenol dissociation is 2.2. There is therefore, very approximately, half a negative charge localised on the phenolate oxygen in the transition state in agreement with the conclusion that transition-state hydrogen-bond formation may contribute to the observed catalysis. The elucidation at a molecular level of the principles that control cooperativity in the biocatalysed ester-hydrolysis reaction represents the first step towards a level of understanding of the concept of cooperativity that may eventually allow us to design tailor-made enzymes for chemical reactions not catalysed by nature.

Keywords: catalysts · de novo design · helical structures · histidine

Introduction

The design of folded-polypeptide catalysts provides a new approach to the study of enzymes, and opens up new routes to engineered non-natural biocatalysts with tailored specificities. Natural enzymes function through a wide range of catalytic mechanisms and show unrivalled selectivities and efficiencies, in spite of the fact that their structure and function is due, in principle, to amino acids of only modest chemical reactivity. One of the hallmarks of native enzymes is the concept of cooperativity. A necessary step towards the understanding of how to design new catalysts is therefore to elucidate the principles that govern the formation of powerful cooperative catalysts from the twenty common amino acid residues. The elucidation of the complex relationship between structure and function is, however, very difficult in natural enzymes where essentially every residue has multiple functions, and more virgin model proteins are clearly needed. Recently complex scaffolds or templates have become available where reactive-site geometries can be systematically altered through the developments in the field of de novo protein design.[1, 2] A number of proteins with non-natural sequences and well-defined tertiary structures are now available, for example four-helix bundles,[3–5] a triple-helix bundle,[6] monomeric triple-stranded β sheets[7, 8] and $\beta\beta\alpha$ motifs,[9, 10] and high-resolution NMR structures have been reported for most of them.[4–7, 9, 10] Instead of using a single-chain primary structure the template-assembled synthetic protein (TASP) concept[11] provides a promising alternative strategy to the design of stable polypeptide structures with protein-like properties.[12, 13] The engineering of reactive sites in a large variety of model proteins, with or without bound cofactors, is therefore now possible.

Structure determination of folded motifs provide the opportunity for correlating structure at the atomic level with function, but designed polypeptides that do not fold into well-ordered structures have also demonstrated catalytic efficiency in the decarboxylation[14] and peptide-ligation reactions.[15] The introduction of the cofactor pyridoxamine into a protein scaffold[16] and into a designed 23-residue peptide[17] has been shown to lead to catalysis of the transamination reaction, a key step in the biosynthesis of amino acids. We have focussed

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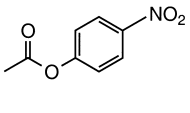
on the implementation of catalytic functions into folded four-helix bundle catalysts and demonstrated the catalysis of hydrolysis, transesterification,[18] amidation,[19] decarboxylation[20] and transamination reactions.[21] We have emphasised detailed structural and reaction-mechanistic studies in the design of new biocatalysts with a particular focus on the structure of the reactive sites[18] and their complexes, and on the implementation of cooperative mechanisms in catalysis.[22]

The hydrolysis of reactive esters, such as *p*-nitrophenyl acetate (**I**) , was catalysed by the 42-residue peptide KO-42 with rate enhancements of more than three orders of



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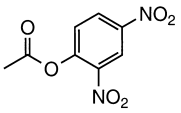

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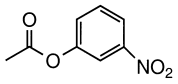


(III)

magnitude over that of the 4-methylimidazole-catalysed reaction. KO-42 was designed to fold into a helix-loop-helix motif that dimerises to form a four-helix bundle and was suggested after kinetic investigations to follow a reaction mechanism that includes cooperative nucleophilic and general-acid catalysis.[18] Structures and pK_a values are given in Figure 1 and Table 1. The design of the reactive sites was based on the concept of bringing together unprotonated and protonated histidine residues in close proximity since the imidazoyl side chain is a good nucleophile in aqueous solution at around neutral pH, and protonated His side chains are known to provide strong hydrogen bonds in enzymatic catalysis. The evidence in favour of cooperative catalysis was mainly the pH dependence of the second-order rate constant and the observation of a kinetic solvent isotope effect of 2.[18]

Abstract in Swedish: *Andra ordningens hastighetskonstanter för hydrolys av nitrofenylestrar katalyserade av designade polypeptider har bestämts och visats vara upp till 1900 gånger större än de för motsvarande 4-metylimidazol katalyserade reaktioner. Hastighetsökningarna är mycket större än vad som kan förväntas av pK_a sänkningarna av de nukleofila histidinerna. Kinetiska lösningsmedelsisotopieffekter observerades vid pH värden lägre än pK_a för de lämnande grupperna, vilket antyder att generell syrakatalys bidrar i pH intervallet där den lämnande gruppen huvudsakligen är protonerad. Å andra sidan observerades inga isotopieffekter vid pH över pK_a för den lämnande gruppen. Ett Hammett ρ värde på 1.4 bestämdes för den peptidkatalyserade hydrolysreaktionen genom att variera substituenterna på den lämnande fenolen. Det motsvarande värdet för den imidazolkatalyserade reaktionen är 0.8 och för fenoldissociation är värdet 2.2. Mycket approximativt är det därför en halv negativ laddning lokaliserat på fenolatsyret i övergångstillståndet vilket överensstämmer med slutsatsen att bildning av en vätebindning i övergångstillståndet bidrar till den observerade katalysen. Klargörandet på en molekylär nivå utav principerna som styr kooperativitet i biokatalyserad ester hydrolys reaktion representerar det första steget mot en förståelse av koncepetet kooperativitet, som så småningom kan göra det möjligt för oss att designa skräddarsydda enzymer för kemiska reaktioner som inte katalyseras i naturen.*

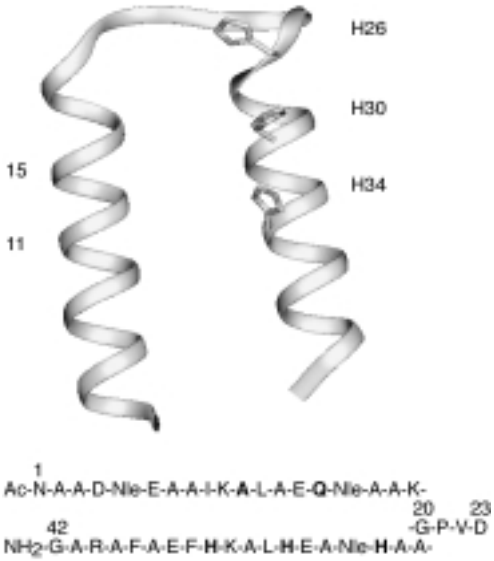


Figure 1. The modelled and primary structure of JN, the peptide that JNI, JNII, JNIIRO and JNIII are based upon. By replacing one of the three histidines in JN with "inactive" residues, JNI, JNII and JNIII were created. For JNI residue 34 is alanine and for JNII and JNIIRO residue 26 is glutamine. In addition, JNIIRO has arginine and ornithine residues in positions 11 and 15, respectively, in order to provide interhelical binding to anionic substrates. For JNIII residue 30 is glutamine. The catalytically active polypeptide is the dimer, but only the monomer is displayed for reasons of clarity. The residues that are varied are shown in bold.

Table 1. The histidine pK_a values and amino acid substitutions for the peptides investigated and referred to in this study. Positions not shown are the same as in Figure 1. The Footnotes indicate the references where the peptides first appeared.

Peptide	11	15	19	26	30	34
SA-42[a]	A	H (6.5)	K	Q	Q	A
KO-42[b]	H (6.9)	H (5.4)	H (7.0)	H (7.2)	H (5.3)	H (5.2)
JNI[c]	A	Q	K	H (6.9)	H (5.6)	A
JNII[c]	A	Q	K	Q	H (5.6)	H (5.6)
JNIIRO[d]	Orn	R	K	Q	H	H
JNIII[c]	A	Q	K	H (6.8)	Q	H (5.4)

[a] Reference [25]. [b] Reference [18]. [c] Reference [22]. [d] Reference [24].

The 42-residue peptides JNI, JNII and JNIII, based on the same structural template as KO-42 but with histidine residues only in positions 26 and 30 (JNI), 30 and 34 (JNII), and 26 and 34 (JNIII), were subsequently used to examine the mechanism of hydrolysis and transesterification of mono-*p*-nitrophenyl fumarate and *p*-nitrophenyl acetate (**I**)[22] first demonstrated in KO-42. JNI and JNII both have two histidine residues separated by four residues within a helix in the helix-loop-helix motif. The pK_a values of JNI were measured and found to be 6.9 and 5.6 for His 26 and 30, respectively. For JNII both His 30 and 34 had pK_a values of 5.6, and JNII is a factor of 5.5 more efficient than JNI in the hydrolysis of mono-*p*-nitrophenyl fumarate at pH 5.1 and 290 K.[22] A model to explain this difference in reactivity based on cooperative nucleophilic [Eq. (1)] and general-acid catalysis [Eq. (2)] was proposed, based on the Brønsted equations,

where k_2 is the second-order rate constant and the coefficients α and β describe the sensitivity of the rate constants to changes in pK_a of the catalysts. Close proximity and depressed pK_a values of the histidine residues were crucial for efficient catalysis to occur and the underlying argument is described in the following paragraph.

$$\log k_2 = A + \beta \mathrm{p}K_a \quad (1)$$

$$\log k_2 = A - \alpha \mathrm{p}K_a \quad (2)$$

The Brønsted coefficient is 0.8 for imidazole-catalysed hydrolysis of **I**.[23] At a pH below its pK_a a nucleophilic histidine residue with a lower pK_a is a more reactive nucleophile than one with a higher pK_a, since the decrease in the concentration of reactive unprotonated His at low pH is directly related to pK_a whereas the decrease in the reactivity of the unprotonated His residue is related to pK_a multiplied by 0.8. In other words the decrease in reactivity with pK_a is overcompensated for by the increase in the concentration of unprotonated nucleophile. Also, a protonated histidine residue with a low pK_a is a better general-acid catalyst than one with a higher pK_a provided that both are protonated to large extents. JNII (His-30, His-34) is thus suggested to be more efficient than JNI (His-26, His-30) because His-30 is as efficient a nucleophile as His-34, as they have identical pK_a values, but His-34 is a more efficient general-acid catalyst than His-26 because it has a lower pK_a. The proposed reaction mechanism for the hydrolysis of *p*-nitrophenyl esters catalysed by the HisH$^+$–His pair of JNII is outlined in Scheme 1. The catalytic efficiency is thus due to pK_a depressions of the catalytic residues as well as to the formation of hydrogen bonds. Using the Brønsted coefficient of 0.8 and the degree of protonation at pH 5.1 JNII can be calculated to be a factor of 24 more efficient as a catalyst in the hydrolysis of **I** than a hypothetical imidazole with the same pK_a, and this is most likely owing to general-acid catalysis by the flanking protonated HisH$^+$ residue.

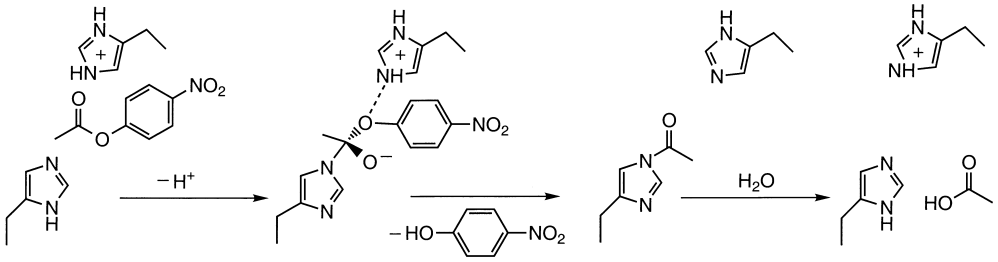
However, the need for general-acid catalysis may seem surprising since the *p*-nitrophenolate anion is a very good leaving group. Since the question of whether general-acid catalysis contributes to the observed reactivity is an important one in understanding cooperativity as well as transition state structure, we have now further investigated under what conditions this mechanism contributes to the observed catalysis. The pH profiles, kinetic solvent isotope effects and the Hammett ρ value for nitrophenyl leaving groups have been determined and these results demonstrate what the requirements are for general-acid catalysis to contribute to the hydrolysis of reactive esters by HisH$^+$–His pairs in folded-polypeptide and protein catalysts. This is a first step towards the understanding of how to introduce cooperative reaction mechanisms in designed catalysts.

Results

We have determined the reactivity of the designed polypeptides JNII and JNIIRO (Figure 1) towards the highly reactive substrate 2,4-dinitrophenyl acetate (**II**) and towards the less reactive *m*-nitrophenyl acetate (**III**) to gain further knowledge of the mechanism of polypeptide-catalysed hydrolysis of activated esters by comparison with their reactivities towards **I**.

The design and characterisation of the peptides used in this work have been described previously.[22, 24] In short the sequences are based on those of the template peptide SA-42[25] and the derived catalyst KO-42[18] that were designed, and found to form helix-loop-helix motifs that dimerise into four-helix bundles. SA-42 and KO-42 were thoroughly examined by NMR and CD spectroscopy and by equilibrium sedimentation ultracentrifugation. The reported mean residue ellipticities at 222 nm were -25000 ± 1000 deg cm^2 dmol^{-1} for SA-42 and -24000 ± 1000 deg cm^2 dmol^{-1} for KO-42, and correspond to helical contents of 60–70 %.[26, 27] The peptides in this study differ from SA-42 or KO-42 in five positions or less and they also exhibit CD spectra typical of α-helical structures with minima at 222 and 208 nm and mean residue ellipticities of -20000 to -25000 ± 1000 deg cm^2 dmol^{-1} at 222 nm. It is therefore assumed that they also form four-helix bundles. The pH and concentration dependencies of the mean residue ellipticities at 222 nm have now been examined for JNII and JNIIRO, and have been shown to vary between -18000 and -25000 ± 1000 deg cm^2 dmol^{-1} between pH 3.1 and pH 8.0, with concentrations in the range from 0.1 mM to 0.4 mM. The lower values were found for the lower pH. The dimer structures thus remain relatively constant throughout the range of experimental conditions and rate differences should not depend on structural changes to a large extent. The fact that the observed reactivity is linear with the concentration of peptide shows that the dimer is the catalytic species. The peptide JNIIRO loses a substantial fraction of its helical content at pH 3.1 where the mean residue ellipticity is only -12000 deg cm^2 dmol^{-1}, a behaviour that was also previously observed for KO-42.

The second-order rate constants for the hydrolysis of **II** and **III** catalysed by the peptides JNII and JNIIRO at pH 5.1 and 290 K have been determined and are presented in Table 2. The second-order rate constants of 7.3 and 7.2 M^{-1}s^{-1} for the



Scheme 1. The proposed reaction mechanism for cooperative nucleophilic and general-acid catalysis of activated esters by the HisH$^+$–His reactive site.

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Table 2. Second-order rate constants for the hydrolysis of ester substrates **I**, **II** and **III**, catalysed by designed peptides and 4-MeIm in aqueous solution at pH 5.1 and 290 K.

Catalyst	k_2 (**I**) [$M^{-1}s^{-1}$]	k_2 (**II**) [$M^{-1}s^{-1}$]	k_2 (**III**) [$M^{-1}s^{-1}$]
JNI	–	2.2	–
JNII	0.048[a]	7.3	–
JNIIRO	0.056[b]	7.2	0.013
JNIII	–	10.4	–
4-MeIm	0.00072	0.0081	4.2×10^{-4}

[a] Reference [22]. [b] Reference [24].

hydrolysis of **II** at pH 5.1 are three orders of magnitude larger than the 4-methylimidazole-catalysed reaction. The rates of hydrolysis of **II** catalysed by 4-methylimidazole (pK_a 7.9), imidazole (pK_a 6.95), 4-hydroxyimidazole (pK_a 6.45) and benzimidazole (pK_a 5.4) at pH 5.1 and 290 K were determined, and a Brønsted β value of 0.45 was calculated for the reaction (Figure 2). From the JNII pK_a values of 5.6 and the β value of 0.45 it was calculated that the JNII-catalysed hydrolysis of **II** is a factor of 68 more efficient than that of a hypothetical imidazole with a pK_a of 5.6 at pH 5.1 and 290 K. The Brønsted β value of 0.8 for the hydrolysis of **I**[23] was confirmed in the present investigation at pH 5.1 and 290 K using the same nucleophiles (data not shown). The kinetic solvent isotope effects for the hydrolysis of **II** catalysed by JNII and JNIIRO at pH 3.1 and 5.1 at 290 K were determined (Table 3). At pH 5.1 no effect was found and at pH 3.1 the kinetic isotope effects were found to be 1.5 and 2.5 for JNIIRO and JNII, respectively. The second-order rate constants for the hydrolysis of **I**, **II** and **III** at pH 5.1 and 290 K catalysed by JNIIRO (Table 2) were used to determine a Hammett ρ value for the reaction at pH 5.1 and 290 K (Figure 3). The second-order rate constant for the hydrolysis of **II** by JNII was measured in the pH range from 3.1 to 6.5 at 290 K (Figure 4) to determine the pH dependence for the reaction. A function describing the dissociation of a monoprotonic acid was fitted to the experimental data and the pK_a was found to be 5.6. Also, the second-order rate constant for the JNIIRO-catalysed hydrolysis of **II** was determined in the presence of 5 vol% trifluoroethanol at pH 5.1 and 290 K. It was the same as that of the corresponding reaction in aqueous solution within experimental error.

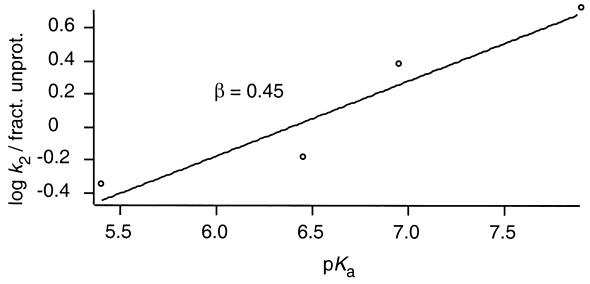

Figure 2. Brønsted plot for imidazoyl-catalysed hydrolysis of **II** at pH 5.1 and 290 K.

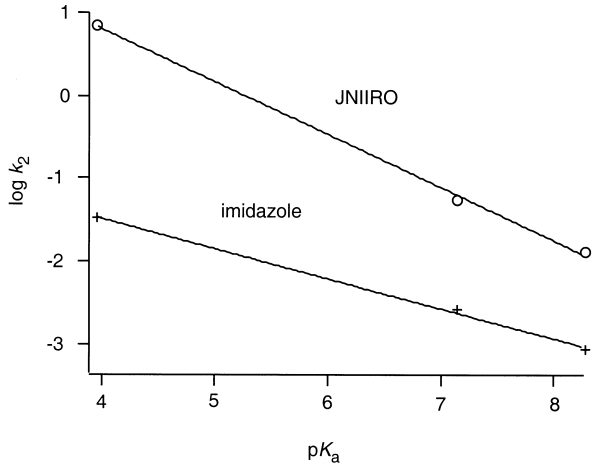

Figure 3. Hammett plot for the reaction of JNIIRO and imidazole with 2,4-dinitrophenyl acetate (**II**), *p*-nitrophenyl acetate (**I**) and *m*-nitrophenyl acetate (**III**) at pH 5.1 and 290 K. The pK_a values of the phenols are 3.96, 7.15 and 8.28, respectively.

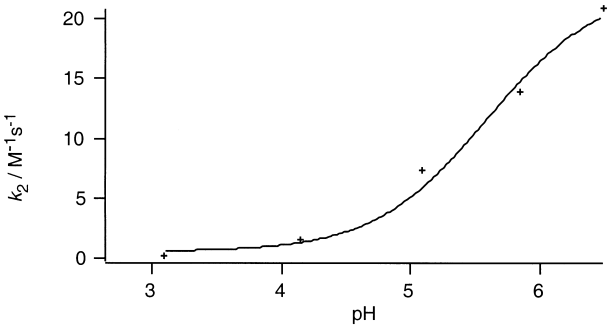

Figure 4. The pH profile for JNII-catalysed hydrolysis of **II** at 290 K. The function describing the dissociation of a monoprotonic acid with a pK_a of 5.6 is fitted to the experimental data.

Discussion

Our ability to engineer new biocatalysts depends on the design of catalytic functions that work cooperatively. In contrast to most catalysts that are currently in use in organic chemistry, biocatalysis is based on the use of only mildly reactive residues that become powerful when combined. The establishment of the principles that govern cooperative catalysis is therefore of intense interest and importance in catalyst design, and ultimately, in the engineering of new enzymes. We have previously reported on histidine-based four-helix bundle catalysts that are capa-

Table 3. The second-order rate constants for the hydrolysis of **II** at pH 3.1 and 5.1 in aqueous, deuturated and trifluoroethanol (TFE) solutions at 320 K.

Catalyst	k_2 (pH 3.1) [$M^{-1}s^{-1}$]	k_2 (pD 3.1) [$M^{-1}s^{-1}$]	k_2 (pH 5.1) [$M^{-1}s^{-1}$]	k_2 (pD 5.1) [$M^{-1}s^{-1}$]	k_2 (pH 5.1, 5% TFE) [$M^{-1}s^{-1}$]
JNII	0.18	0.084	7.3	–	–
JNIIRO	0.049	0.032	7.2	7.8	6.5
4-MeIm	9.9×10^{-5}	–	0.0081	–	–

ble of catalysing the hydrolysis of reactive esters with second-order rate constants that are more than three orders of magnitude larger than those of the uncatalysed reactions.[18] In addition, it was demonstrated that the catalysts were capable of substrate binding, substrate discrimination and to a modest degree chiral recognition.[28] The reactivity could be explained by a model based on cooperative nucleophilic and general-acid catalysis, but the concept of cooperative catalysis is such an important one that further investigations were warranted. The questions of why and under what circumstances general-acid catalysis becomes important in the hydrolysis of very reactive substrates such as *p*-nitrophenyl esters, are of particular relevance. We have therefore determined the reactivity of the four-helix bundle catalysts towards a more reactive substrate than **I** and towards a less reactive substrate than **I**, to probe the importance of the pK_a value of the leaving group and to provide a role for general acids in the hydrolysis reaction. The catalytic power is substantial as the second-order rate constant of the peptide-catalysed reaction is almost a factor of 2000 larger than that of the 4-methylimidazole-catalysed one at pH 3.1, partly owing to pK_a depression of the His nucleophile but mainly owing to other factors.

In the JNII-catalysed hydrolysis of the more reactive substrate **II** the second-order rate constant was found to be 7.2 $M^{-1}s^{-}$ at pH 5.1 and 290 K (Table 2) which is almost three orders of magnitude larger than that of the 4-methylimidazole-catalysed reaction. The JNI-catalysed second-order rate constant for the hydrolysis of **II** was found to be 2.34 $M^{-1}s^{-}$ a factor of three smaller than that of the JNII catalyst. The model proposed in previous work[22] for the catalytic efficiency of JNII relative to JNI appears to be valid also in the hydrolysis of **II** even if the effect is not as pronounced. The Brønsted coefficient β for the hydrolysis of **II** catalysed by imidazole derivatives at pH 5.1 and 290 K was determined to investigate whether general-acid catalysis contributed also to the observed rate enhancements for the more reactive substrate. The measured value was 0.45 (Figure 2), and the rate acceleration for the JNII-catalysed hydrolysis of **II** was therefore a factor of 68 larger than that of a hypothetical imidazole derivative with a pK_a value of 5.6. The catalytic efficiency in the absence of effects on pK_a is larger for the more reactive substrate and contributions from factors other than nucleophilic catalysis are therefore substantial.

The second-order constants for JNII-catalysed hydrolysis of **II** were determined (Figure 4) at several pH values, and at pH 4.1 and 290 K the second-order rate constant is a factor of 1600 larger than that of the 4-methylimidazole-catalysed reaction. The pH dependence shows that the catalysis is dependent on an unprotonated species with a pK_a of 5.6, the value found for both of the histidine residues. The catalysis therefore depends on the presence of an unprotonated His residue, but the rate enhancement is larger than what is expected from a single nucleophilic His residue. The question of whether general-acid catalysis contributed was therefore further investigated using isotopic solvents.

The kinetic solvent isotope effect was measured for the JNIIRO-catalysed hydrolysis of **II** at pH 5.1 and 290 K (Table 3). No effect was observed in contrast to what was found in the JNII-catalysed hydrolysis of **I** where a value of 1.5 was obtained at pH 5.1.[22] The catalytic efficiency of the peptides towards the highly reactive substrate **II** at pH 5.1 in the absence of pK_a effects was calculated to be larger than that towards **I**, and larger than that calculated for nucleophilic catalysis by unprotonated His residues of similar reactivities. From the absence of kinetic solvent effects under the experimental conditions one can conclude that although considerable catalytic efficiency is provided by the catalysts, it does not include that by general acids at that pH.

There is a change in reaction mechanism as the substrates are changed, and the most obvious difference between the two substrates are the pK_a values of the leaving groups, the pK_a of *p*-nitrophenol is 7.15 whereas that of 2,4-dinitrophenol is 3.96. There is thus no need for the latter when expelled to abstract a proton at pH 5.1, whereas the *p*-nitrophenolate ion will to a large extent become protonated in aqueous solution. At pH 3.1 the kinetic solvent isotope effects in the JNIIRO- and JNII-catalysed hydrolysis of **II** were measured and found to be 1.5 and 2.5, respectively, and at pH 3.1 the rate enhancement was almost a factor of 2000 over that of the 4-methylimidazole-catalysed reaction. The pK_a of 2,4-dinitrophenol is 3.96 and at pH 5.1 it exists predominantly in solution in its unprotonated form, whereas at pH 3.1 it is mainly protonated. The observation of a kinetic solvent isotope effect at a pH below the pK_a of the leaving group is therefore strong evidence for the participation of general-acid catalysis at that pH. At a pH where the leaving group is protonated, the need for general-acid catalysis arises.

In order for this comparison to be valid there must not be a change in the rate-limiting step as the substrate is changed to a more reactive one. In enzymatic catalysis there are classic examples of how changes in rate-limiting step occurs when the reactivity of the substrate is changed, for example in the chymotrypsin-catalysed hydrolysis of amides and esters.[29] The pH dependence of the second-order rate constant, Figure 4, clearly shows that catalysis depends on the unprotonated form of a His residue, in agreement with a mechanism for the hydrolysis of **I**, that involves the rate-limiting formation of an acyl intermediate. To ensure that the introduction of the 2,4-dinitrophenyl leaving group does not give rise to a change in rate-limiting step, the second-order rate constant was determined in the presence of an efficient nucleophile, trifluoroethanol. The transesterification reaction of **II** catalysed by JNIIRO in 5 vol% trifluoroethanol to form the trifluoroethyl ester showed no rate enhancement over that of the hydrolysis reaction in aqueous solution. If the rate-limiting step is the breakdown, rather than the formation, of the acyl intermediate the reaction rate should increase with the addition of the nucleophile trifluoroethanol. Since this is not observed we conclude that the rate-limiting step in the hydrolysis of the reactive ester **II**, as in the hydrolysis of **I**, is the formation of the acyl intermediate. The absence of a kinetic solvent isotope effect at pH 5.1 is therefore not owing to a change in rate-limiting step. It is therefore most likely that the hydrolysis of **I** and **II** follow similar reaction mechanisms but with different pH dependencies. At a pH below their pK_as there is cooperative nucleophilic general-acid catalysis but at a pH above their pK_a nucleophilic catalysis dominates with

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little or no contribution to catalysis from the flanking, protonated $HisH^+$ residue.

A Hammett plot for the JNIIRO-catalysed hydrolysis of the substrates **I**, **II** and **III** was obtained (Figure 3) and a slope of -0.6 was found, corresponding to a ρ value of 1.4. In addition, the ρ value for the hydrolysis by 4-methylimidazole, imidazole and 4-hydroxymethylimidazole was measured and found to be 0.8. The degree of charge localisation on the developing phenolate oxygen is considerably less than that on a phenolate ion, the ρ value for phenol dissociation is 2.23.[30] There is thus a substantial charge localised on the phenolate oxygen in the transition state of the peptide-catalysed reaction which provides a rationale for why hydrogen bonding contributes to the rate enhancement. It stabilises the partial negative charge on the oxygen by a hydrogen bond.

Since the hydrolysis of **II** also appears to follow the two-step mechanism that is predominant in imidazole-catalysed reactions of active esters, the localisation of what is considerably less than a negative charge on the phenolate oxygen strongly supports the formation of a tetrahedral transition state on the reaction pathway in the peptide-catalysed reaction. The question of whether there is a tetrahedral intermediate, and if the formation of the intermediate or its breakdown is rate limiting has not been addressed, so far, and does not affect the conclusions drawn here.

The second-order rate constants for the JNIIRO and JNII-catalysed hydrolyses of **II** were measured at pH 5.1 and 290 K (Table 2) and were found to be the same within experimental error. The histidine residues of both peptides have identical pK_a values and in contrast to what was observed for the hydrolysis of **I**,[22] no extra binding energy arises from introducing negatively charged residues in the helix. It appears that there is little charge on the carbonyl oxygen and that therefore the putative tetrahedral intermediate may not occur along the reaction pathway. We have, however, not investigated this particular feature in detail and the conclusion is clearly preliminary. An alternative interpretation might be that the structure of the transition state is different from those of the hydrolyses of *p*-nitrophenyl esters and that therefore the developing oxyanion is not directed towards the flanking groups.

Interestingly the hydrolysis of **II** using JNIII was the most efficient with a second-order rate constant of $10.3\,M^{-1}s^{-}$ at pH 5.1 and 290 K. This peptide has previously been shown to catalyse the hydrolysis of *p*-nitrophenyl valerate with a second-order rate constant of $0.097\,M^{-1}s^{-}$, whereas mono-*p*-nitrophenyl fumarate was hydrolysed with a k_2 of only $0.007\,M^{-1}s^{-}$. For JNIII the nucleophilic catalysis by the low pK_a histidine residue in position 34 is probably accompanied by transition state binding of the hydrophobic substrate substituents or by binding of the substrate by hydrophobic interactions to provide a proximity effect. Substrate binding in the mM range has previously been observed in the hydrolysis of mono-*p*-nitrophenyl fumarate by a designed helix-loop-helix motif.[28]

The observation of a large catalytic efficiency by JNIII in the hydrolysis of **II** (Table 2) although no general-acid mechanism can operate suggests that in this reaction, like in the hydrolysis of *p*-nitrophenyl valerate by JNIII, there is binding of the substituents of the substrate by the peptide, in the transition state or in a precomplexation. It is suggested that such binding is the source of catalysis in the hydrolysis of **II** at pH 5.1 where no general-acid catalysis is observed.

An important consequence of identifying cooperative nucleophilic general-acid catalysis is that the structure of the transition state becomes considerably less difficult to predict and thus to model. In nucleophilic catalysis a covalent bond is formed, or being formed, and the distance between the nucleophilic nitrogen and the carbonyl carbon of the substrate is well defined. Unfortunately, in the absence of other bonds between catalyst and substrate a large number of conformations are still possible, which makes it very difficult to predict the location of the substituents and introduce binding interactions. In terms of the chemistry, the catalyst is also less likely to be capable of significant amounts of selectivity. If nucleophilic catalysis is supplemented with general-acid catalysis by a residue that has a very well-defined spatial location relative to the nucleophile, a second bond is formed between catalyst and substrate, and the positions of all substituents are more restricted in space. The introduction of cooperativity in reactive sites is therefore important for selectivity as well as for efficiency and a necessary step in the design of new enzymes. Although we are still far from being able to design tailor-made enzymes the foundations for this important endeavour are now being laid, and the elucidation of the rules that govern cooperativity is one of them.

The implications of the results obtained here are that a prerequisite for general-acid catalysis to occur is that the leaving group should have a pK_a that is higher than the pH at which the reaction is carried out, but also that general-acid catalysis becomes important even for reactive esters under these conditions. The observation that the catalytic efficiency decreases with increasing pK_a of the leaving group is surprising but may reflect the fact that the hydrogen-bond strength depends at least partly on the difference in pK_a of the hydrogen-bond donor and that of the acceptor. The fact that we are now learning the "rules" for designing efficient and selective biocatalysts for chemical reactions of reactive substrates clearly means that in the future we will be able to apply these concepts to less reactive, and more interesting substrates.

Experimental Section

The peptides were synthesised and purified as described previously.[22, 24] The kinetic experiments were performed using Varian Cary 100 or 500 spectrophotometers equipped with Varian temperature controllers. Peptide stock solutions were prepared by weighing, by assuming a water content of 25%, at concentrations of 0.3 or 0.4 mM. After the kinetic runs the concentrations of the stock solutions were determined by quantitative amino acid analysis and found to agree within $\pm 10\%$ with the estimated values. The pH was adjusted and readjusted after centrifugation if necessary, and peptide solutions for kinetic measurements were prepared by diluting the stock solution with buffer. 4-Methylimidazole, imidazole and 4-hydroxymethylimidazole solutions with typical concentrations of 50, 100 and 150 mM were prepared in a similar way. Due to the low solubility of benzimidazole this derivative was used at concentrations of 5-15 mM.

The buffers used were phosphate (100 mM, pH 3.1), acetate (100 mM, pH 4.1 – 5.85) and Bis-Tris (50 mM, pH 6.5). The reaction solutions (270 μL)

were transferred to 1 mm cuvettes and temperature equilibrated at 290 K for twenty minutes in the cell compartment of the spectrophotometer. The substrate (5 μl, 7.15 mM in acetonitrile) was added to give typically a substrate concentration of 0.13 mM. The substrates used were the commercially available 2,4-dinitrophenyl acetate and *p*-nitrophenyl acetate, and *m*-nitrophenyl acetate which was synthesised by a standard ester formation procedure by reacting equimolar amounts of *m*-nitrophenol and acetic anhydride. The release of the 2,4-dinitrophenolate ion was followed at 360 nm and that of *p*-nitrophenol was followed at 320 nm. The rate of disappearance of *m*-nitrophenyl acetate was followed at 255 nm.

The pseudo-first-order rate constants were obtained by fitting a single-exponential function to the experimental data using Igor Pro software and plotted as a function of peptide or imidazole concentration to provide the second-order rate constants from linear regression analysis. The error limits for the second-order rate constants are estimated to be ±10% due to the combined errors in the amino acid analysis and in the linear regression analysis.

Kinetic solvent isotope effects were measured using a standard pH electrode to determine uncorrected pH values under the usual assumption that the isotope effect on the dissociation constants of the histidine side chain cancels that on the reading of the pH meter.

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