Binding Site Optimisation for Artificial Enzymes by Diffusion NMR of Small Molecules

Catherine E. Atkinson, Abil E. Aliev,* and William B. Motherwell[a]

Abstract: A binding site optimisation protocol for the design of artificial enzymes based on "small molecule–small molecule" binding studies by diffusion NMR is presented. Since the reaction chosen was the hydrolysis of ester **1** ([4-(4-carboxy-1-oxobutyl)-aminobenzyl]-phenethyl ester), an analogous phosphonate ester **2** ([4-(4-carboxy-1-oxobutyl)-aminobenzyl]-phosphonic phenethyl ester) was selected as a suitable transition state analogue (TSA). The key objective of the NMR studies was to find a unit with functional groups capable of binding to the acidic sites of the TSA. Nine dipeptides, mainly with basic and hydroxyl groups, were used and their affinity to the TSA was studied by measuring the change in the diffusion coefficient, D_{pep}, upon binding by pulse field gradient NMR. The value of D_{pep} at 298 K in D_2O at pD 5, 7 and 10 was measured both in free solution, and mixtures containing one dipeptide and the TSA. As both components are low molecular weight species with $M < 500$, a TSA-to-dipeptide ratio of 10:1 was used to detect significant changes in D_{pep}. The results revealed that dipeptides with basic residues show higher affinity to the TSA than those with hydroxyl or aliphatic side chains in aqueous solutions. The dipeptide showing the most significant relative change in D_{pep} was H-Arg-Arg-OH, and the binding constant was estimated to be $86\ L\,M^{-1}$ by measuring D_{pep} at varying concentrations of the TSA. In addition, binding of the TSA to a new water-soluble polymer with a polyallylamine backbone and randomly distributed Arg-Arg binding sites was examined, and the binding constant was estimated to be $\geq 1500\ L\,M^{-1}$. As confirmed by further catalytic activity tests, polymers containing Arg-Arg as a binding site are capable of significant rate accelerations in the hydrolysis of ester **1**.

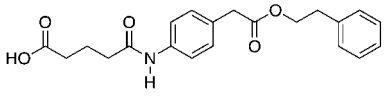
Keywords: diffusion · enzyme models · ester hydrolysis · peptides · transition states

Introduction

It is now well established through extensive studies of naturally occurring enzymes that the acceleration of reaction rates is most effectively accomplished when the enzyme active site is capable of binding a species intermediate between the substrate and the product.[1] As an automatic corollary it follows that artificial enzyme-like properties can be achieved by designing an active site structure that suitably binds the transition state for a given reaction. However, in view of the short lifetime of any given transition state it is generally impossible to preselect a matching binding unit, and hence the highly successful alternative strategy based on the use of a stable transition state analogue (TSA) is often employed. The seminal work of Lerner and Schultz in developing catalytic antibodies is centred around this concept.[2, 3]

Nevertheless, if some degree of complexity is required in designing and constructing a catalytic domain, the development of a reliable and quantitative assay for estimation of binding between the TSA and even a small structural portion of the ultimate artificial enzyme is a highly desirable objective. The purpose of the present paper is to outline a useful NMR method for the estimation of binding strength between two small molecules of similar molecular weight. Since even the cumulative effect of a limited number of small intermolecular non-covalent interactions does not manifest itself in a substantial gain in energy, such binding studies, especially in aqueous media, are often considered to be problematic.

Thus, whilst investigating the design of a new artificial enzyme capable of catalysing the hydrolysis of ester **1**, we



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required an efficient method of identifying small molecular weight species that would bind the transition state of the reaction. Since phosphonates are well known to mimic the tetrahedral geometry found in the transition state of the reaction, the phosphonate **2** was therefore chosen as the TSA.

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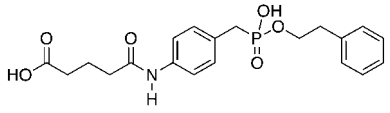

2

For the design of the catalyst, a low molecular weight unit with the ability to bind the TSA was required. The unit should in principle be capable of binding both the carboxylate and the phosphonic acid sites of TSA **2**, and also possibly of forming a hydrogen bond with the amide bridge. The choice of peptide units derived from natural amino acids as potential binding units is appropriate, as they are a constituent part of enzymes and contain functional groups that are potentially capable of binding to the TSA **2**. In addition, if a dipeptide was chosen to bind with the acid sites of the TSA **2**, the peptide bond should also be appropriately placed to form a hydrogen bond with the amide group of the TSA **2**. Thus, various dipeptides with basic and hydroxyl groups were selected for binding site optimisation. As both the TSA and the chosen dipeptides have a molecular weight $M < 500$, a technique was required that could identify binding between two small molecules.

A variety of techniques have been reported for studying binding interactions, and the majority of these involve NMR. Binding studies by NMR rely on the fact that when two molecules are involved in simple "association $\rightleftharpoons$ dissociation" equilibrium, changes are observed in NMR parameters, such as chemical shifts, relaxation rates and diffusion coefficients.[4] The use of diffusion for binding studies in solution is of particular interest since the diffusion coefficient is molecular weight dependent: when two molecules associate in solution, the apparent molecular weight increases, and this can be observed as a slowing down of the translational diffusion in solution. Diffusion NMR can also be superior to the techniques based on T_2 relaxation,[5] as well as ^{1}H NMR chemical shift measurements,[6] especially when complexation is associated with proton exchange between binding components, and there is a possibility of confusing acid–base chemistry with binding phenomena.[7] As a result diffusion NMR has been widely applied for binding studies.[6–25] However, the majority of the reported applications involve binding between a small ligand and a large receptor. Monitoring binding of two small molecules of similar molecular weight is in general a more difficult task since the observed changes in the NMR parameters are expected to be small.

In this work we have studied the binding properties of various dipeptides to the TSA **2** by diffusion NMR. Both components in this case are low molecular weight species of a similar molecular mass. As shown below a simple approach based on the use of an excess of one of the components allows us to detect significant changes in the diffusion coefficient of the minor component and hence allows comparative analysis of binding properties of a number of small molecular weight binding partners. The affinity of the TSA **2** to nine dipeptides at three different pD values was studied in this way and the results are reported below. Unlike techniques based on screening of soluble compound mixtures,[16, 19] separate solutions of each dipeptide with the TSA **2** were used in order to exclude possible bindings of different dipeptides to each other. In addition, for one of the dipeptides the binding constant has also been determined using variable concentration measurements. Finally, the binding properties of a new water-soluble polymer incorporating the highest affinity dipeptide as judged by NMR results has been examined with a view to probing the efficiency of binding as a function of macromolecular environment.

Results and Discussion

Dipeptides and choice of concentrations: Nine commercially available dipeptides were chosen for use in binding studies (see below). Of them, four, H-Arg-Arg-OH, H-Ala-Arg-OH, H-βAla-Lys-OH and H-βAla-His-OH (L-carnosine) contain one or more basic groups. Two dipeptides, H-Gly-Tyr-OH and H-Gly-Thr-OH contain hydroxyl residues, and one more dipeptide, H-Ser-His-OH contains both basic and hydroxyl residues. For comparison, two dipeptides, H-Ala-Gly-OH and H-βAla-Leu-OH, which contain neither basic, nor hydroxyl groups, were also included in this set.

The translational diffusion coefficients of the dipeptides and the TSA **2** were measured both in free solution and in a mixture containing one dipeptide and the TSA. Initially when a 1:1 ratio was used, only very small changes in the diffusion coefficients were observed. As the binding of the TSA with dipeptides in solution is an equilibrium process, by Le Chatelier's principle, the use of an excess of one of the components is expected to push the equilibrium to the right, towards the formation of the TSA $\cdots$ Pep complex.

$$\mathrm{TSA} + \mathrm{Pep} \rightleftharpoons \mathrm{TSA} \cdots \mathrm{Pep} \quad (1)$$

Thus, a 10:1 ratio of concentrations of TSA-to-dipeptide was used, and, as shown below, this was sufficient for the observation of significant changes in the diffusion coefficients of the low molecular weight dipeptides studied. A control experiment was also carried out for a 10 : 1 dipeptide-to-TSA solution for one of the dipeptides.

NMR parameters, pD dependence of proton chemical shifts: The ^{1}H NMR chemical shifts and $^3J_{\alpha\beta}$ coupling constants for 3 mM solutions of dipeptides in D_2O at three different pD values are listed in Table 1. The chemical shifts of the α-protons of the first H-Pep residue in each dipeptide vary considerably as the pD is varied, that is when increasing pD from 5 to 10 an increase of 0.41–0.65 ppm in $\delta(H^\alpha)$ is observed.

The observed changes can be explained using pK_a values of dissociating groups in free amino acids and peptides.[1] For the α-amino group pK_a is about 9–10 in amino acids and about 7.7 in peptides. On increasing pD from 5 to 10 the concentration of the unprotonated R-NH_2 form is increasing, and as the -NH_2 group is less electron withdrawing than -NH_3^+, a significant shift to lower frequencies (relative to R-NH_3^+) is observed for the α-protons of the first residue. For α-carboxyl group pK_a is about 2 in amino acids and about 3.6 in peptides, hence at pD 5, 7 and 10 a carboxyl group is predominantly present as the carboxylate ion. As a result, no significant

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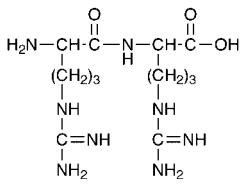
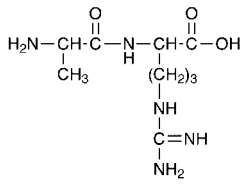
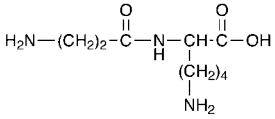
Dipeptides with one or more basic residues



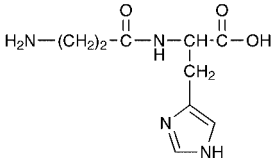
H-Arg-Arg-OH



H-Ala-Arg-OH

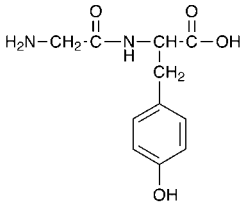


H-βAla-Lys-OH

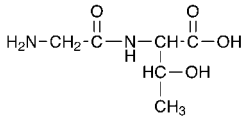


H-βAla-His-OH

Dipeptides with a hydroxyl residue

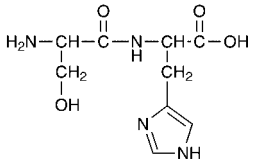


H-Gly-Tyr-OH



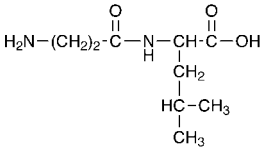
H-Gly-Thr-OH

Dipeptides with both a basic and hydroxyl residue

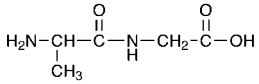


H-Ser-His-OH

Dipeptides without a basic or a hydroxyl residue



H-βAla-Leu-OH



H-Ala-Gly-OH

chemical shift changes are observed for the α-protons of the second Pep-OH residue as pD is changed from 5 to 10.

Chemical shift changes of the side chain protons can also be explained using the corresponding pK_a values. For instance, the pK_a of the ε-amino group of Lys is about 10.5 in peptides and therefore at pD 5 and 7 these groups are in the protonated form [H_3N^+–CH_2–]. At pD 10 a considerable amount of the unprotonated form [H_2N–CH_2–] is expected, hence a shift to the low frequency is observed for the adjacent H^ε protons. The other significant chemical shift changes observed for the aromatic His and Tyr protons can be explained in a similar manner using corresponding pK_a values (6 – 7.4 for imidazole of His and 9.5 – 10.5 for phenolic hydroxyl of Tyr).

The ^{1}H NMR chemical shifts and $^3J_{\alpha\beta}$ coupling constants for 3 mM dipeptide mixed with 30 mM TSA at three different pD values are listed in Table 2. For the majority of dipeptides no significant chemical shift changes are observed when comparing values measured for 3 mM dipeptide (Table 1) and those for 3 mM dipeptide mixed with 30 mM TSA solution (Table 2).

Some significant changes of chemical shifts are observed for the aromatic protons of His in H-Ser-His-OH (at pD 5 and 7) and H-βAla-His-OH (at pD 7). These are likely to be caused by the alteration of the populations of the ionised dipeptide species, which is in turn caused by the change of the pK_a value for His in the (D_2O+TSA) environment. Peptide pK_a variations depending on the environment are common, and, interestingly, variations as much as three pH units are often encountered at the active sites of enzymes.[1] Finally, Table 3 summarises the ^{1}H chemical shifts for solution of the TSA **2** in D_2O at three different pD values. As the TSA **2** is expected to be a relatively strong acid with low pK_a, no significant pD dependence of the chemical shifts is observed. The largest change of only -0.09 ppm is observed for the CH_2 protons adjacent to the COOH group as a result of pD increase from 5 to 10.

Diffusion measurements for the TSA and dipeptides: The translational diffusion coefficients of the dipeptides and the TSA **2** were measured both in free solution and in a mixture containing one dipeptide and the TSA in D_2O at pD 5, 7 and 10. As an illustration, the result of measurements for 3 mM H-βAla-Leu-OH at pD 10 is shown in Figure 1. The measured value of the diffusion coefficient was $(4.85 \pm 0.02) \times 10^{-10}\,m^2\,s^{-1}$. For the mean value and mean deviation calculations diffusion coefficients measured from four different experiments for five different peak areas in each experiment were used. The overall mean deviation for all of the samples used in this work was in the range ± 0.01 – $\pm 0.10 \times 10^{-10}\,m^2\,s^{-1}$.

The diffusion coefficient of the TSA **2** (D_{TSA}) in 30 mM aqueous solution was measured to be $3.48 \pm 0.03 \times 10^{-10}\,m^2\,s^{-1}$ at pD 5, $3.38 \pm 0.01 \times 10^{-10}\,m^2\,s^{-1}$ at pD 7, and $3.33 \pm 0.02 \times 10^{-10}\,m^2\,s^{-1}$ at pD 10. The lowest value of D_{TSA} at pD 10 maybe the result of increased hydration or self-association of the TSA molecules. It is relevant to note that lysozyme aggregation has been studied as a function of pH and it has been shown that the aggregation species shift from dimer to higher oligomer as the pH is increased.[5] In the presence of any of the dipeptides, it was expected that the value of D_{TSA} would not significantly change, since even if a very stable complex formed, only 10% of the TSA molecules

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Table 1. ^{1}H NMR parameters of 3 mM dipeptide solutions in D_2O at pD 5, 7 and 10 ($T = 298$ K). The standard atom numbering convention for peptides is used.

		^{1}H NMR chemical shift, δ [ppm]					J_{HH} [Hz]	
Dipeptide	pD	H^{α}	H^{β}	H^{γ}	H^{δ}	H^{ε}	$^3J_{\alpha\beta}$(HPep)	$^3J_{\alpha\beta}$(PepOH)
H-Arg-Arg-OH	5	4.04 HArg; 4.19 ArgOH	1.63–1.95	1.63–1.95	3.21; 3.22	–	6.3; 6.3	5.8; 7.9
	7	3.99 HArg; 4.19 ArgOH	1.63–1.93	1.63–1.93	3.21; 3.22	–	6.3; 6.3	5.8; 7.9
	10	3.43 HArg; 4.19 ArgOH	1.60–1.85	1.60–1.85	3.17; 3.19	–	6.3; 6.3	5.2; 8.0
H-Ala-Arg-OH	5	4.09 Ala 4.16 Arg	1.55 Ala 1.74; 1.84 Arg	1.62 Arg	3.21 Arg	–	7.1 Ala	5.8; 7.8 Arg
	7	3.86 Ala 4.17 Arg	1.43 Ala 1.73; 1.84 Arg	1.62 Arg	3.21 Arg	–	7.1 Ala	5.5; 7.8 Arg
	10	3.51 Ala 4.18 Arg	1.26 Ala 1.72; 1.85 Arg	1.59 Arg	3.19 Arg	–	7.0 Ala	5.1; 8.0 Arg
H-βAla-Lys-OH	5	3.27 βAla 4.26 Lys	2.73 βAla 1.75; 1.87 Lys	1.44 Lys	1.69 Lys	3.00 Lys	scss[a]	5.2; 8.4 Lys
	7	3.26 βAla 4.14 Lys	2.71 βAla 1.70; 1.81 Lys	1.42 Lys	1.68 Lys	3.00 Lys	scss[a]	5.3; 8.2 Lys
	10	2.86 βAla 4.14 Lys	2.42 βAla 1.65; 1.78 Lys	1.33 Lys	1.43 Lys	2.58 Lys	scss[a]	4.8; 8.8 Lys
H-βAla-His-OH	5	3.23 βAla 4.54 His	2.69 βAla 3.12; 3.26 His	–	7.27 His	8.59 His	scss[a]	5.3; 8.2 His
	7	3.19 βAla 4.44 His	2.63 βAla 2.95; 3.11 His	–	6.92 His	7.68 His	scss[a]	4.7; 8.7 His
	10	2.75 βAla 4.46 His	2.33 βAla 2.91; 3.12 His	–	6.90 His	7.63 His	scss[a]	4.5; 9.3 His
H-Ser-His-OH	5	4.10 Ser 4.50 His	3.97 Ser 3.15; 3.25 His	–	7.25 His	8.53 His	4.9; 4.9 Ser	5.7; 7.5 His
	7	3.89 Ser 4.46 His	3.86 Ser 3.06; 3.17 His	–	7.06 His	8.06 His	4.0; 5.7 Ser	5.2; 7.8 His
	10	3.45 Ser 4.44 His	3.66 Ser 2.98; 3.11 His	–	6.91 His	7.64 His	5.2; 5.2 Ser	4.8; 8.0 His
H-Gly-Tyr-OH	5	3.70; 3.80 Gly 4.52 Tyr	2.90; 3.15 Tyr	–	7.16 Tyr	6.85 Tyr	(^2J −16.1 Gly)	5.0; 8.8 Tyr
	7	3.64; 3.74 Gly 4.43 Tyr	2.85; 3.12 Tyr	–	7.15 Tyr	6.84 Tyr	(^2J −16.2 Gly)	4.9; 8.9 Tyr
	10	3.22; 3.24 Gly 4.38 Tyr	2.81; 3.05 Tyr	–	7.01 Tyr	6.62 Tyr	(^2J −16.9 Gly)	4.9; 8.4 Tyr
H-Gly-Thr-OH	5	3.91 Gly 4.26 Thr	4.30 Thr	1.19 Thr	–	–	–	scss[a]
	7	3.88 Gly 4.17 Thr	4.24 Thr	1.18 Thr	–	–	–	4.1 Thr
	10	3.39 Gly 4.17 Thr	4.24 Thr	1.16 Thr	–	–	–	3.8 Thr
H-βAla-Leu-OH	5	3.27 βAla 4.24 Leu	2.72 βAla 1.61 Leu	1.64 Leu	0.89; 0.93 Leu	–	scss[a]	scss[a]
	7	3.24 βAla 4.16 Leu	2.70 βAla 1.57 Leu	1.61 Leu	0.87; 0.91 Leu	–	scss[a]	scss[a]
	10	2.90 βAla 4.18 Leu	2.44 βAla 1.56 Leu	1.60 Leu	0.87; 0.90 Leu	–	scss[a]	scss[a]
H-Ala-Gly-OH	5	4.12 Ala 3.84; 3.92 Gly	1.55 Ala	–	–	–	7.1 Ala	(^2J −17.5 Gly)
	7	4.10 Ala 3.73; 3.85 Gly	1.53 Ala	–	–	-	7.1 Ala	(^2J −17.2 Gly)
	10	3.50 Ala 3.74; 3.77 Gly	1.26 Ala	–	–	–	7.0 Ala	(^2J −17.3 Gly)

[a] scss: strongly coupled spin system.

Table 2. ^{1}H NMR parameters of 3 mM dipeptide mixed with 30 mM TSA in D_2O at pD 5, 7 and 10 ($T = 298$ K). The standard atom numbering convention for peptides is used.

		^{1}H NMR chemical shift, δ [ppm]					J_{HH} [Hz]	
Dipeptide	pD	H^{α}	H^{β}	H^{γ}	H^{δ}	H^{ε}	$^3J_{\alpha\beta}$(HPep)	$^3J_{\alpha\beta}$(PepOH)
H-Arg-Arg-OH	5	4.03 HArg 4.17 ArgOH	1.60–1.95	1.60–1.95	3.15; 3.17	–	6.3; 6.3	5.8; 7.9
	7	4.02 HArg 4.17 ArgOH	1.61–1.93	1.61–1.93	3.18; 3.16	–	6.3; 6.3	5.6; 7.9
	10	3.44 HArg 4.18 ArgOH	1.59–1.83	1.59–1.83	3.16; 3.14	–	6.3; 6.3	5.3; 8.0
H-Ala-Arg-OH	5	4.08 Ala 4.15 Arg	1.54 Ala 1.72; 1.83 Arg	1.60 Arg	3.16 Arg	–	7.1 Ala	5.6; 7.8 Arg
	7	3.94 Ala 4.15 Arg	1.48 Ala 1.72; 1.83 Arg	1.60 Arg	3.16 Arg	–	7.1 Ala	5.5; 7.9 Arg
	10	3.49 Ala 4.17 Arg	1.25 Ala 1.71; 1.84 Arg	1.57 Arg	3.15 Arg	–	7.0 Ala	5.1; 8.0 Arg
H-βAla-Lys-OH	5	3.25 βAla 4.14 Lys	2.70 βAla 1.70; 1.80 Lys	1.41 Lys	1.67 Lys	2.97 Lys	scss[a]	5.2; 8.3 Lys
	7	3.29 βAla 4.15 Lys	2.74 βAla 1.72; 1.83 Lys	1.44 Lys	1.70 Lys	3.00 Lys	scss[a]	5.1; 8.4 Lys
	10	2.88 βAla 4.14 Lys	2.41 βAla 1.65; 1.78 Lys	1.33 Lys	1.43 Lys	2.59 Lys	scss[a]	4.8; 8.8 Lys
H-βAla-His-OH	5	3.24 βAla 4.51 His	2.64 βAla 3.11; 3.25 His	–	7.29 His	8.54 His	scss[a]	5.3; 8.3 His
	7	3.21 βAla 4.47 His	2.66 βAla 3.04; 3.18 His	–	7.12 His	8.20 His	scss[a]	5.1; 8.4 His
	10	2.75 βAla 4.45 His	2.33 βAla 2.91; 3.12 His	–	6.90 His	7.63 His	scss[a]	4.5; 9.3 His
H-Ser-His-OH	5	4.07 Ser 4.48 His	3.95 Ser 3.12; 3.23 His	–	7.20 His	8.40 His	4.9; 4.9 Ser	5.4; 7.5 His
	7	3.96 Ser 4.46 His	3.90 Ser 3.07; 3.18 His	–	7.22 His	8.20 His	4.6; 5.3 Ser	5.3; 7.7 His
	10	3.44 Ser 4.43 His	3.64 Ser 2.97; 3.10 His	–	6.90 His	7.63 His	5.2; 5.2 Ser	4.9; 8.0 His
H-Gly-Tyr-OH	5	3.66; 3.77 Gly 4.44 Tyr	2.85; 3.12 Tyr	–	7.15 Tyr	6.84 Tyr	(^2J −16.1 Gly)	5.0; 9.0 Tyr
	7	3.64; 3.76 Gly 4.43 Tyr	2.85; 3.12 Tyr	–	7.14 Tyr	6.83 Tyr	(^2J −16.1 Gly)	5.0; 9.0 Tyr
	10	3.21; 3.25 Gly 4.38 Tyr	2.82; 3.07 Tyr	–	7.08 Tyr	6.76 Tyr	(^2J −16.8 Gly)	5.0; 8.5 Tyr
H-Gly-Thr-OH	5	3.90 Gly 4.18 Thr	4.24 Thr	1.18 Thr	–	–	–	4.1 Thr
	7	3.89 Gly 4.17 Thr	4.24 Thr	1.18 Thr	–	–	–	4.1 Thr
	10	3.41 Gly 4.18 Thr	4.26 Thr	1.17 Thr	–	–	–	3.8 Thr
H-βAla-Leu-OH	5	3.26 βAla 4.17 Leu	2.70 βAla 1.58 Leu	1.61 Leu	0.88; 0.92 Leu	–	scss[a]	scss[a]
	7	3.19 βAla 4.17 Leu	2.65 βAla 1.58 Leu	1.61 Leu	0.88; 0.92 Leu	–	scss[a]	scss[a]
	10	2.89 βAla 4.18 Leu	2.43 βAla 1.57 Leu	1.61 Leu	0.88; 0.91 Leu	–	scss[a]	scss[a]
H-Ala-Gly-OH	5	4.11 Ala 3.75; 3.85 Gly	1.54 Ala	–	–	–	7.0 Ala	(^2J −17.2 Gly)
	7	4.09 Ala 3.72; 3.84 Gly	1.52 Ala	–	–	-	7.1 Ala	(^2J −17.2 Gly)
	10	3.51 Ala 3.74; 3.77 Gly	1.25 Ala	–	–	–	7.0 Ala	(^2J −17.6 Gly)

[a] scss: strongly coupled spin system.

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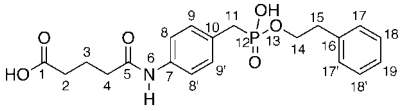
Table 3. ^{1}H NMR chemical shifts of TSA in D_2O at 298 K. The following numbering of protons is used in the Table.



Proton	δ [ppm] at pD 5	δ [ppm] at pD 7	δ [ppm] at pD 10
2-CH_2	2.33	2.26	2.24
3-CH_2	1.94	1.92	1.91
4-CH_2	2.43	2.42	2.40
8,8′-CH	7.28	7.30	7.27
9,9′-CH	7.13	7.14	7.12
11-CH_2	2.91	2.91	2.90
14-CH_2	3.95	3.96	3.94
15-CH_2	2.84	2.85	2.83
17,17′-CH	7.24	7.25	7.23
18,18′-CH	7.34	7.35	7.33
19-CH	7.27	7.28	7.25

would be involved in binding. We were therefore interested in observing the change in diffusion coefficients of the dipeptides, since dipeptides showing the strongest binding should show the greatest decrease in diffusion coefficient. The measured diffusion coefficients are summarised in Table 4. The parameter R_D in Table 4 reflects the relative change in diffusion coefficient as percentage and is defined as:

$$R_D = 100 \times \frac{D_{pep} - D'_{pep}}{D_{pep}} \quad (2)$$

where D_{pep} is the diffusion coefficient observed for the pure dipeptide solution and D'_{pep} is the diffusion coefficient observed for dipeptide mixed with the TSA **2**.

We first consider the diffusion coefficients of the free dipeptides. To the first approximation, comparison of D_{pep} values shows that diffusion coefficients of the free dipeptides correlate well with their molecular weights. The self-association ability of each dipeptide, which is expected to be pD dependent, may in principle interfere with its binding to TSA **2**. For the two dipeptides containing an Arg residue, D_{pep} values at the three different pD values are the same within the uncertainties of measurements involved. For the rest of dipeptides, D_{pep} values decrease by about 2 – 13 % with the increase of pD from 5 to 10. The largest single step change is observed for H-Gly-Tyr-OH, from 4.97×10^{-10} at pD 7 to 4.54×10^{-10} at pD 10. This is likely to be caused by the change in the protonation state of the phenolic OH group, the pK_a of which is known to be in the range 9.5 – 10.5 in proteins and 10.1 for tyrosine. Hence, at pD 7 the phenolic OH groups are predominantly in the protonated form, whereas at pD 10 a significant amount of the unprotonated form is present. The observed decrease of D_{pep} at pD 10 can then be assigned to the possibility that in its unprotonated phenolic form Gly-Tyr either self-associates or solvates better than that in the corresponding protonated form. Considering the relatively large change in D_{pep} as a function of pD, self-association, rather than solvation, is the likely primary factor. The influence of the Gly amino protonation state (pK_a 9.8 for glycine) is expected to be negligible for H-Gly-Tyr-OH, since no strong pD dependence of D_{pep} is observed for H-Gly-Thr-OH (Table 4). The other two dipeptides showing a moderate decrease of D_{pep} (by ca. 5 %) with increasing pD are H-βAla-Lys-OH and H-βAla-Leu-OH.

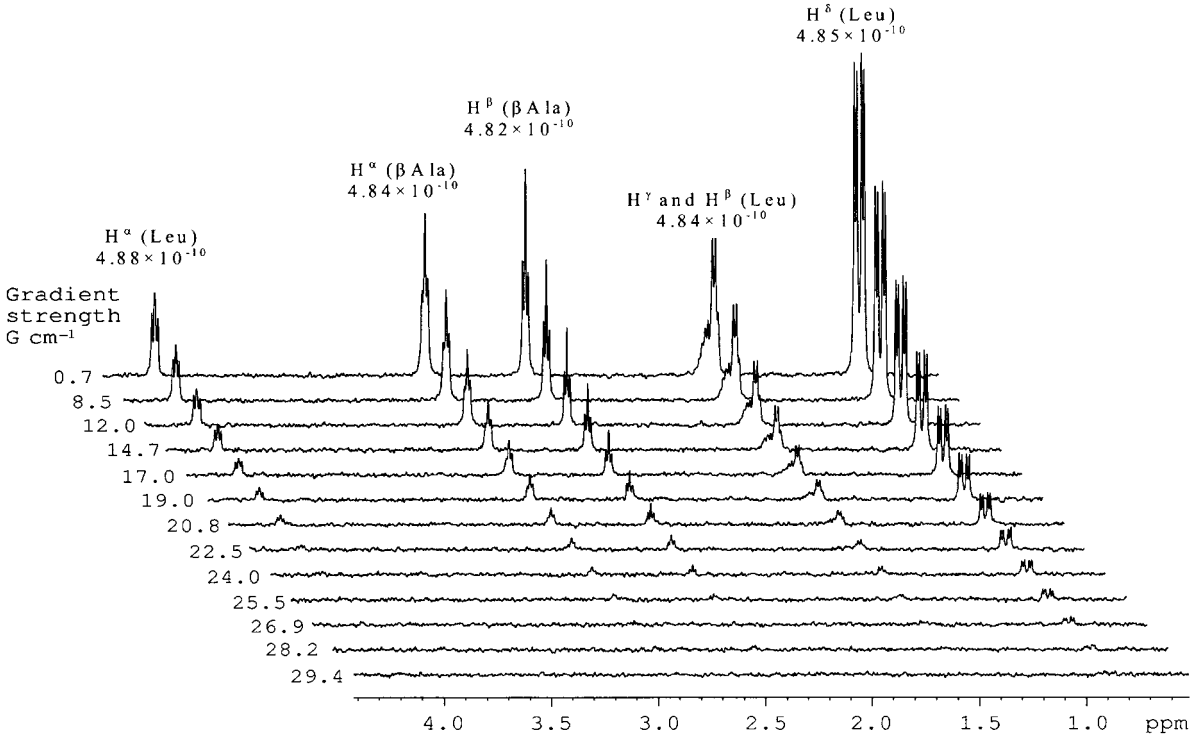


Figure 1. ^{1}H-BPPLED NMR spectra measured for 3 mm H-βAla-Leu-OH in D_2O at pD 10. The spectra were acquired at 298 K with $\Delta = 200$ms, $\delta = 2.8$ ms and g ranging from 0.7 G cm^{-1} to 32.9 G cm^{-1}. Sixteen BPPLED spectra were acquired as a function of g and the first thirteen of these are shown. Diffusion coefficients measured for five different peak areas are also shown.

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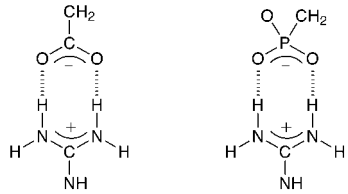
Table 4. Observed diffusion coefficients of dipeptides in D_2O at 298 K: D_{pep} for 3 mM solutions of dipeptide and D'_{pep} for mixtures containing dipeptide (3 mM) and TSA (30 mM). The parameter R_D in the Table reflects the relative change in diffusion coefficient.

Dipeptide	pD 5			pD 7			pD 10		
	D_{pep} [$\times 10^{-10}$ m^{2}s^{-1}]	D'_{pep} [$\times 10^{-10}$ m^{2}s^{-1}]	R_D [%]	D_{pep} [$\times 10^{-10}$ m^{2}s^{-1}]	D'_{pep} [$\times 10^{-10}$ m^{2}s^{-1}]	R_D [%]	D_{pep} [$\times 10^{-10}$ m^{2}s^{-1}]	D'_{pep} [$\times 10^{-10}$ m^{2}s^{-1}]	R_D [%]
H-Arg-Arg-OH	4.08 ± 0.03	3.46 ± 0.05	15	4.10 ± 0.04	3.40 ± 0.04	17	4.14 ± 0.02	3.68 ± 0.05	11
H-Ala-Arg-OH	4.76 ± 0.03	4.23 ± 0.04	11	4.76 ± 0.05	4.18 ± 0.08	12	4.77 ± 0.03	4.41 ± 0.07	8
H-βAla-Lys-OH	4.93 ± 0.03	4.57 ± 0.04	7	4.91 ± 0.03	4.39 ± 0.04	11	4.69 ± 0.03	4.32 ± 0.07	8
H-βAla-His-OH	5.22 ± 0.03	4.74 ± 0.06	9	5.18 ± 0.03	4.71 ± 0.05	9	5.05 ± 0.05	4.56 ± 0.06	10
H-Ser-His-OH	4.98 ± 0.03	4.81 ± 0.09	3	4.89 ± 0.03	4.46 ± 0.05	9	4.87 ± 0.04	4.72 ± 0.07	3
H-Gly-Tyr-OH	5.23 ± 0.07	4.55 ± 0.07	13	4.97 ± 0.04	4.64 ± 0.07	7	4.54 ± 0.03	4.35 ± 0.08	4
H-Gly-Thr-OH	5.56 ± 0.04	5.26 ± 0.05	5	5.54 ± 0.04	5.19 ± 0.04	6	5.47 ± 0.03	5.20 ± 0.05	5
H-βAla-Leu-OH	5.09 ± 0.03	4.87 ± 0.04	4	4.97 ± 0.08	4.62 ± 0.09	7	4.85 ± 0.02	4.59 ± 0.04	5
H-Ala-Gly-OH	6.16 ± 0.04	5.70 ± 0.10	7	6.08 ± 0.05	5.72 ± 0.08	6	6.00 ± 0.05	5.50 ± 0.10	8

We now consider the change in the diffusion coefficients of the dipeptides mixed with TSA. As the binding constant measurements require lengthy variable concentration studies, the relative change in the diffusion coefficient, R_D, is used to compare affinity of different dipeptides to the TSA **2**. Overall, the largest changes in R_D are observed at pD 7, hence in the following discussion we mainly concentrate on R_D values measured at neutral pD. All of the dipeptides used are unprotected, and therefore contain at least one free amino group at their N-terminus. Therefore, all dipeptides should be capable of forming a complex, however weak, between the amine of the dipeptide and the carboxylic acid group of the TSA **2**. In all cases a decrease of the diffusion coefficient upon mixing with the TSA is expected ($D'_{pep} < D_{pep}$). As can be seen from Table 4, this was indeed the case. As there are only two amino acid residues in each dipeptide, the functionalities of the side chains are important in determining differences in physical and chemical behaviour of the dipeptides. Based on preliminary information obtained from the Cambridge Structural Database, it was expected that the dipeptides containing basic, or hydroxyl residues should show the strongest affinity to the TSA **2**, since the terminal amino group could interact with the carboxylic acid group, and the hydroxyl or basic residue could bind with the phosphonic acid. As shown in Table 4, at pD 7 the five dipeptides containing one or more basic residues show the largest change in diffusion coefficient upon mixing. Dipeptides containing hydroxyl residues, H-Gly-Tyr-OH and H-Gly-Thr-OH, did not appear to show good affinity for the TSA **2**, and their R_D are comparable to those observed for the two dipeptides that contained neither a basic nor a hydroxyl residue. This may be due to the aqueous medium used, which interferes strongly with possible hydrogen bonds formed between the dipeptide hydroxyl groups and the TSA functional groups. Nevertheless, for H-Gly-Tyr-OH, $R_D = 13\,\%$ at pD 5, although R_D decreases significantly as pD is increased. Based on the discussion above, for H-Gly-Tyr-OH the self-association is less at low pD, hence better binding at pD 5 than at pD 7 and 10 is expected due to the competitive nature of the binding and self-association processes.

Of the five dipeptides containing basic residues, H-βAla-His-OH (L-Carnosine) and H-Ser-His-OH showed slightly weaker affinity to the TSA **2**, when compared to the others. This can be related to the fact that the basic side chain of His has a much lower pK_a (pK_a ca. 6 for imidazole, 6.5 – 7.4 in proteins) when compared with Arg (pK_a > 12) and Lys (pK_a > 10.5). Nevertheless, the affinity for the TSA **2** of dipeptides containing a His residue was better than that of dipeptides with no basic groups. Of all the dipeptides studied, the two that showed the greatest change were H-Ala-Arg-OH, and H-Arg-Arg-OH. The side chain of arginine contains a guanidine fragment, which is known to form complexes with carboxylate groups,[26] stabilised by hydrogen bonding and electrostatic interactions as shown schematically below. It is possible that the phosphonic acid site of TSA **2** might be able to interact in a similar manner.



In conclusion, the results show that dipeptides with basic residues show higher affinity to the TSA **2** than others. The presence of hydroxyl groups in the side chain of the peptide residues does not improve binding capabilities of dipeptides to the TSA **2** in aqueous solutions. To the first approximation, ignoring differences in hydrophobic properties of the dipeptides, the pK_a of the sidechain correlates well with the binding capability of the dipeptide to acidic sites of the TSA **2**, that is the stronger the dipeptide sidechain base the better a binder it is. Overall, compared to other dipeptides R_D is significantly higher for the H-Arg-Arg-OH at pD 7. It is possible that this dipeptide is acting as a bidendate ligand, that is both guanidine groups are involved in binding to the TSA functional groups.

Binding constant measurements: As the largest change in the diffusion coefficient is found for H-Arg-Arg-OH, this dipeptide was chosen for the binding constant (K_a) measurements. The binding constant for a bimolecular process is given by:

$$K_a = \frac{[\mathrm{TSA}\cdots\mathrm{Pep}]}{[\mathrm{TSA}][\mathrm{Pep}]} \quad (3)$$

Assuming that the equilibrium process is fast in the NMR timescale, the observed diffusion coefficient for the dipeptide

mixed with the TSA **2** (D'_{pep}) is the mole fraction weighted average of the diffusion coefficients observed for the free molecule (D_{pep}) and for the complexed molecule (D''_{pep}):

$$D'_{pep} = x D_{pep} + x'' D''_{pep} \quad (4)$$

On the assumption that there are only free and bound dipeptide species, the sum of the mole fractions of free (x) and bound dipeptide (x'') is 1. We note that no separate NMR peaks were observed due to free and bound dipeptides for any of solutions studied, indicating that the equilibrium process is fast in the NMR chemical shift timescale. As the diffusion delay used is sufficiently long (200 ms), the fast "association $\rightleftharpoons$ dissociation" equilibrium in the NMR chemical shift timescale will also be fast in the diffusion NMR timescale.[8]

In systems where the host is considerably larger than the ligand, the diffusion coefficient of the host should not be affected upon complexation, and hence D''_{pep} is assumed to be equal to the diffusion coefficient of the free host. The mole fractions can therefore be found from Equation (4), and the binding constant K_a can be calculated. In cases, where the two molecules are similar in size, such an assumption is not valid, and K_a can be determined by measuring the diffusion coefficients for a series of differing concentrations of one of the components, as shown by binding measurements using ^{1}H NMR chemical shifts.[4] For the binding constant measurement, the concentration of H-Arg-Arg-OH was kept constant (1 mM), and the concentration of the TSA **2** was varied from 0 to 55 mM. Neutral pD was used, since the overall aim was to synthesise a water soluble polymer that would catalyse ester hydrolysis under neutral conditions. As the concentration of H-Arg-Arg-OH is relatively low, the integral intensity of the well-separated signal due to four H^δ protons at about 3.2 ppm was used for the diffusion coefficient measurements.

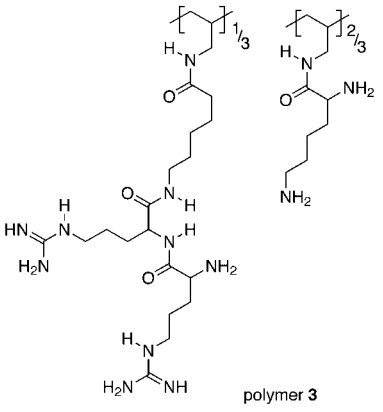
As can be seen from Table 5, the diffusion coefficient of H-Arg-Arg-OH gradually decreases with increasing TSA concentration. This agrees well with the presence of a binding equilibrium between H-Arg-Arg-OH and the TSA **2**. The measured diffusion coefficients of the H-Arg-Arg-OH were plotted against the concentration of the TSA, and the binding constant K_a was calculated using the non-linear least squares fitting.[27, 28] The estimated value of K_a was found to be $86 \pm 15\ L\,M^{-1}$ ($D'_{pep} = 3.24 \pm 0.07 \times 10^{-10}\ m^2 s^{-1}$). The small difference in the diffusion coefficients of H-Arg-Arg-OH and the TSA is likely to be the main source of the relatively high error in the K_a determination. The binding constants calculated from the chemical shifts of H^δ (Table 5) was $82 \pm 19\ L\,M^{-1}$. Note that concentration-dependent changes of chemical shifts observed for H^α are very small (Table 5) and cannot be used for a reliable prediction of the binding constant. More pronounced concentration-dependent chemical shift changes for H^δ than for H^α (Table 5), as well as comparison of the chemical shift changes for different protons in the (H-Arg-Arg-OH $\times$ TSA) mixture (Tables 1 and 2), suggests that the guanidine moieties are likely to interact with the carboxylate and phosphonate groups of the TSA **2**, hence larger shifts for the near-to-guanidine protons H^δ are observed when the TSA concentration is increased.

Table 5. Diffusion coefficients and ^{1}H NMR chemical shifts of H-Arg-Arg-OH as a function of TSA concentration in D_2O solution at pD 7 and $T = 298$ K. The concentration of H-Arg-Arg-OH was 1 mM. The mean deviation was in the range $\pm 0.03 - \pm 0.08 \times 10^{-10}\ m^2 s^{-1}$ for the diffusion coefficient measurements and ca. ± 0.003 ppm for the chemical shift measurements.

Concentration of TSA [mM]	D'_{pep} [$\times 10^{-10}\ m^2 s^{-1}$]	δ (H^α) [ppm]	δ (H^δ) [ppm]
0	4.16 (D_{pep})	4.189	3.210
1	4.10	4.187	3.204
2	4.02	4.185	3.198
4	3.88	4.183	3.200
7	3.84	4.180	3.190
9	3.76	4.181	3.183
16	3.68	4.178	3.175
19	3.60	4.175	3.170
28	3.55	4.173	3.168
38	3.47	4.172	3.165
55	3.37	4.169	3.159

Weak binding generally has a value of K_a less than $10\ L\,M^{-1}$, and strong binding a value of K_a greater than $10^5\ L\,M^{-1}$.[4] In our case binding constants determined from the diffusion coefficients and the chemical shifts show that H-Arg-Arg-OH has a moderate affinity for the TSA **2**. Strong binding in an aqueous solution was not expected, since the use of water as a solvent interferes with much of the hydrogen bonding between the guanidine group of H-Arg-Arg-OH and the acidic sites of the TSA **2**. Electrostatic interactions between ionised species are in general effective over greater distances than are hydrogen bonds. However, these interactions are also weakened by water, as the strength of electrostatic interaction is inversely proportional to the dielectric constant of the medium.

Binding properties of a synthetic polymer: The information obtained from the diffusion rate measurements indicated that H-Arg-Arg-OH should be able to act as a binding site for the transition state of the ester hydrolysis reaction. However, binding is also dependent on the surrounding environment. Therefore, a soluble polymer **3** containing randomly distributed Arg-Arg (attached to ca. ⅓ of repeat units) and Lys (attached to ca. ⅔ of repeat units) fragments was synthesised from polyallylamine,[29] in order to establish if the unit could



still bind in a polymer environment. Lysine residues were included simply to move the free amine groups of the polymer away from the polymer backbone. Polymers containing only

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lysine residues showed little or no interaction with the TSA, and thus these amine groups were not expected to participate in the binding.

6-Amino caproic acid (ACA) was also introduced as a linker between the polymer backbone and the Arg-Arg unit in order to ensure a high conformational flexibility of the dipeptide fragment, and consequently to enhance its binding capabilities. A control experiment was also carried out for 20 mM H-Arg-Arg-OH and 2 mM TSA free solutions and their mixture at pD 7.

The results of diffusion coefficient measurements are shown in Table 6. A decrease of D_{TSA} ($R_D = 10\,\%$) was observed for the control (2 mM TSA+20 mM H-Arg-Arg-OH) solution. On passing we note that D_{TSA} for the 30 mM free solution ($3.38 \times 10^{-10}\,m^2s^{-1}$) was less than that for 2 mM solution. The observed concentration dependence of D_{TSA} can be explained by increased hydration or self-association at higher concentrations.[30, 31] A similar concentration dependence of D was also observed for H-Arg-Arg-OH ($3.77 \times 10^{-10}\,m^2s^{-1}$ for the 20 mM solution, $4.10 \times 10^{-10}\,m^2s^{-1}$ for the 3 mM solution and $4.16 \times 10^{-10}\,m^2s^{-1}$ for the 1 mM solution).

Table 6. Observed diffusion coefficients of the TSA (D_{TSA}), H-Arg-Arg-OH (D_{pep}) and polymer **3** (D_{pol}) in D_2O solutions at pD 7 and $T = 298$ K. The mean deviation was in the range $\pm 0.03 - \pm 0.09 \times 10^{-10}\,m^2s^{-1}$.

Solution	D_{TSA} [m^2s^{-1}]	D_{pol} or D_{pep} [m^2s^{-1}]
2 mM TSA	3.60×10^{-10}	–
4.5 mg mL^{-1} Polymer **3**	–	1.36×10^{-11}
20 mM H-Arg-Arg-OH	–	3.77×10^{-10}
2 mM TSA+4.5 mg mL^{-1} Polymer **3**	7.28×10^{-11} (D'_{TSA})	1.36×10^{-11} (D'_{pol})
2 mM TSA+20 mM H-Arg-Arg-OH	3.22×10^{-10} (D'_{TSA})	3.74×10^{-10} (D'_{pep})

When the TSA **2** is mixed with polymer **3**, the observed diffusion coefficient of the TSA decreased by about 80 % (Table 6). This is a significant change, and suggests a strong binding between the TSA **2** and the polymer **3**. As the molecular weight of the polymer is considerably larger than that of the TSA **2**, the normal assumption can be made that the diffusion coefficient of the complex (D''_{TSA}) is the same as that for the polymer measured and shown in Table 6, and thus the percentage of bound TSA **2** can be calculated using the following expressions:[19]

$$x'' = \frac{D_{TSA} - D'_{TSA}}{D_{TSA} - D'_{pol}} \quad (5)$$

$$K_a = \frac{x''}{(1 - x'')(c_p - cx'')} \quad (6)$$

where c and c_p are the total concentrations of the TSA **2** and the functional polymer repeat unit. Substituting the values shown in Table 6 into Equation (5), the mole fraction, x'', was found to be 0.83. Unfortunately, the exact molecular weight of the polymer is unknown, and hence it is not possible to measure an exact binding constant K_a. Nevertheless, assuming that polymer **3** is monodisperse, a recently described relationship between D_{pol} and molecular weight M of a polymer ($D_{pol} = 10^{-7.62}\,M^{-0.62}$, with D_{pol} in units of m^2s^{-1})[32] gives an approximate value of $M \approx 172\,300$. For comparison, if every amino group of the starting polyallylamine ($M \approx 42\,900$) were functionalised by Arg-Arg-ACA (attached to ca. ⅓ of repeat units) and Lys (attached to ca. ⅔ of repeat units), the maximum molecular weight for polymer **3** would be about 214 000. The lower limit of the K_a value can then be assessed using the following three possibilities: i) assuming that there are still free -[CH_2-$CH(CH_2NH_2)$]- fragments in polymer **3** not substituted by Arg-Arg-ACA and Lys, $M \approx 172\,300$ gives $K_a \approx 1500\,L\,M^{-1}$; ii) if all the amino groups of the starting polyallylamine are substituted by Arg-Arg-ACA and Lys, $M \approx 172\,300$ gives $K_a \approx 4000\,L\,M^{-1}$, and iii) assuming full substitution of the starting polyallylamine by Arg-Arg-ACA and Lys, $M \approx 214\,000$ gives $K_a \approx 1500\,L\,M^{-1}$. Hence, $K_a \geq 1500\,L\,M^{-1}$ and the corresponding Gibbs free energy of binding $|\Delta G| \geq 18\,kJ\,mol^{-1}$. Note that in general a higher binding energy is favoured, as it will lower the energy difference between the ground state and the transition state of the reaction, hence leading to reaction rate acceleration.

Although these are only estimates, the results nevertheless indicate that the TSA **2** has a relatively strong affinity for the polymer **3**. If a polymer containing Arg-Arg has a strong affinity for the TSA, then it is expected to bind the transition state in the ester hydrolysis, and hence enhance the efficiency of the catalyst. Indeed, as confirmed by HPLC kinetic studies,[29] a polymer containing Arg-Arg as a binding site showed a significant rate acceleration for the hydrolysis reaction of ester **1** whilst the "blank" lysine polymer without predesigned binding sites, was entirely without effect.

Conclusions

In this work we have shown how the binding of small molecules to a TSA can be modelled and optimised using diffusion NMR. The approach undertaken mainly focuses on modelling studies aimed at improved binding of a transition state analogue to dipeptides, and examines whether a random distribution of peptide side chains on a polymer will indeed improve its affinity to a TSA. For this purpose, dipeptides with basic, hydroxyl and aliphatic side chains were used, and their affinity to the TSA with three different functional groups was studied by measuring the change in the diffusion coefficient upon binding by diffusion NMR. As both components of the binding equilibrium are low molecular weight species, a 10:1 TSA-to-dipeptide ratio was used to detect sufficient changes in D_{pep}. The results showed that dipeptides with basic residues show higher affinity to the TSA **2** than others. The presence of hydroxyl groups in the side chain of the peptide residues did not improve binding capabilities of dipeptides to the TSA **2** in D_2O solutions. The dipeptide showing the most significant relative change in D_{pep} was H-Arg-Arg-OH. Based on these results, a new water-soluble polymer with polyallylamine backbone and randomly distributed Arg-Arg and Lys side chains was synthesised, and the diffusion NMR measurements confirmed that the TSA **2** has a strong affinity for this polymer. Subsequent catalytic activity tests have shown that polymers containing the Arg-Arg unit as a binding site are indeed capable of significant rate accelerations in the hydrolysis of ester **1**.

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Experimental Section

3 mM Solutions of each dipeptide in D_2O and a 30 mM solution of the TSA **2** in D_2O were prepared with pD 5, 7 and 10. The pD of solutions were adjusted using NaOD and DCl. Sample mixtures containing one dipeptide and the TSA **2** were also made up so that the concentration of the dipeptide was 3 mM, and the concentration of the TSA **2** was 30 mM. Eleven samples were prepared for binding constant measurements at pD 7, all with a fixed 1 mM concentration of H-Arg-Arg-OH and with a TSA concentration varying from 0 to 55 mM. The upper limit of the TSA concentration in these studies was limited by its solubility in D_2O. To probe the binding properties of polymer **3** (described in "Results and Discussion"), three solutions at pD 7 were used for the diffusion measurements: 4.5 mg of polymer in 1 mL of D_2O; 2 mM solution of the TSA **2** in D_2O, and 4.5 mg of polymer in 1 mL of a 2 mM solution of the TSA **2**.

All NMR experiments were carried out on a Bruker AVANCE500 NMR spectrometer operating at 500.13 MHz for ^1H observation, and equipped with pulse field z gradients. A 5 mm broadband probe with a z gradient actively shielded coil was used. Based on the comparative analysis of various pulse sequences for diffusion coefficient measurements,[33] the BPPLED pulse sequence was chosen,[34] and diffusion coefficients of the dipeptides and the TSA **2**, both in the pure samples, and in their mixtures were measured. The pulse sequence used contains a longitudinal eddy current delay at the end of the pulse sequence (11 ms in our experiments), which avoids artefacts arising from residual eddy currents. The use of the BPPLED sequence is also known to minimise J modulation[33, 35] and background gradient effects.[36]

In the BPPLED experiments, the attenuation of the NMR resonance depends on gradient areas q according to:

$$I = I_0 \exp\left[-Dq^2\left(\Delta - \frac{\delta}{3} - \frac{\tau}{2}\right)\right] \quad (7)$$

where I is the signal intensity in the NMR spectrum measured for a given q, I_0 is the signal intensity in the absence of gradient pulses, Δ is the diffusion delay (200 ms in our experiments),[34] D is the diffusion coefficient, and τ is the delay between the bipolar gradient pulse pair (0.1 ms). For the sine shaped gradients used in this work:

$$q = \frac{2}{\pi} g \gamma \delta \quad (8)$$

where γ is the gyromagnetic ratio, g is the amplitude of the bipolar gradient pulse pair and δ is the duration of the bipolar gradient pulse pair (2.4–2.8 ms for dipeptides, the TSA **2** and their mixture, and 12 ms for polymer **3**). In each experiment, a set of 16 or 32 separate BPPLED spectra was acquired as a function of gradient amplitude, which ranged from 2 to 95% of the maximum sine shaped gradient strength, corresponding to 0.7–32.9 G cm^{-1}. Up to six experiments were carried out for each solution in order to assess the uncertainties of diffusion coefficient measurements. The maximum gradient field strength (54.4 G cm^{-1}) was calibrated by measuring the diffusion coefficient of residual HOD in D_2O (>99.9 at % D, purchased from GOSS Scientific Instruments) at 298 K and using a value of $(1.902 \pm 0.003) \times 10^{-9}$ m^2 s^{-1}[37] for backward calculation of the gradient strength. This was additionally verified by measuring the diffusion coefficient of de-ionised H_2O at 298 K (measured value $(2.30 \pm 0.01) \times 10^{-9}$ m^2 s^{-1}, reported value for distilled water $(2.299 \pm 0.003) \times 10^{-9}$ m^2 s^{-1}[38]) and tetradecane (>99%, Aldrich) at 298 K (measured value $(5.54 \pm 0.01) \times 10^{-10}$ m^2 s^{-1}, reported values $(5.50 \pm 0.01) \times 10^{-10}$ m^2 s^{-1} and $(5.54 \pm 0.01) \times 10^{-10}$ m^2 s^{-1}[39]). The latter was chosen as a secondary standard since its diffusion coefficient is of the same order as those of the TSA **2** and dipeptides studied.

To avoid any possible vibrational instabilities, as well as spinning sidebands all measurements were performed on a static sample. All of the diffusion measurements were performed for solutions in D_2O at 298 K. The temperature was stabilised using a high airflow of 400–670 L h^{-1}. As shown by detailed studies recently, no convection effects are observed at 298 K in D_2O solutions,[40] mainly due to the relatively high viscosity of this solvent. Our control experiment for D_2O and for 3 mM H-βAla-Leu-OH solution in D_2O using convection compensated pulse sequence[41] revealed essentially the same diffusion coefficients as those measured by BPPLED sequence confirming that the convection effects are unimportant for aqueous solutions at 298 K. As a control of a possible solution viscosity change, the diffusion coefficient of HOD, which should have a value of 1.902×10^{-9} m^2 s^{-1} at 298 K for residual HOD in D_2O,[37] was monitored in each experiment. In our experiments a value between 1.89×10^{-9} and 1.93×10^{-9} m^2 s^{-1} was always observed.

The BPPLED measurements were carried out with and without solvent presaturation. The purpose of solvent presaturation was to suppress the residual HOD peak in order to minimise possible baseline distortions near about 4.8 ppm and to improve the reliability of the diffusion coefficient measurements for nearby peaks due to dipeptide H^{α}-protons, as well as to optimise the spectrometer receiver setting for the dipeptide and the TSA signals.

Data acquisition and processing was performed using standard Bruker XwinNMR software (version 2.6). The NMR spectra were acquired with typically 16–32 K complex points, with a spectral width of 6 kHz. The baseline was fitted to a zero-order polynomial. ^1H Chemical shifts were measured relative to sodium salt of 3-(trimethylsilyl)-1-propane sulfonic acid (Aldrich). The diffusion coefficients were determined from Equation (7) using T_1/T_2 utility of XwinNMR. The Associate program, which employs the non-linear least squares Levenberg–Marquardt method to fit parameters to equilibrium complexation models, was used to determine the binding constant K_a.[27, 28]

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