Comparing dendritic with linear esterase peptides by screening SPOT arrays for catalysis†‡

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Fluorescence screening of a 96-membered SPOT library of histidine containing dendritic and linear peptides revealed the remarkable esterolytic activity of short histidine oligomers that show catalytic proficiencies within one order of magnitude of histidine-containing esterase peptide dendrimers.

Enzyme catalysis arises by cooperativity between amino acid side chains upon folding of the polypeptide, which enhances the catalytic power of functional groups and enables substrate binding by formation of a macromolecular receptor.[1] In the context of enzyme model studies,[2] catalytic peptides with linear,[3] cyclic[4] or dendritic topologies[5] offer a privileged platform to reconstruct enzyme catalysis in simplified systems. In our studies of dendritic esterase mimics,[6] a combinatorial approach by split-and-mix synthesis[7] led us to histidine containing peptide dendrimers catalysing the hydrolysis of fluorogenic esters in aqueous medium with substrate binding and multiple turnover. In particular catalysts for esterolysis of 1-acetoxypyrene-3,6,8-trisulfonate **1** exhibit catalytic proficiencies (k_{cat}/K_M) that are 10^2–10^5-fold larger than the 2nd order rate constant k_2 with the reference small molecule catalyst 4-methyl imidazole (4MeIm) (Fig. 1).[8] These catalysts include peptide dendrimers using cooperativity between multiple histidine side chains for binding and catalysis (*e.g.* (Ac–HisX)$_8$(*Dap*HisX)$_4$(*Dap*HisX)$_2$*Dap*HisX–NH$_2$, *Dap* = L-2,3-diaminopropionic acid branching, **A3B**: X = β-alanine, $(k_{cat}/K_M)/k_2$ = 28 000 at pH 5.5 with **1**, **A3C**: X = Thr, $(k_{cat}/K_M)/k_2$ = 13 000 at pH 5.5 with **1**),[9] and peptide dendrimers with a single catalytic site, *e.g.* **RG3** (Ac–TyrThr)$_8$(*Dap*TrpGly)$_4$(*Dap*ArgSer)$_2$*Dap*HisSer–NH$_2$ ($(k_{cat}/K_M)/k_2$ = 46 at pH 5.5 with **1**).[10]

In **RG3** the core catalytic histidine residue is assisted for substrate binding by a pair of cationic arginines in the first generation branch and multiple aromatic residues in the outer dendrimer branches. More recently cyclic peptides with multiple lysines and a single histidine residue were shown to catalyse the same reaction thereby acting as ATP and heparin sensors,[4] and octapeptides bearing four histidine residues were shown to catalyse the hydrolysis of **1**.[11] We therefore became interested in directly comparing the esterolytic activity of histidine containing peptides and peptide dendrimers towards **1**.

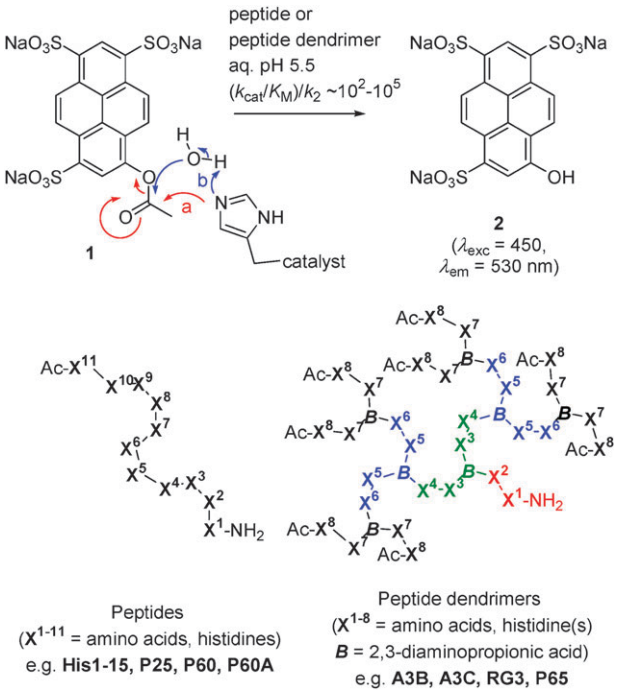

Fig. 1 Ester hydrolysis reaction catalysed by histidine containing peptides and peptide dendrimers. The imidazole group acts either as a nucleophile (path a) or general base (path b). See text for amino acid sequences of individual peptides and peptide dendrimers.

Herein we report the screening of a focused 96-membered SPOT peptide array library[12] on cellulose support leading to the identification of linear and dendritic peptide catalysts with remarkable catalytic proficiencies for the hydrolysis of **1**. These experiments show for the first time that screening SPOT-peptide arrays directly on solid support is an efficient method to discover peptide catalysts, and reveal the transition from catalysis by an isolated functional group to cooperative binding and catalysis in linear peptide and peptide dendrimer enzyme models.

With the aim of exploring focused sequence variations of known peptide dendrimer catalysts, we set out to screen a small library prepared by SPOT synthesis on cellulose using the fluorogenic hydrolysis of **1** as test reaction. A 96-membered library was designed featuring variations of esterase dendrimers **A3B**, **A3C**, **RG3** and related analogs. The variations included sequence shuffling as well as repositioning and exchange of *Dap* branching against alanine all the way to linear peptides within a sequence of 11 consecutive couplings. The library contained linear undecapeptides and 1st, 2nd and 3rd generation dendrimers with up to 54 amino acids (Tables S1 and S2, ESI‡). SPOT synthesis was performed using automated Fmoc synthesis

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on cellulose paper support with a non-cleavable linker in a customized 96-spot format. All sequences were capped by acetylation of the N-termini, and the side-chain protecting groups were removed by global acidic treatment. Following extensive washing, the peptide SPOTs were punched out and transferred to a 96-well plate. Activity screening was performed by immersing the paper disks carrying each library member in a buffered aqueous solution of substrate **1** (80 μM in 5 mM citrate pH 5.5) and recording the formation of 1-hydropyrene-3,6,8-trisulfonate **2** in the solution by fluorescence.

Analysis of the screening data (Table S2 and Fig. S1, ESI‡) showed a 45-fold difference in fluorescence intensity reading between the most and the least active library member. The least active readings came from dendritic sequences (Ac–HisX)$_8$(*Dap*GlyGly)$_4$(*Dap*GlyGly)$_2$*Dap*GlyGly– (X = Phe, Leu, Pro, Thr, Ala, Tyr, Gly, Ser, linked to cellulose), and sequences containing multiple anionic residues, which inhibit substrate binding,[7e] including a simple catalytic triad model peptide Ac–DSHLDSHLDSH–. The most active reading came from the histidine undecapeptide **His11**, followed by 3rd generation dendrimers related to **A3B/C** carrying multiple histidines throughout the branches. When considering the activity per histidine residue, the most active sequences were (Ac–TyrGly)$_8$(*Dap*TyrSer)$_4$(*Dap*XX)$_2$*Dap*ThrHis– for X = Lys (**P65**) and X = Arg (**P75**), which carry only a single catalytic histidine residue at the core. They were followed by the histidine undecapeptide **His11**, Ac–HTHTHTHTHTH– **P18**, Ac–HKHKHKHKHKH– **P25**, Ac–HRHRHRHRHRH– **P26**, and Ac–HTAHTAHTAHT– **P60**.

The strong apparent activity of linear peptides in the array might reflect a higher synthetic efficiency compared to the dendrimers. Nevertheless, a series of hits and related sequences were prepared for closer characterization including linear histidine oligomers up to 15 residues **His1–His15**, the cationic His/Lys peptide **P25**, **P60** and its alanine analog Ac–HAAHAAHAAHA–NH$_2$ (**P60A**), and peptide dendrimer **P65** which showed the highest apparent activity per histidine residue. Products were obtained by Fmoc synthesis on Rink-amide resin and purified by RP-HPLC (Table S3, ESI‡).

Catalytic parameters were determined for the hydrolysis of ester **1** (Table 1, Fig. 2). The activities of purified peptides and dendrimers in dilute aqueous buffer paralleled the SPOT array data, suggesting that multivalency on the solid support did not enhance catalysis during the screening. **His1** and **His2** behaved as small molecule catalysts with second order kinetics. All other peptides starting with **His3** obeyed Michaelis–Menten kinetics with preequilibrium substrate binding and saturation of catalysis. The strongest catalyst was the pentadecapeptide **His15**, with 6000-fold rate acceleration over background k_{cat}/k_{uncat}, corresponding to a 2200-fold larger relative catalytic proficiency $(k_{cat}/K_M)/k_2$ compared to 4MeIm. The His–Lys oligomer **P25** also showed a remarkable catalytic proficiency $(k_{cat}/K_M)/k_2$ = 395 due to its stronger substrate binding (K_M = 47 μM, see below). Dendrimer **P65** showed the highest activity per His residue with $(k_{cat}/k_{uncat})/N_{His}$ = 620 and relative catalytic proficiency $(k_{cat}/K_M)/k_2$ = 190. However both **P25** and **P65** underwent slow acylation at the lysine side-chains ($t_{1/2}$ ≈ 4–6 h) and therefore did not behave as true catalysts.

Table 1 Catalytic parameters for selected peptides and dendrimers

			k_{cat}/k_{uncat}		$(k_{cat}/K_M)/k_2$[c]	
Cpd[a]	N_{His}[b]	K_M/μM	/cat	/His	/cat	/His
His1	1	—	—	—	3	3
P65[d]	1	120	620	620	190	190
His2	2	—	—	—	27	14
His3	3	280	1050	350	130	43
His4	4	180	1100	275	220	55
P60	4	145	770	190	203	51
P60A	4	155	835	210	203	51
His5	5	180	1300	260	270	54
His6	6	140	1500	250	380	63
P25[d]	6	47	2950	490	2370	395
His7	7	100	2500	355	790	115
His8	8	100	3000	375	950	120
His9	9	90	3030	335	1090	120
His10	10	100	3800	380	1230	125
His11	11	95	3500	320	1210	110
His12	12	110	5200	435	1540	130
His13	13	100	4900	375	1560	120
His14	14	100	5200	370	1690	120
His15	15	86	6030	400	2200	145

[a] All products were prepared by Fmoc-SPPS on TGR resin and purified by preparative RP-HPLC. [b] N_{His} = number of histidine residues. [c] k_2 is the 2nd order rate constant with 4-methyl imidazole, k_2 = 1.0 M^{-1} min^{-1}. The spontaneous background reaction is k_{uncat} = 3.58 × 10^{-5} min^{-1}; /cat = per catalyst; /His = per histidine residue. Conditions: aq. 5 mM citrate, pH 5.5, 34 °C, 58.5–1000 μM **1**. Catalyst concentration: **His1–His4**: 20 μM; **His5–His6**, **P60**, **P60A**: 15 μM; **His7–His14**: 5 μM, **His15**, **P25**, **P65**: 3.75 μM. 120 μL assays in microtiterplate wells were followed by fluorescence (λ_{exc} = 450 ± 25 nm, λ_{em} = 530 ± 12 nm). All kinetics were run in triplicate with error ±5–20%, see Table S3 (ESI†) for complete data. [d] **P25** and **P65** underwent slow acylation at the lysine side-chain with $t_{1/2}$ ≈ 4–6 h.

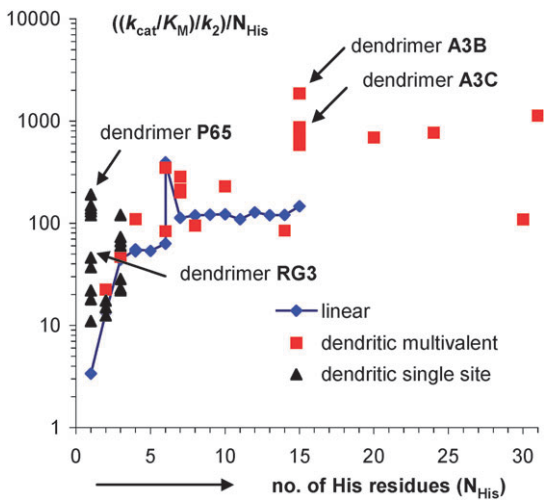

Fig. 2 Overview of catalytic proficiencies of linear and dendritic esterases for the hydrolysis of **1** at pH 5.5. The reactivity increase of peptide and peptide dendrimer catalysts over 4-methylimidazole per histidine residue as a function of the number of histidine residues N_{His} in the catalysts. Data from this work and ref. 9 and 10.

The oligohistidine peptides showed very good activities but reached a plateau of relative proficiency per histidine for the heptamer **His7**. Further oligomerization did not significantly increase catalytic proficiency per histidine up to **His15**, which remains one order of magnitude less active than peptide

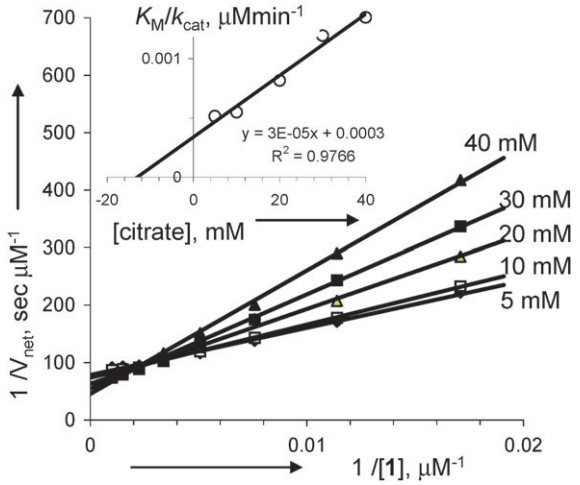

Fig. 3 Inhibition of **His15** catalysis of ester **1** hydrolysis by citrate buffer at pH 5.5. Main plot: Lineweave–Burk plots with increasing citrate. Inset: Dickson replot of K_M/k_{cat}. The 8-fold increase in citrate from 5 to 40 mM causes a 5-fold increase in K_M, a 2-fold increase in k_{cat}, and a 1.2-fold increase in k_{uncat}. Assay conditions as in Table 1.

dendrimers such as **A3B** and **A3C** which also carry 15 histidines.[9] Note that the activity per His residue in peptide dendrimer **A3B** represents a remarkable multivalency effect which is not outperformed by dendrimers with higher number of histidines (Fig. 2).

Part of the increase in catalytic proficiency in the histidine linear peptides was caused by stronger binding of substrate **1**, which increased gradually by up to three-fold between the smallest enzyme model **His3** (K_M = 280 μM) and the histidine oligopeptides **His7–His15** (K_M ≈ 100 μM) or the peptide dendrimer **P65** (K_M = 120 μM). The even lower K_M of the polycationic peptide **P25** (K_M = 42 μM) suggested an electrostatic component in substrate binding, presumably a salt bridge between cations on the catalyst and the sulfonate groups of substrate **1**, as previously described for multivalent histidine peptide dendrimer such as **A3C**.[9] Electrostatic substrate–catalyst interactions were evidenced for **His15** by the observation of a 2-fold increase in K_M and 2-fold decrease in k_{cat} upon addition of 0.5 M KCl to the reaction buffer (Table S4, ESI‡). In addition, catalysis by **His15** was inhibited by citrate (K_i = 13 mM, Fig. 3), and by the tetraanion pyrene-1,3,6,8-tetrasulfonate (K_i = 280 μM, Fig. S2, ESI‡).

Acid–base titration of **His15** showed an apparent pK_a of ~5.4, which implies that eight of the fifteen histidines are protonated and available for electrostatic binding of the pyrene trisulfonate substrate **1** at the reaction pH of 5.5, while seven occur as free base to catalyse the ester cleavage reaction. The gradual lowering of the histidine pK_a upon histidine oligomerization pK_a(**His1**) ≈ 6.4 > pK_a(**His2**) ≈ 6.1 > pK_a(**His3**) ≈ 6.0 > pK_a(**His8**) ≈ 5.7 > pK_a(**His15**) ≈ 5.4 indicates that a larger fraction of the histidines are available as free bases to catalyse the reaction as the number of histidine residues increases. This effect explains the 3-fold higher catalytic activity of **His1** (pK_a ≈ 6.4) over 4-methyl imidazole (pK_a ≈ 7.5) in the hydrolysis of **1** and probably contributes to the higher catalytic efficiency per histidine residue $(k_{cat}/k_{uncat})/N_{His}$ observed in the larger histidine oligomers independent of the increased binding to substrate **1**.

In summary, the experiments above represent to our knowledge the first example of catalyst discovery by screening a SPOT library on solid support. The library screening showed that multivalency effects previously observed in peptide dendrimers for the hydrolysis of pyrenesulfonate ester **1** also occur in linear peptides. While histidine and its dipeptide behave as small molecule catalysts, the histidine tripeptide and longer oligomers show preequilibrium substrate binding by electrostatic interactions, with catalytic proficiencies 10^2–10^3-fold higher than 4-methyl imidazole. Their catalytic proficiency per histidine residue reaches a plateau at the level of the heptamer **His7**, with values within one order of magnitude of previously identified peptide dendrimers such as **A3B** and **A3C** for the same reaction.

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