Noncovalently Bound Cofactors for Chemical Nucleases[+]

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A large range of amino acids, including protected ones, were tested as cofactors in the Eu^{III}-catalyzed hydrolysis of the biocidic bi(nitrophenyl)phosphate (BNPP) and of plasmid DNA. Serine, histidine, and aspartic acid – and combinations of these – were found to be the most effective acids. An azacrown with two attached imidazoles led to more pronounced effects, resulting, in the presence of dicarboxylic acids, in increases of up to k_{rel} = 5 in comparison with the effect of the metal alone. Incorporation of imidazole and carboxylic group within a single synthetic cofactor resulted in the largest Eu^{III} catalysis rate increase so far observed with plasmid DNA.

Introduction

Cofactors in the form of functional amino acids play an essential role at the catalytic site of most enzymes, but have until now scarcely been used in enzyme-analogue supramolecular catalysts. We therefore wanted to explore usable functions as cofactors for the hydrolysis of biocidic phosphate esters and of nucleic acids. Application of metal cations with high charge density has already resulted here in remarkable catalytic activities;[1] these are, however, still significantly far off the efficiency of natural nucleases. These achieve their high turnover numbers by means of the simultaneous action of several metal ions in combination with several amino acid functions, which have been identified for a number of such enzymes.[2] Earlier reports[3−5] on the efficiency of cyclic peptides containing Asp, His, and Ser as functional amino acids in many hydrolases were subsequently disputed.[6−8] Testing combinatorial approaches to catalysis, Menger et al.[9] used a library of polyallylamines substituted with eight different carboxylic acids. In combination with metals such as Mg^{2+}, Zn^{2+}, or Fe^{3+}, significant accelerations in the hydrolysis of the model phosphate **BNPP** were observed; they were, however, more than 10 times smaller than those obtained with, for example, Eu^{3+} salts alone, ***without*** any cofactors. More recently, Berkessel and Herault[1i] have screened a combinatorial library of 625 different peptide undecamers containing Arg, His, and Trp; the best combination was tested with *p*-nitrophenylphosphate, and in the presence of Zr^{IV} salts showed a maximum rate increase factor of five, in absence of saturation kinetics.

We have explored the efficiency of functions that typically occur in metal-based hydrolases by measuring rates in the presence of large numbers of corresponding amino acids, some small peptides, and corresponding derivatives (Scheme 1). The activity of these potential cofactors was determined not only with a biocidic model phosphate (**BNPP**), but also, for the first time, with plasmid DNA, using methods described earlier.[10] We thus obtained a library of cofactors, with no geometric constraints imposed by peptide chains, but at the same time without any predisposition towards any optimal orientation, of the type present in the active sites of enzymes. Because of this, and of the low molarity of the simple cofactors in the absence of a substrate-binding pocket, one cannot, of course, expect large catalytic effects. Out of four amino acids that we used in various combinations, the results selected in Table 1 represented moderate rate enhancements, and only so with DNA; all other combinations gave decreased efficiency relative to the metal cation Eu^{3+} alone (Table 2). In fact, serine (**Ser**), histidine (**His**), and aspartic acid (**Asp**) are active components in many hydrolases, with frequently described mechanisms.[2,11] In contrast, arginine showed no or very small rate enhancements in any combination (Table 3), with a maximum factor k_{rel} = 1.5[11] for **BNPP** with an **Arg** + **His** combination.

For **BNPP** hydrolysis, the presence of the highly charged europium(III) ion in combination with **Asp** often led either to precipitation or to biphasic curves in the hydrolysis. The same problem arose in experiments with carboxylic acids like capronic acid (**3**) or glutaric acid (**4**), which give (small) rate enhancements only in the presence of the above-mentioned amino acids. If biphasic curves occurred, only those parts for which an increase of extinction was observed were evaluated; all analyzed reactions showed perfect fits to pseudo-first order reaction curves, with deviations below 2% when run in duplicate.

Catalysis by the simple use of metal ions and cofactors is limited by the low concentrations of a productive complex between the (at least three) components, which must include the substrate. As a next step to improve this situation, we attached the imidazole to an azacrown, which in the case of **2** binds the lanthanide ion in water with affinities of log *K* = 1.23 (in water/MeOH = 8:2). In this way, the concentration of a productive complex can be enhanced, and

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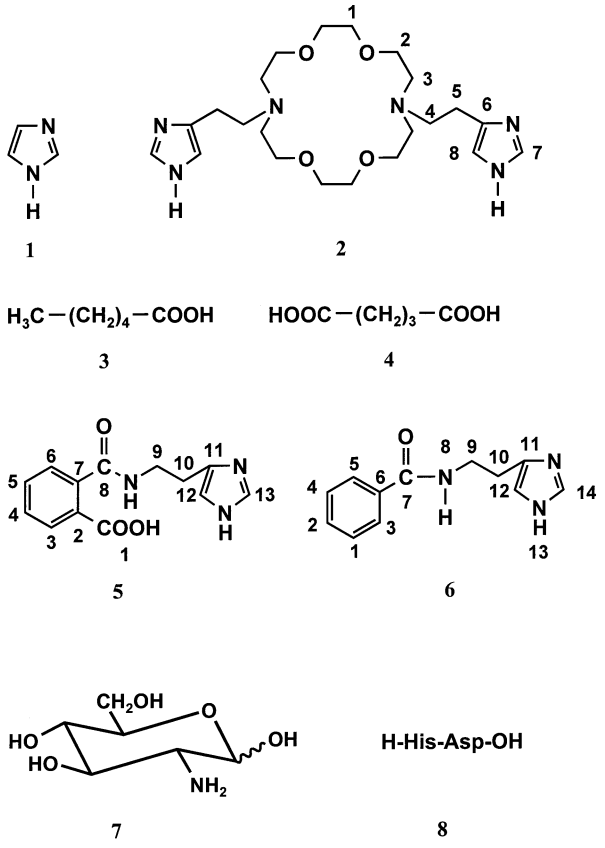


Scheme 1. Ligands and cofactors

results with derivatives like **2** are promising: the hydrolysis rate of **BNPP** was enhanced by a factor of 3.4, increasing to $k_{rel} = 5$ with the addition of dicarboxylic acid **4**. With DNA, the effect was still remarkable (2.8); in the presence of other dicarboxylic acids, however, a decrease was found. This should not be unexpected, though, in view of the reduced electrostatic attraction between the metal complex and the negatively charged DNA groove. A separate experiment in which a large concentration of the **BNPP** substrate was used showed no rate decrease (Table 1), providing evidence for a true catalytic process without any significant product inhibition.

In order to analyze the effect of the functional side chains without interference by the terminal N and C functions of the free amino acids, we also investigated rates using N- and C-protected amino acids (Table 4). For **BNPP** hydrolysis, introduction of the Z group at the N-terminus always resulted in a pronounced decrease ($k_{rel} = 0.04$ to 0.12). This is in line with known effects of positively charged groups in phosphoric ester hydrolysis.[12] Noticeably, however, Z_α-Lys, which still bears a positive charge, did show a rate enhancement. On the other hand, DNA hydrolysis is less affected (Table 4, k_{rel} up to 1.6), also in line with the more positive effects seen when using free amino acids alone (Table 1). Removal of the negative charge by the use of C-protected amino acids (Table 4) led with **BNPP** to rate enhancements of up to $k_{rel} = 3.1$, complementary to the results with the Z-protected acids as discussed above. In contrast, DNA

Table 1. Relative rate enhancements k_{rel} in the hydrolysis of BNPP and DNA with Eu^{3+} and addition of different cofactors

Cofactors	k_{rel} BNPP[a][b]	k_{rel} DNA[a][c]
–	≡1	≡1
Ser	0.72	2.2
1+His+Ser	0.24	1.5
1+Asp+Ser+**3**	[d]	1.8
1+Asp+Ser+**4**	[d]	1.4
8	[d]	0.3
5+His+Ser	[e]	5.2
5+His+Asp	[e]	5.2
5+Asp	[e]	6.3[f]
2[g]	3.4	2.8
2[g]+**4**	5.0	0.6
5	3.2	0.9
6	0.71	2.9
5+Lys[h]	3.7	1.6
5+**7**[i]	0.57	2.6

[a] $k_{rel} = k_{obs}/k_m$, with $k_m = 1.67 \cdot 10^{-4}$ s^{-1} (for **BNPP**) and $k_m = 0.59 \cdot 10^{-4}$ s^{-1} (for DNA) with $[Eu^{3+}] = 5 \cdot 10^{-3}$ M. No rate enhancements are observed with: dipeptide H-His-Asp-OH, tripeptide H-Asp-Asp-Asp-OH and other combinations of the amino acids His, Asp, Ser, Lys, Arg. – [b] [cofactor] = $5 \cdot 10^{-3}$ M, $[Eu^{3+}] = 5 \cdot 10^{-3}$ M in 0.01 M EPPS, pH = 7.0, $T = 50$ °C, [**BNPP**] = $3.76 \cdot 10^{-5}$ M; error in $k = \pm 2\%$; unless noted otherwise. – [c] [cofactor] = $5 \cdot 10^{-3}$ M, $[Eu^{3+}] = 5 \cdot 10^{-4}$ M in 0.01 M EPPS, pH = 7.0, $T = 37$ °C, incubation time 2 h, [DNA] = $1.9 \cdot 10^{-5}$ M (bp); error in $k = \pm 2\%$; unless noted otherwise. – [d] Only decrease of absorption detected. – [e] Precipitation. – [f] Largest known rate for supercoiled DNA with lanthanides (also some RF III apparent, see Figure 1) – [g] For better solubility with 7% methanol in the final solution. – [h] [**5**]:[Lys] = 1:5. – [i] [**5**]/[**7**] = 5:1.

Table 2. Rate constants with different combinations of the amino acids L-His, L-Asp, and L-Ser

Amino acid	BNPP[a] $k_{obs}[s^{-1}] \times 10^4$	DNA[b] $k_{obs}[s^{-1}] \times 10^4$
His	1.31	0.84
Asp	0.13	0.28
Ser	1.20	1.31
His+Asp	0.04[c]	0.39
Asp+Ser	0.12[c]	0.35
His+Ser	0.88	0.75
His+Asp+Ser	0.04[c]	0.40
Eu^{3+}	1.67	0.59

[a] [**5**]:[**7**] = 5:1. – [b] [amino acid] = $5 \cdot 10^{-3}$ M, $[Eu^{3+}] = 5 \cdot 10^{-3}$ M in 0.01 M EPPS, pH = 7.0, $T = 50$ °C, [**BNPP**] = $3.76 \cdot 10^{-5}$ M, error in $k = \pm 2\%$. – [c] [amino acid] = $5 \cdot 10^{-3}$ M, $[Eu^{3+}] = 5 \cdot 10^{-4}$ M in 0.01 M EPPS, pH = 7.0, $T = 37$ °C, incubation time 2 h, [DNA] = $1.9 \cdot 10^{-5}$ M (bp), error in $k = \pm 2.5\%$.

cleavage was characterized by smaller rates, in line with the positive effect of the carboxylic functions here (Table 1). That one observes a rate depression with DNA and the C-protected amino acids is probably the result of blocking of phosphate by the excess of positively charged ligands, which is no longer counteracted by the amino acid carboxylate – an effect observed with many amines.[10a,10c,10d]

As another way to increase the concentration of productive complexes, we *combined* the two most useful functions found by screening single amino acids within one cofactor **5**. With **BNPP**, this ligand in combination with amino acids

Table 3. Rate constants with different combinations of the amino acids L-His, L-Asp, L-Ser, and L-Arg

Amino acid	BNPP[a] $k_{obs}[s^{-1}] \times 10^4$	DNA[b] $k_{obs}[s^{-1}] \times 10^4$
Arg	1.44	0.60
Arg+His	2.45	0.52
Arg+Asp	0.22	0.43
Arg+Ser	2.12	0.58
Arg+His+Asp	0.18	0.50
Arg+Asp+Ser	1.77	0.46
Arg+His+Ser	0.20	0.75
Arg+His+Asp+Ser	0.19	0.70
Eu^{3+}	1.67	0.59

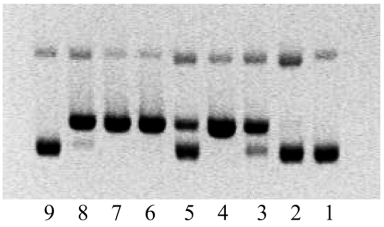
[a] Calculated after increase of extinction (2nd phase), see text. – [b] [amino acid] = $5 \cdot 10^{-3}$ M, [Eu^{3+}] = $5 \cdot 10^{-3}$ M in 0.01 M EPPS, pH = 7.0, T = 50 °C, [**BNPP**] = $3.76 \cdot 10^{-5}$ M, error in k = ± 2%.

Table 4. Rate constants with different protected amino acids

Amino acid	BNPP[a] $k_{obs}[s^{-1}] \times 10^4$	DNA[b] $k_{obs}[s^{-1}] \times 10^4$
Z-Asp	0.21	1.21
Z-Asp+His	0.19	0.66
Z-Asp+Ser	0.07	0.78
Z-Asp+His+Ser	0.12	0.89
AspOMe	3.60[c]	0.09
AspOMe+His	3.13[d]	0.13
AspOMe+Z-His	0.42[d]	0.17
AspOMe+Ser	3.57[c]	0.18
AspOMe+His+Ser	2.80[d]	0.16
AspOMe+Z-His+Ser	0.40[c]	0.16
$Asp(OMe)_2$	5.13[c]	0.12
$Asp(OMe)_2$+His	0.40[c]	0.21
$Asp(OMe)_2$+Z-His	0.94[d]	0.22
$Asp(OMe)_2$+Ser	0.73	0.17
$Asp(OMe)_2$+His+Ser	3.25[c]	0.17
$Asp(OMe)_2$+Z-His+Ser	0.75	0.22
Z_α-Lys	2.58	0.95
Eu^{3+}	1.67	0.59
Asp+Z-His	0.27	0.11
Lys	1.45	0.74
Z-His	0.89	0.17

[a] [amino acid] = $5 \cdot 10^{-3}$ M, [Eu^{3+}] = $5 \cdot 10^{-4}$ M in 0.01 M EPPS, pH = 7.0, T = 37 °C, incubation time 2 h, [DNA] = $1.9 \cdot 10^{-5}$ M (bp), error in k = ± 2.5%. – [b] [amino acid] = $5 \cdot 10^{-3}$ M, [Eu^{3+}] = $5 \cdot 10^{-3}$ M in 0.01 M EPPS, pH = 7.0, T = 50 °C, [**BNPP**] = $3.76 \cdot 10^{-5}$ M, error in k = ± 2%. – [c] [amino acid] = $5 \cdot 10^{-3}$ M, [Eu^{3+}] = $5 \cdot 10^{-4}$ M in 0.01 M EPPS, pH = 7.0, T = 37 °C, incubation time 2 h, [DNA] = $1.9 \cdot 10^{-5}$ M (bp), error in k = ± 2.5%. – [d] Calculated from 0–100 min (initial phase, see footnote [c] in Table 1 and text). –

unfortunately always led to precipitation, but with plasmid DNA and lanthanide complexes we found the *largest* rate enhancements reported until now with Eu^{3+} complexes (Table 1).[13] The hydrolysis is so efficient that one also observes formation of the open-form RF III, by cleavage of the first hydrolysis product RF II (Figure 1). Experiments with the related ligand **6**, bearing only one imidazole unit and lacking the carboxylic acid function, confirmed that the presence of a carboxylic acid group is essential for effective catalysis. This is also obvious from saturation kinetics observed with **5** and varying concentrations of aspartic acid as additional co-substrate (Figure 2). Saturation kinetics with **5** and Eu^{3+} alone show $k_{cat} = 5.2 \cdot 10^{-5} s^{-1}$ and K_A = 8800 M^{-1}.



1, 9: DNA
2: + His
3: + Asp
4: + Ser
5: + His + Asp
6: + Asp + Ser
7: + His + Ser
8: + His + Asp+ Ser

Figure 1. Gel electrophoresis of plasmid DNA hydrolysis with **5** and different combinations of amino acids (conditions: see Table 1)

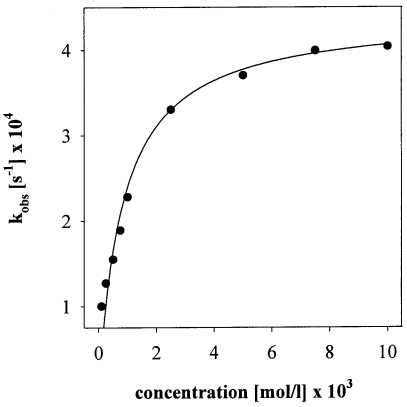


Figure 2. DNA saturation kinetics with [Eu^{3+}] = $5 \cdot 10^{-4}$ M and varying [**5**] and [Asp]

The results demonstrate how one can select cofactors from a library of simple compounds, which should bring the efficiency of synthetic nucleases closer to that of natural enzymes. We have also shown how the incorporation of several functions in simple synthetic arrays can enhance the rates. Further steps will need an increase in the association constants between metal ions, cofactors, and substrate, and arrangement of the functions in proper geometries around the substrate.

Experimental Section

Hydrolysis Kinetics with BNPP: As described earlier,[10c,10e] the rate of *p*-nitrophenolate release was monitored at 400 nm (ε = 6430 $M^{-1}cm^{-1}$) with a Varian Cary 1 Bio UV/Vis spectrophotometer at 75.0 °C. Rate constants were calculated from nonlinear, least-squares fitting to the first order rate law.

DNA Cleavage Experiments: In line with published procedures, supercoiled plasmid DNA samples (pBR 322) were incubated at 37 °C for 2 h in 10 μL samples.[10d] After quenching by addition of 2 μL of a buffer containing 40 wt.% saccharose, TRIS (0.89 M), boric acid (0.89 M), EDTA (1.0 M), and a trace of bromophenol blue, electrophoresis was performed on 0.9% agarose in a horizontal gel apparatus at 70 V for 2 h, using a buffer with TRIS (0.89 M), boric acid (0.89 M), EDTA (2 mM), and ethidium bromide (0.5 μg mL^{-1}). The electrophoresis results were assessed using "Zero-Dscan" software from Scanlytics with an "Eagle Eye II" densitometry system from Stratagene, with corrections for the proportion of RF II already present in the starting material.

Table 5. NMR spectroscopic data

Compound **2**								
	1	2	3	4	5	6	7	8
^{1}H	3.21 (s, 16 H)		230 (s, 8 H)	2.70# (t, 4 H)	2.80# (t, 4 H)		7.75 (s, 2 H)	7.10 (s, 2 H)
J				7.2	7.2			
^{13}C[a]	70.65	70.01#	54.33	50.10	30.99	127.89*	136.30*	122.30

Compound **5**						
	1	2	3	4	5	6
^{1}H			(m, 4 H)	7.25		
^{13}C[a]	175.00	131.98#	130.68#	129.87#	128.55*	120.51

	7	8	9	10	11	23	13
^{1}H			3.60 (t, 2 H)	3.02 (t, 2 H)		7.62 (s, 1 H)	8.38 (s, 1 H)
J			6.2	6.2			
^{13}C[a]	118.95	162.3	42.37	28.27	139.11*	128.47*	132.93*

Compound **6**							
	1	2	3	4	5	6	7
^{1}H	7.44−7.54 (m, 6 H)						
^{13}C[a]	127.04	128.16#	128.22#	127.04#	128.22#	129.01#	166.10

	8	9	10	11	12	13	14
H	11.86 (s, 1 H)	3.48 (t, 2 H)	2.7u6 (t, 2 H)		7.44−7.54 (m, 6 H)	6.83 (s, 1 H)	7.85 (s, 1 H)
J		7.5	7.4				
^{13}C[a]		38.96	26.85	134.63*	130.94*		131.20*

[a] Shifts in [ppm] from internal TMS; measured in D_2O at ambient temperature; signal numbering see Scheme 1; exchangeable signals are denoted by # or * signs.

NMR spectra were taken at 400.13 MHz for 1-H, and 100.61 MHz for 13-C with a Bruker AM 400 system, under conditions as given in Table 5.

10,16-Bis[2-(4-imidazolyl)ethyl]-1,4,10,13-tetraoxa-10,16-diazacyclooctadecane (2): The compound was obtained analogously to a literature procedure[14] by adding a solution of 1-bromo-2-(1*H*-imidazol-4-yl)ethane (430 mg, 2.0 mmol) in acetonitrile (25 mL) to a mixture of the commercially available diazacrown (260 mg, 1.00 mmol) and anhydrous Na_2CO_3 (530 mg, 5.00 mmol) in acetonitrile (50 mL) and refluxing for 16 h; after filtration, removal of the solvent, dissolving the residue in $CHCl_3$, washing with water, and drying, 210 mg (47%) of an oil were obtained. This was not purified further, but showed no impurities in the NMR spectra (Table 5).

***N*-[2-(1*H*-Imidazol-4-yl)ethyl]phthalamide (5):** In analogy to literature procedures,[15] a solution of histamine (free base, 500 mg, 4.50 mmol) in acetone (100 mL) was added dropwise to a solution of phthalic acid anhydride (670 mg, 4.50 mmol) in acetone (50 mL); after stirring for 1 h at room temperature the resulting phthalamide was filtered off and washed 3 times with 50 mL acetone; colorless crystals (640 mg, 55%), decomposition above 130 °C; NMR spectroscopic data, see Table 5. − $C_{13}H_{13}N_3O_3$ (259.26): calcd. C 60.22, H 5.5, N 16.20; found C 60.37, H 5.57, N 15.55 [%].

***N*-[2-(1*H*-Imidazol-4-yl)ethyl]benzamide (6):** Preparation according to ref.[16] NMR spectroscopic data see Table 5.

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[12] Relative rates k_{rel} are given as the ratio between observed rate constants in the presence of the cofactor and the rate without cofactor, always with $[Eu^{3+}] = 5 \cdot 10^{-3}$ M and the same molarity of cofactors.

[13] See J. Rammo, H.-J. Schneider, *Inorg. Chim. Acta* **1996**, *251*, 125 and references cited therein

[14] As reported in ref. [1f], [1g], and [1i], Zr^{IV} and Ce^{IV} salts are very effective, but in our hands proved to be insoluble at pH 7.0, even after addition of EDTA, aspartic acid, or γ-cyclodextrin, and therefore could not be used.

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