enabled broad-range optical access for the first time in the near- and mid-IR region even in a scH_2O atmosphere. Aqueous solutions of pinacol (0.40 M) and cyclohexanone oxime (0.15 M) were introduced into the system with flow rates in the range of 0.07 to 2.0 mL min^{-1} (the reaction time varied from 4 to 215 s) using a high-pressure liquid pump, and pressure control was achieved by a back-pressure regulator. The temperature was then raised to the desired value. Once the system had stabilized, 50 spectra (4 cm^{-1} resolution) were summed in one destination file. Each file was normalized against the spectrum of pure H_2O under the same conditions. Furthermore, all of the products were identified qualitatively by ^1H NMR spectroscopy and GC-MS.

The frequency precision at the band center of the Raman OH symmetric stretch (ν_1) in the spectrum depends on changes in the optical pixel width of the charge-coupled device, and the resulting frequency error of the ν_1 mode is within 2.0 cm^{-1}.[8]

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A 10^{10} Rate Enhancement of Phosphodiester Hydrolysis by a Dinuclear Aminopeptidase—Transition-State Analogues as Substrates?[**]

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The studies of metal complex based chemical models for hydrolytic metalloenzymes[1–3] have provided insight into the mechanistic roles of the metal ion(s) at the active-site and the coordinated nucleophilic water molecule in those enzymes.[4] However, these model complexes lack specific recognition and catalysis toward peptide substrates over phosphoester substrates, or vice versa, as a result of the absence of a well-defined active site. Conversely, enzymes have evolved to recognize one particular type of substrate and are able to stabilize the corresponding transition state,[5] such as the tetrahedral transition state in peptide hydrolysis which is very different from the trigonal bipyramidal transition state in phosphoester hydrolysis.[4] As a result each class of hydrolytic metalloenzymes can perform only one type of hydrolysis despite the presence of a highly activated ($>10^7$ in terms of pK_a) coordinated water molecule that is ready for nucleophilic attack on the substrate. Moreover, anionic tetrahedral phosphoesters,[6] phosphonates,[7] phosphoamidates,[8] and semiacetals,[9] resemble the transition-state *gem*-diolate of peptides, esters, and amides formed during hydrolysis and can serve as potential inhibitors for the corresponding enzymes. Thus, it seems unlikely that an enzyme with a single active site can hydrolyze both phosphoester and peptide substrates because the specific recognitions and hydrolytic pathways for these two types of substrates are quite different.

Herein we report a unique example of an “alternative” enzyme catalysis in which a dizinc aminopeptidase from *Streptomyces griseus* (sAP) exhibits remarkable hydrolytic activities toward both peptide and phosphodiester substrates. Thus, this enzyme can serve as an alternative dinuclear model system to provide further insight into the catalytic mechanism of metal-centered dinuclear hydrolysis.

The activity profiles obtained during the purification of the sAP[10, 11] and its thermo-deactivation at 69 °C show that the activity toward the hydrolysis of the phosphodiesterase substrate bis-*p*-nitrophenylphosphate (BNPP)[12] is always parallel to the activities toward the aminopeptidase substrates Leu-*p*-nitroanilide (Leu-*p*NA) and Lys-*p*NA (see data in ref. [13] and Table 1). This observation suggests that these two different types of hydrolytic reactions are possibly carried out by a single enzyme, and the decrease in both activities is attributable to thermo-denaturation of the sAP. Both activities are competitively inhibited by the dizinc aminopeptidase inhibitors bestatin,[14] 1-aminobutylphosphonate,[7] and Leu-

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Table 1. The hydrolyses of aminopeptidase substrates and BNPP by sAP (the first active fractions eluted from the ion-exchange column) in 0.1 M HEPES buffer at pH 8.0 in the presence of 2 mM Ca^{2+} at 30 °C.

	Heat deactivation[a]	K_i [μM][b]			Kinetic parameters[c]		
substrate	7 h [%]	bestatin[d]	ABP[d]	Leu-NHOH[e]	k_{cat} [s^{-1}]	K_m [mM]	k_{cat}/K_m [$M^{-1}s^{-1}$]
BNPP	22	1.3 ± 0.1	1.5 ± 0.2	40 ± 13	0.45 ± 0.02	4.5 ± 0.4	100
Leu-*p*NA	21	1.5 ± 0.2	1.9 ± 0.2	38 ± 10	657 ± 54	0.45 ± 0.42	1.46×10^6
Ala-*p*NA	19	1.2	1.9	30	3.6 ± 0.4	5.0 ± 0.4	720
Lys-*p*NA	26	2.1	1.9	52	1.5 ± 0.2	7.1 ± 0.8	211

[a] BNPP, 10 mM; Leu-*p*NA, 1 mM; Ala-*p*NA, 10 mM; and Lys-*p*NA, 10 mM in 0.1 M HEPES at pH 8.0 and 69 °C. The activities are reported with respect to untreated controls. [b] Obtained from three different inhibitor concentrations.[5b] The K_i value of BNPP in the hydrolysis of Leu-*p*NA is 5.1 mM. [c] The substrate concentrations were BNPP: 1.0–20 mM; Leu-*p*NA: 0.1–2.0 mM; Ala-*p*NA: 1–20 mM; and Lys-*p*NA: 1–20 mM. The initial rates were obtained from the change of absorbance at 405 nm for peptide substrates ($\varepsilon = 10600\,M^{-1}cm^{-1}$) and for BNPP ($\varepsilon = 17500\,M^{-1}cm^{-1}$). [d] 1, 2, and 4 μM, pre-incubated with sAP for 3 hours; ABP = 1-aminobutylphosphonate. [e] 25, 50, and 100 μM, without incubation.

hydroxamate[15] (Table 1). Moreover, the "slow substrate" BNPP itself serves as a competitive inhibitor ($K_i = 5.1$ mM) toward the hydrolysis of Leu-*p*NA by sAP. We conclude on the basis of the competitive inhibition patterns that the hydrolysis of BNPP occurs in the dinuclear active site of sAP.

We have previously demonstrated that Co^{2+} binds sequentially to the two metal binding sites of apo-sAP.[11] The hydrolysis of BNPP by zinc-free sAP exhibits a selective Co^{2+} activation profile (trace –□–, Figure 1) similar to the hydrolysis of a peptide substrate[11] (trace –●–), which suggests that both hydrolyses are carried out in the dinuclear active site of sAP. The sequential binding of Co^{2+} to the dinuclear metal site of apo-sAP has also been concluded by NMR spectroscopy (see insets in Figure 1).[12] The hydrolysis of BNPP by any phosphodiesterase contaminant can thus be completely excluded from this stoichiometric metal-activation study.

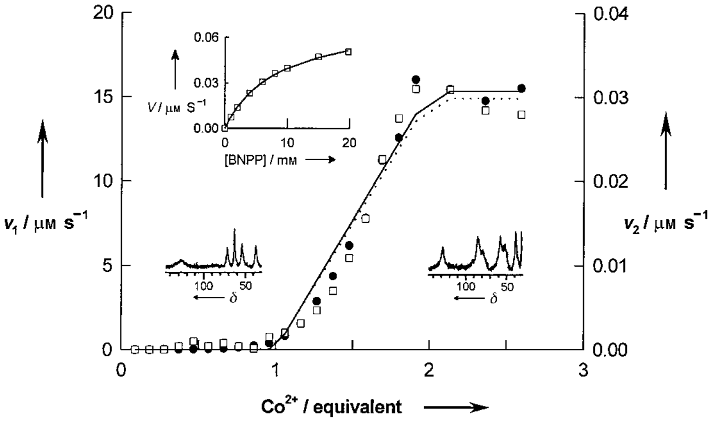

Figure 1. Activation of apo-sAP (0.112 mM in 0.1 M MES buffer at pH 6.1) by Co^{2+} towards the hydrolysis of Lys-*p*NA (2.5 mM, ●, left axis v_1) and BNPP (5 mM, □, right axis v_2) in 0.1 M HEPES buffer at 30 °C and pH 8.0 (HEPES = 2[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid). The solid (Lys-*p*NA) and dotted (BNPP) lines are obtained by fitting the data to an ideal selective metal binding mode. The inset is a Michaelis–Menten kinetic plot for BNPP hydrolysis, which gives $k_{cat} = 0.45\,s^{-1}$ and $K_m = 4.5$ mM. The inset ^1H NMR spectra (Bruker AMX360 at 360.13 MHz, 298 K, 90° pulse, and presaturation for solvent suppression) demonstrate the binding of one (left) and two (right) Co^{2+} ions to apo-sAP.

A complete Michaelis–Menten kinetic analysis of BNPP hydrolysis by sAP has been carried out (see inset in Figure 1 and Table 1). It is very interesting to note that the second-order rate constant of BNPP hydrolysis by sAP ($100\,M^{-1}s^{-1}$ in terms of k_{cat}/K_m) under mild conditions (30 °C and pH 8.0) exceeds those of synthetic Zn^{2+}-containing chemical models (which typically lie in the range $1.3-12.7 \times 10^{-5}\,M^{-1}s^{-1}$ at 35 °C and pH 8.6–10.5) by at least 10^6 times.[2, 16] The specific activity towards the hydrolysis of 1 mM BNPP was also determined as 33.7 nmol $min^{-1}mg^{-1}$. This value is higher than that for the hydrolysis of 1 mM Gly-*p*NA by sAP ($\lesssim$19.3 nmol $min^{-1}mg^{-1}$, calculated from ref. [10b]), and surpasses the activities of several phosphoesterases toward the hydrolysis of 1 mM BNPP. For example, the specific activities of the alkaline phosphatase from calf intestine, chicken intestine, and *Escherichia coli* are 41.5, 16.5, and 11.1 nmol $min^{-1}mg^{-1}$, respectively, and of wheat germ acid phosphatase 1.9 nmol $min^{-1}mg^{-1}$.[12] The activities of 5′-nucleotide phosphodiesterase from bovine intestine and snake venom, 3′-nucleotide phosphodiesterase from bovine spleen, and cAMP phosphodiesterase from bovine heart are 2450, 82.3, 0.8, and 0.3 nmol $min^{-1}mg^{-1}$, respectively.[12]

Despite the fact that the phosphodiesterase substrate BNPP is highly activated by the *p*-nitro group toward hydrolysis, its auto-hydrolytic rate is still extremely slow, with a first-order rate constant of $3 \times 10^{-10}\,s^{-1}$ at pH 7.0 and 50 °C,[2b] and $6.3 \times 10^{-8}\,s^{-1}$ at pH 10.0 and 100 °C.[17] The pseudo-first-order rate constant of BNPP hydrolysis by sAP can be extrapolated from the Michaelis–Menten kinetics at low substrate concentrations, the conditions used in many chemical modeling studies.[1–3] The value of $1.0\,s^{-1}$ obtained at pH 8 and 30 °C is about 10^{10} times larger than the first-order rate constant of the auto-hydrolysis of this substrate under similar conditions. This rate enhancement is quite significant when we take into account that this phosphodiester is a transition-state analogue in peptide/ester hydrolysis,[6] and is not supposedly hydrolyzed by peptidases. Moreover, the rate of uncatalyzed phosphodiester hydrolysis is generally lower than that of peptides by a factor of about 10^4.[18] The much smaller k_{cat}/K_m value for the hydrolysis of BNPP than for the hydrolysis of the best peptide substrate Leu-*p*NA by sAP can thus be reasonably expected (Table 1). The slower rate of phosphodiester hydrolysis is attributed to kinetic inertness[19] since the nucleophilic attack by hydroxide ions on the negatively charged phosphate group is not favorable as a result of charge repulsion. Nevertheless, a dinuclear center such as in sAP can neutralize the charge on the phosphate moiety quite effectively.

The mechanism for BNPP hydrolysis by sAP can be postulated (Figure 2) from the X-ray crystal structure of sAP,[20] and the inhibitor-bound complexes of bovine lens leucine aminopeptidase in which the amino group is coordinated to one zinc center and one *gem*-diolate-like $C-O^-$ group bridges the two zinc ions.[7a, 9, 21] Since BNPP and Leu-*p*NA bind to sAP competitively, they may bind to the active site in a similar manner despite the absence of the amino group in BNPP. In this model the phosphorus center of BNPP is accessible to nucleophilic attack by a coordinated water molecule.

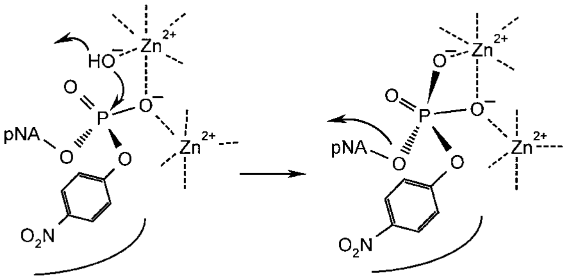

Figure 2. A plausible mechanism for the hydrolysis of BNPP by dinuclear *Streptomyces* aminopeptidase, in which the bridging $P-O^-$ unit mimics the bridging carbonyl group of a peptide substrate while the amino group of the peptide substrates is missing in BNPP. Presumably one coordinated hydroxide ion on one Zn^{2+} center attacks the phosphorus center at the position *trans* to the leaving group. An alternative binding mode might be that the $P=O$ moiety mimics the amino group of peptide substrates that bind to one Zn^{2+} ion.

We have described here a unique and unprecedented case that a transition-state analogue phosphodiester is indeed a substrate for a dizinc aminopeptidase, and is hydrolyzed with an enormous rate acceleration of approximately 10^{10} times. Dinuclear mechanisms have also been proposed in several other hydrolytic multinuclear enzymes, including urease, nucleases, and ribozymes.[4a, 22] This dinuclear metalloprotease sAP, which exhibits this unique alternative catalysis, can thus serve as a dinuclear model system for the further investigation of dinuclear hydrolysis of both peptides and phosphodiesters.

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[13] The crude enzyme mixture exhibits significant activities (nmol min^{-1} mg^{-1}) toward several substrates of aminopeptidase and phosphoesterase: 1 mM Leu-*p*NA, 9300; 2.5 mM Lys-*p*NA, 57; 5 mM *p*-nitrophenylphosphate, 5.2; and 5 mM BNPP, 4.8. After incubation of the mixture for 7 h at 69°C the hydrolysis toward 10 mM *p*-nitrophenylphosphate was not detectable; Leu-*p*NA (1 mM), 67% retained; Lys-*p*NA (10 mM), 28%; Ala-*p*NA (10 mM), 49%; BNPP (10 mM), 42%. During the chromatographic steps (gel filtration and DEAE ion exchange) the phosphodiesterase activity is parallel to the aminopeptidase activity. These procedures afford a pure aminopeptidase whose activity can be eliminated by demetalation, and reactivated to different extents by several different metal ions.

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