P1−S1 Interactions Control the Enantioselectivity and Hydrolytic Activity of the Norleucine Phenylesterase Catalytic Antibody 17E8

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The catalytic antibody 17E8 is an esterase that catalyzes the hydrolysis of *N*-acyl amino acid phenyl esters.[1] This catalytic antibody was generated against the norleucine phenyl phosphonate analog **1**, and one of the best substrates for the enzyme is *N*-formyl norleucine phenyl ester **2** (Figure 1). The crystal structure of the 17E8 Fab complexed with the phosphonate analog **1** has been solved to 2.5 Å resolution.[2] This structure shows that 17E8 has similar active site features to natural triad-based hydrolases. In addition, the structure shows that **1** is buried deeply in the antigen combining site, and there are separate and well-defined binding pockets for the phenyl group and *n*-butyl side chain of **1**. The system is thus a simple model of proteolytic enzymes containing a single, isolated S-subsite pocket for recognition of a P1 hydrophobic side chain of the amino acid substrate.

In our initial kinetic studies with 17E8, we observed that the hydrolysis reaction was catalyzed in an enantioselective manner.[1] The synthesis of substrate **2** resulted in Cα racemization, making it unsuitable for preparing enantiomerically pure substrates with either *R* or *S* Cα configuration. However, we were able to obtain enantiomerically enriched Cα *R* and *S* samples and found that the *S*-enriched substrate afforded a higher specific activity than the *R*-enriched substrate by the expected value assuming complete *S*-enantioselectivity for 17E8-catalyzed hydrolysis. Additional support for the hydrolytic enantioselectivity was obtained from the 17E8-**1** crystal structure which showed that the enantiomer of **1** with the same Cα configuration as the *S* substrate was bound in the active site even though racemic **1** was used in the crystallization experiment.[2]

An alternative synthetic route involving esterification of *N*-Boc amino acids was developed to prepare enantiomerically pure 17E8 substrates.[3] We now report results that unambiguously confirm the 17E8 *S*-enantioselectivity of hydrolysis and show that enantioselectivity and catalytic activity are controlled by hydrophobic interactions between the substrate P1 side chain and enzyme S1 pocket.

The enantioselectivity of the 17E8-catalyzed hydrolysis reaction was examined using the *S* and *R* substrate as well as the racemic mixture (Table 1). The (*S*)-**2** and racemic **2** are effective substrates for 17E8, whereas the hydrolysis of (*R*)-**2** was not catalyzed by 17E8. A steady-state kinetic analysis was performed with (*S*)-**2** and racemic **2** to obtain the kinetic constants k_{cat} and K_M.[4] The magnitudes of these constants are consistent with an *S*-enantioselective mode of hydrolysis: the same k_{cat} value is observed for (*S*)-**2** and racemic **2**, but the K_M value for racemic **2** is approximately twice that of (*S*)-**2**. Taken together, these results unambiguously establish that 17E8 catalyzes the enantioselective hydrolysis of norleucine phenyl substrates that have the *S* configuration at Cα, and the enantiomeric *R* substrates are not processed by the enzyme.

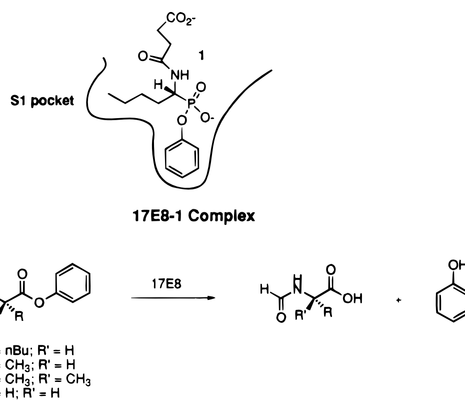

Figure 1. Schematic of 17E8-**1** active site structure and hydrolysis reaction catalyzed by 17E8.

Table 1. Enantioselectivity of 17E8 Catalysis

substrate	Cα configuration	$[\alpha]_D$[a]	k_{cat} (s^{-1})[b]	K_M (mM)[b]
N-for-Nle-OPh (**2**)	*S*	−14.0°	2.1 ± 0.1	0.10 ± 0.01
	R	+13.7°		
	racemic	0	2.0 ± 0.1	0.18 ± 0.04
N-for-Ala-OPh (**3**)	*S*	−36.0°	0.9 ± 0.1	11 ± 4
	R	+ 35.6°		
	racemic	0	0.8 ± 0.1	9 ± 2
N-for-Aib-OPh (**4**)	nonstereogenic	0		
N-for-Gly-OPh (**5**)	nonstereogenic	0		
phenyl hexanoate[c]	nonstereogenic	0	2.2 ± 0.6	0.6 ± 0.2

[a] Optical activity measurements were performed on a Perkin-Elmer 241 polarimeter. All samples were prepared as dichloromethane solutions, and optical density measurements were made at 25 °C. [b] Activity assays were performed in 50 mM borate, 150 mM NaCl, pH 8.7 at 24.5 °C. Steady-state kinetic constants were obtained from nonlinear fits of *v* vs [S] data to the Michaelis−Menten equation using the Kaliedagraph program. [c] The kinetic data for this substrate were obtained at pH 9.5. For comparison, the racemate of the natural substrate **2** gives a k_{cat} of 3.7 s^{-1} and K_M of 0.4 mM at pH 9.5.

The serine proteases show similar S-enantioselectivity of hydrolysis.[5] A simple explanation to account for the enantioselectivity of serine proteases is a three-locus model where the three non-hydrogen groups attached to the stereogenic α-carbon make stabilizing interactions with specifically oriented active site groups in the hydrolysis transition state.[6] In the case of chymotrypsin, an H-bond between the NH of the α-N-acyl group and an active site acceptor has been identified as a critical interaction for controlling enantioselectivity.[7] Replacement of the *N*-acetyl group with a hydrogen atom in phenylalanine ester substrates leads to a 4000-fold decrease in k_{cat} and a 9000-fold decrease in k_{cat}/K_M. In contrast, the analogous experiment with 17E8 shows that the substrate *N*-acyl group is not particularly important in catalysis. Phenyl hexanoate, which contains a hydrogen atom in place of the *N*-formyl group, gives values for k_{cat} and K_M similar to those of the norleucine substrate **2** (Table 1), suggesting that the enantioselectivity of 17E8 is not controlled by an H-bonding interaction between the α-*N*-acyl group of substrate and an active site acceptor.

The alanine, Aib, and glycine substrates (**3**, **4**, and **5**) were examined to probe the contribution of the substrate P1 side chain

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(2) Zhou, G. W.; Guo, J.; Huang, W.; Fletterick, R. J.; Scanlan, T. S. *Science* **1994**, *265*, 1059.

(3) Castro, B.; Evin, G.; Selve, C.; Seyer, R. *Synthesis* **1977**, 413. Both the *R* and *S* enantiomers of the norleucine (**2**) and alanine (**3**) were prepared by this route. The α-amino isobutyric acid (Aib, **4**) and glycine (**5**) substrates, which are nonstereogenic at Cα, were prepared by direct esterification of the corresponding *N*-formyl amino acid. Phenyl hexanoate, a substrate that possesses the *n*-butyl P1 side chain but does not contain the α-formylamino substituent, was prepared by esterification of hexanoic acid.

(4) The kinetic constants k_{cat} and K_M were obtained from a nonlinear fit of the *v* vs [S] data to the Michaelis−Menten equation using the Kaleidagraph program.

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Table 2. Comparison of P1 Catalytic Effects Between Chymotrypsin and 17E8

enzyme	substrate	no. at P1 C's[a]	k_{cat} (s^{-1})	K_M (mM)	ΔP1 C's[b]	Δk_{cat}	ΔK_M	$\Delta\Delta G_b^{\ddagger}$ (kcal/mol)[c]
chymotrypsin	*N*-Ac-L-Nle-OMe (**6**)	4	16.1[d]	5.37[d]				
chymotrypsin	*N*-Ac-L-Tyr-OEt (**7**)	7	192[d]	0.663[d]				
chymotrypsin	**7**/**6**				3	12-fold	8-fold	−2.7
chymotrypsin	*N*-Ac-L-Phe-OMe (**8**)	7	97.1[d]	0.93[d]				
chymotrypsin	**8**/**6**				3	6-fold	6-fold	−2.1
17E8	(*S*)-**3**	1	0.9	11.0				
17E8	(*S*)-**2**	4	2.1	0.10				
17E8	(S)-**3**/(S)-**2**				3	2-fold	110-fold	−3.3

[a] Refers to the number of carbon atoms contained in the P1 side chain. [b] Refers to the difference in the number of carbon atoms between the P1 side chains of the two substrates. [c] $(k_{cat}/K_M)_a/(k_{cat}/K_M)_b = \exp(-\Delta\Delta G_b^{\ddagger}/RT)$ as defined by Fersht.[12] [d] Kinetic constants for chymotrypsin taken from ref 9.

to catalytic enantioselectivity (Table 1). Hydrolysis of the glycine phenyl ester substrate **5**, which does not contain a P1 substituent, is not catalyzed by 17E8. The alanine phenyl ester **3**, which bears a P1 methyl substituent, is a 17E8 substrate, and hydrolysis of **3** proceeds with the same *S*-enantioselectivity observed for the norleucine substrate **2**.[8] Hydrolysis of the Aib phenyl ester **4** is not catalyzed by 17E8, suggesting that the enzyme cannot accommodate substrates with Cα substituents larger than hydrogen in the pro-*R* position. Taken together, the results support a model where 17E8 hydrolytic activity and enantioselectivity are controlled by (1) hydrophobic P1−S1 interactions, (2) a substituent no larger than an H atom at the pro-*R* position of Cα, and (3) hydrophobic and electrostatic interactions between the carbophenoxy substituent and the phenyl ester binding pocket of the enzyme.

Similar P1-selectivity experiments using *N*-acyl amino acid ester substrates have been carried out with chymotrypsin, which prefers substrates with aromatic P1 side chains, but will also accept substrates with unbranched aliphatic P1 side chains.[9] With the data available, a comparison can be made between chymotrypsin-catalyzed hydrolysis of *N*-acetyl Tyr, Phe and Nle esters, and 17E8-catalyzed hydrolysis of *N*-formyl Nle and Ala esters **2** and **3** (Table 2).[10] The comparisons are made between the enzyme's "natural" substrate, where P1−S1 binding interactions are maximized, and an inferior substrate that has a P1 side chain three carbon atoms smaller than that of the natural substrate. The kinetic data show that 17E8 has a higher specificity than chymotrypsin for the natural substrate over the substrate with the smaller P1 substituent; addition of the three P1 carbon atoms results in 3.3 kcal/mol of catalytically productive binding energy ($\Delta\Delta G_b^{\ddagger}$) for 17E8, whereas 2.1 and 2.7 kcal/mol of binding energy accompanies S1 pocket filling for chymotrypsin with the Phe and Tyr substrates, respectively. Interestingly, the isolated effects on k_{cat} and K_M resulting from increased P1−S1 binding interactions are markedly different for 17E8 and chymotrypsin. In chymotrypsin catalysis, the increase in binding energy is distributed approximately evenly between k_{cat} and K_M, whereas for 17E8 nearly all of the additional binding energy is used to decrease the value of K_M. In chymotrypsin-catalyzed hydrolysis of specific ester and amide substrates, the K_M value generally corresponds to K_s (the dissociation constant for the ES complex). This may not be the case for 17E8, and a better understanding of the catalytic mechanism will be needed to understand how the catalytic antibody uses binding energy along the hydrolytic reaction coordinate.

In conclusion, both the natural enzyme and catalytic antibody are able to use hydrophobic binding interactions remote from the reaction center to direct enantioselectivity and stabilize the hydrolytic transition state. The magnitude of the hydrophobic P1−S1 interactions is greater for 17E8 than for chymotrypsin, and the precise mechanism by which the binding energy is used to accelerate the hydrolysis reaction appears to be different for the natural and unnatural enzyme. The structural simplicity of the 17E8 active site, where the isolated interactions of a single P1 substituent and a single S1 pocket can be examined, may illuminate the more complex multiple P−S and P′−S′ subsite interactions of natural endopeptidases.[11]

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(8) The high K_M, values for **3** make it technically difficult to obtain precise saturation curves, and the expected 2-fold difference in K_M values between the (*S*)-**3** and racemic **3** substrates is not as apparent as with the natural norleucine substrate **2**.

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(10) In this analysis we are comparing the catalytic effects of a substrate that completely fills the S1 pocket to one that has a smaller P1 molecular volume by three C atoms. An assumption in this comparison is that the P1−S1 interactions are largely hydrophobic even though the natural P1 chymotrypsin preference is for aromatic amino acids, whereas the 17E8 natural P1 preference is for aliphatic amino acids. This assumption is supported by P1 specificity studies with chymotrypsin with the homologous substrate series of Gly, Ala, But, Nvl, Nle, and Phe. A plot of $\log(k_{cat}/K_M)$ vs the hydrophobicity constant π shows a linear correlation for the series including the Phe data. Dorovskaya, S.; Varfolomeyev, D.; Kazanskaya, N. F.; Klyosov, A. A.; Martinek, K. *FEBS Lett.* **1972**, *23*, 122.

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