Enzyme Promiscuity

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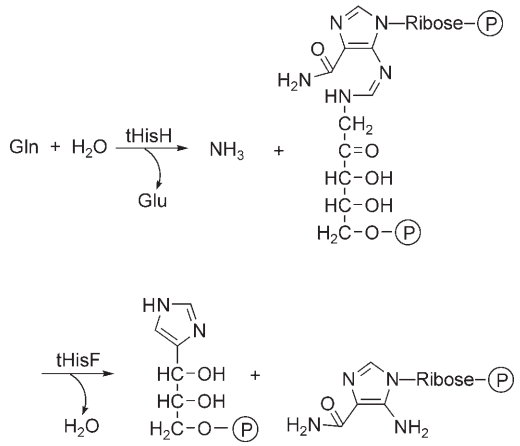
Alternate-Site Enzyme Promiscuity**

*Andreas Taglieber, Horst Höbenreich, J. Daniel Carballeira, Régis J. G. Mondière, and Manfred T. Reetz**

Enzyme promiscuity means, in the broadest terms, the ability of a given enzyme to catalyze distinctly different chemical transformations of natural or nonnatural substrates.[1] Although it was originally thought to be a fairly rare event,[2] research over the last few years has uncovered many more examples. It has also become clear that catalytic promiscuity has implications in evolutionary relationships.[1,3] This intriguing frontier in enzymology has several important theoretical and practical facets. For example, the question of how proteins in nature evolve new functions such as antibiotic resistance or the ability to degrade man-made chemicals, both within months or years, is of fundamental significance and has been studied by applying the methods of directed evolution.[1c,3] Moreover, the discovery of promiscuous behavior of wild-type (WT) enzymes or mutants thereof produced by protein engineering has the potential of expanding the repertoire of synthetic organic methodologies.[1]

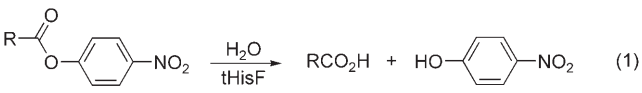
In all studies reported so far,[1–3] the promiscuous (secondary) reaction has been linked to the binding site of the reaction for which the enzyme is primarily known, generally involving some or all of the original catalytically active amino acids or metal centers. Examples are alkaline phosphatase catalyzed hydrolysis of *p*-nitrophenylsulfate,[4] aminopeptidase-catalyzed hydrolysis of phosphoesters,[5] lipase-catalyzed Michael additions of N-,[6] O-,[6] S-,[6] and C-nucleophiles,[7] aldol additions,[8] oligomerization of siloxanes,[9] racemase-catalyzed PLP-dependent aldol additions,[10] and arylmalonate decarboxylase catalyzed aldol additions.[11] Many of these studies involve protein engineering. To the best of our knowledge, no case of enzyme promiscuity has been reported in which the known natural catalytically active site is not involved. Herein we report the first example of this phenomenon, which we call alternate-site enzyme promiscuity.

We discovered alternate-site enzyme promiscuity unexpectedly when screening the thermostable enzyme tHisF from *Thermotoga maritima* for promiscuous behavior. It has a $(\beta/\alpha)_8$-barrel structure and constitutes the synthase subunit of a bienzyme complex involved in the biosynthesis of histidine.[12] Mechanistic studies have demonstrated that ammonia is generated in a first step by hydrolysis of glutamine (Gln), a process catalyzed by the tHisH subunit. The ammonia then enters tHisF at the lower rim of the barrel structure and moves to the relatively broad top rim where the enzyme catalyzes a cyclization reaction in an acid/base manner (Scheme 1). Asp11 and Asp130 were shown by site-directed mutagenesis to be essential for this catalytic step, whereas Asp176 was reported to be important, but not essential.[12]



Scheme 1. Natural reaction of the bienzyme complex tHisH–tHisF.[12]

As acid/base catalysis is involved in the natural function of tHisF, we speculated that this robust enzyme might also show promiscuous hydrolytic behavior at the natural binding site, specifically esterase-like activity.[13] As a model reaction we chose the hydrolysis of *p*-nitrophenyl esters **1a–e** [Eq. (1)]. Activity can be measured easily and accurately by the standard photometric assay (absorption of *p*-nitrophenolate at 405 nm). The rate of the background reaction is relatively



(1)

1 a R = CH_3
b R = C_2H_5
c R = nC_3H_7
d R = nC_4H_9
e R = nC_7H_{15}

2 **3**

[*] A. Taglieber, H. Höbenreich, Dr. J. D. Carballeira, Dr. R. J. G. Mondière, Prof. Dr. M. T. Reetz
Max-Planck-Institut für Kohlenforschung
Kaiser-Wilhelm-Platz 1, 45470 Mülheim/Ruhr (Germany)
Fax: (+49) 208-306-2985
E-mail: reetz@mpi-muelheim.mpg.de

[**] A.T. and H.H. have contributed equally. We thank R. Sterner (Universität Regensburg) for providing the plasmids for tHisF and mutant Cys9Ala/Asp11Cys, N. Dickmann (Max-Planck-Institut für Bioanorganische Chemie) for MALDI-TOF measurements and M. Rusek for performing some of the fermentations. This work was supported by the Fonds der Chemischen Industrie, the Deutsche Forschungsgemeinschaft (SPP 1170, Re 359/13-1), and the EU (IBAAC MCRTN-CT-2003-505020).

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low under the reaction conditions (buffer at pH 7.4). To obtain unambiguous results all experiments were performed with carefully purified protein (see the Supporting Information). To rule out small traces of possible contamination with hydrolytic enzymes (e.g., esterases, lipases, proteases), kinetic parameters were determined before and after treatment with a variety of inhibitors (see the Supporting Information). As no significant differences were observed, contamination can be excluded. Moreover, the observed promiscuity is not due to the classical mechanism of hydrolytic enzymes.[13]

Figure 1 shows two important results, namely, that tHisF is indeed capable of promiscuous esterase-like catalysis

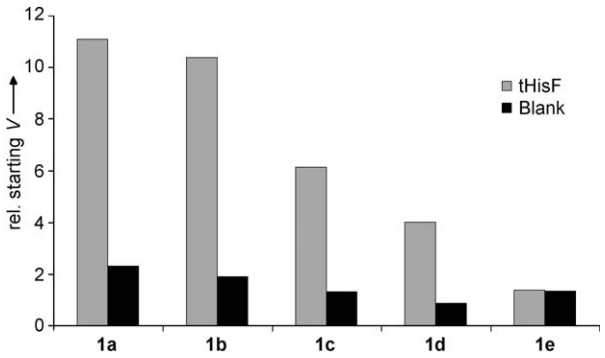
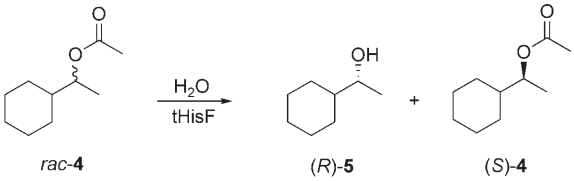


Figure 1. Relative activity of the tHisF-catalyzed hydrolysis of *p*-nitrophenyl esters **1a**–**e**.

(although at lower rates than esterases or lipases),[14] and that there is notable substrate selectivity. The highest activity is observed when the acetate **1a** is subjected to hydrolysis, whereas octanoic acid *p*-nitrophenyl ester (**1e**) hardly undergoes any enzyme-catalyzed acceleration beyond the background reaction. Such a trend is reminiscent of the behavior of esterases.[13]

We then studied the hydrolytic kinetic resolution of the chiral ester *rac*-**4** [Eq. (2)]. In this case, again tHisF was found



(2)

to catalyze ester hydrolysis. Enantioselectivity of this promiscuous reaction turned out to be substantial: the selectivity factor amounted to $E = 29$ in favor of (*R*)-**5**.

Guided by the previous structural and mechanistic information regarding the natural function of tHisF,[12] we set out to identify the essential amino acid residues necessary for the promiscuous hydrolytic reaction. Figure 2 displays the known crystal structure of tHisF,[12a,c] highlighting the amino acids that are essential or important for natural activity (Asp11, Asp130, and Asp176; Scheme 1) in addition to

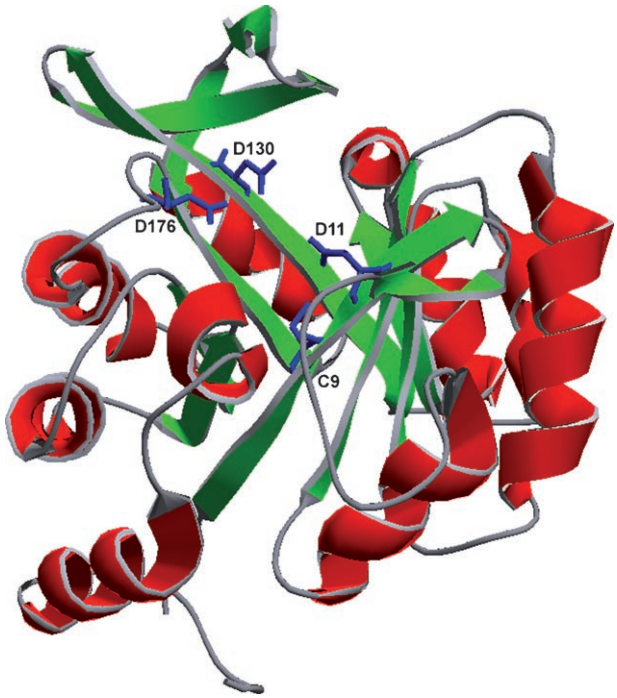


Figure 2. X-ray crystal structure of tHisF[12a,c] showing in blue the natural catalytic amino acids Asp11, Asp130, and Asp176, as well as Cys9.

position Cys9, which could be suspected of contributing to the promiscuous esterase-like activity. By using site-specific mutagenesis, we prepared six mutants and tested them as potentially promiscuous catalysts in the hydrolysis of *p*-nitrophenyl butyrate **1c** (Table 1).[15]

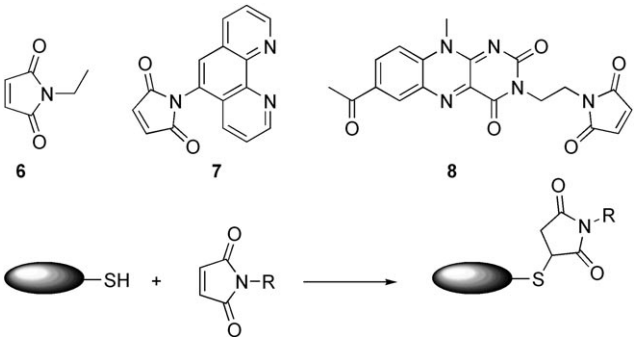
Table 1: Kinetic parameters of the hydrolysis of *p*-nitrophenyl butyrate (**1c**).

tHisF or mutant	K_M [mM]	k_{cat} s^{-1}]	k_{cat}/K_M [s^{-1} M^{-1}]	v_{max} [mM min^{-1}]
WT	1.9	5.4×10^{-4}	0.28	0.0029
Asp11Ala	2.2	5.5×10^{-4}	0.25	0.0030
Cys9Ala/Asp11Ala	1.9	5.0×10^{-4}	0.26	0.0027
Cys9Ala/Asp11Cys	1.5	5.6×10^{-4}	0.39	0.0030
Cys9Ala/Asp11Cys/Asp130Ala	1.4	4.9×10^{-4}	0.34	0.0026
Cys9Ala/Asp11Cys/Asp176Ala	1.6	4.5×10^{-4}	0.28	0.0024
Cys9Ala/Asp11Cys/Asp130Ala/Asp176Ala	1.9	4.1×10^{-4}	0.22	0.0022

The results summarized in Table 1 are surprising because they show that mutations at amino acid positions essential or important for natural tHisF activity do not influence catalytic activity in the promiscuous ester hydrolysis. The values of k_{cat}/K_M remain essentially constant. Thus, the catalytic machinery of tHisF which mediates the natural cyclization reaction (Scheme 1) is not responsible for the observed promiscuous hydrolytic reaction. Moreover, Cys9 can also be excluded as playing an active role in the hydrolysis reaction.

The results also indicate that the promiscuous hydrolytic reaction may not even occur at the binding site relevant in the natural cyclization reaction. Owing to the different steric, electronic, and chemical nature of the newly introduced amino acids of the mutants, one would expect to detect at least some influence on the kinetics of the promiscuous reaction even if other amino acids at the natural binding pocket of tHisF are responsible for catalysis. It should be noted that if an effect had been found, it would not necessarily exclude an alternate site, because mutations remote from the true binding pocket can influence activity or the enantioselectivity of enzymes.[16]

To corroborate the hypothesis regarding the actual binding site in the promiscuous hydrolytic reaction, we synthesized covalent bioconjugates that can be expected to occupy considerable space in the natural binding pocket. In contrast to the WT tHisF, the mutant Cys9 Ala/Asp11 Cys has an appropriately exposed cysteine residue that can be selectively modified with Michael acceptors. Therefore, the maleimide derivatives **6**, **7**, and **8**, which have different spatial properties. were used in the preparation of the respective bioconjugates (Scheme 2). The resulting bioconjugates were analyzed by



Scheme 2. Bioconjugation of the tHisF mutant Cys9 Ala/Asp11 Cys at position Cys11.

MALDI-TOF mass spectrometry, which showed essentially quantitative formation of the desired chemically modified proteins. They were then tested as catalysts in the hydrolysis of butyrate **1c**. Table 2 shows that the promiscuous catalytic

Table 2: Michaelis–Menten kinetics of the hydrolysis of **1c**.

Catalyst	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [$s^{-1}\,M^{-1}$]	v_{max} [mM min^{-1}]
Cys9 Ala/Asp11 Cys (unmodified)	1.5	5.6×10^{-4}	0.39	0.0030
Cys9 Ala/Asp11 Cys-**6**	1.4	4.5×10^{-4}	0.31	0.0024
Cys9 Ala/Asp11 Cys-**7**	1.9	5.7×10^{-4}	0.29	0.0031
Cys9 Ala/Asp11 Cys-**8**	1.8	4.9×10^{-4}	0.27	0.0026

profile does not change substantially from the unmodified mutant Cys9 Ala/Asp11 Cys to the respective bioconjugates.

The graphical representation of the bioconjugates in Figure 3 indicates that the phenanthroline- and flavin-based

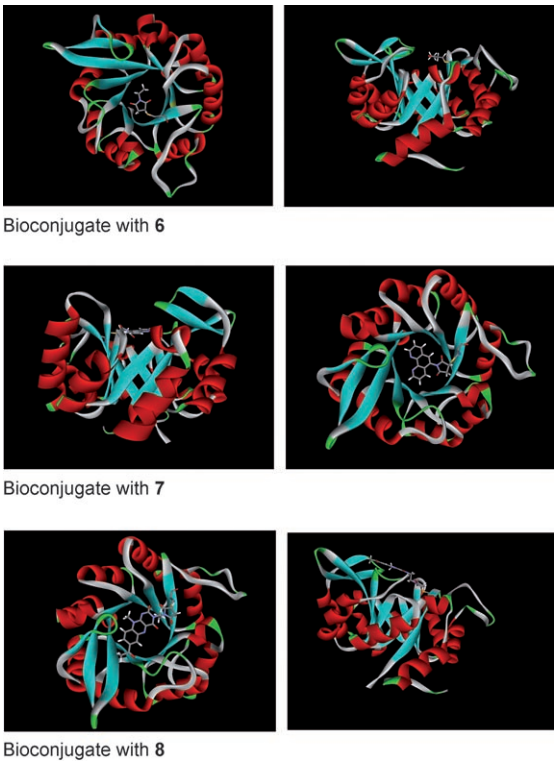


Figure 3. Bioconjugates made by treating mutant Cys9 Ala/Asp11 Cys with Michael acceptors **6**, **7**, and **8**. The putative structures were generated by manual docking of the respective ligands into the tHisF structure.

conjugates contain fairly space-filling organic residues in the natural binding pocket, whereas the simple maleimide-derived analogue leads to a sterically less crowded environment. As no significant effects on the catalytic profile of the hydrolytic reaction were observed, we conclude that promiscuity is not likely to occur in the binding pocket used by the enzyme in the natural cyclization reaction, which means that alternate-site enzyme promiscuity must be operating.[17]

In conclusion, we have discovered the first example of a promiscuous enzyme-catalyzed reaction that does not involve any of the catalytic amino acids of the natural enzymatic process, nor does it appear to occur in the natural binding pocket. Further work is required to identify the actual active center. The present results are significant because, among other things, they raise the question of whether promiscuity as observed for example in the degradation of man-made chemicals evolves in nature solely by mutations at the original active site of the enzyme, as assumed and observed so far.[1–11] This raises a more general question, namely whether nature utilizes, in select cases, alternate-site promiscuity to endow an organism with an advantage for its survival and further evolution.[18]

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[17] It is interesting to note that tHisF has been shown to display significant catalytic activity in a reaction catalyzed by a related enzyme HisA (conversion of *N*′-[5′-phosphoribosyl)-formimino]-5-aminoimidazol-4-carboxamide ribonucleotide into 5′-phosphoribulosyl isomer).[12c] This promiscuous reaction was demonstrated unequivocally to occur at the natural active site of tHisF (upper rim), which is fundamentally different from our finding.

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