Incorporation of a single His residue by rational design enables thiol-ester hydrolysis by human glutathione transferase A1-1

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A strategy for rational enzyme design is reported and illustrated by the engineering of a protein catalyst for thiol-ester hydrolysis. Five mutants of human glutathione (GSH; γ-Glu-Cys-Gly) transferase A1-1 were designed in the search for a catalyst and to provide a set of proteins from which the reaction mechanism could be elucidated. The single mutant A216H catalyzes the hydrolysis of the S-benzoyl ester of GSH under turnover conditions with a kcat/KM of 156 M⁻¹·min⁻¹, and a catalytic proficiency of >10⁷ M⁻¹·s⁻¹ when compared with the first-order rate constant of the uncatalyzed reaction. The wild-type enzyme did not hydrolyze the substrate, and thus, the introduction of a single histidine residue transformed the wild-type enzyme into a turnover system for thiol-ester hydrolysis. By kinetic analysis of single, double, and triple mutants, as well as from studies of reaction products, it was established that the enzyme A216H catalyzes the hydrolysis of the thiol-ester substrate by a mechanism that includes an acyl intermediate at the side chain of Y9. Kinetic measurements and the crystal structure of the A216H GSH complex provided compelling evidence that H216 acts as a general-base catalyst. The introduction of a single His residue into human GSH transferase A1-1 created an unprecedented enzymatic function, suggesting a strategy that may be of broad applicability in the design of new enzymes. The protein catalyst has the hallmarks of a native enzyme and is expected to catalyze various hydrolytic, as well as transesterification, reactions.

The quest for new enzymes extends the boundaries of our understanding of catalysis and protein structure and is expected, when successful, to generate new biocatalysts for reactions that are not catalyzed by nature (1–3). It allows for unprecedented opportunities in chemistry, but the implementation of nonnative catalytic functions in protein scaffolds remains a challenge. The redesign of protein macromolecules provides a powerful strategy for engineering nonnatural reactive sites and determining structure–function relationships. For example, the functional swapping between native enzymes has been instructive (4–8), and new catalytic activities have been introduced by chemical modification of amino acid side chains (9). In addition, the incorporation of amino acid residues in the active sites of native enzymes has been used to alter the fate of the natural substrates or inhibitors (10–14). However, although it is possible in principle to modify the active site of any native enzyme to generate nonnatural activity, this approach has met with only limited success because of the difficulties involved in predicting the effect of sequence modifications on structure and function. Catalytic antibodies based on the properties of the immune system in generating binding sites that are complementary to transition-state analogues have been shown to be efficient catalysts for numerous chemical reactions (15–16). Their catalytic efficiencies, based mainly on transition-state stabilization, have been shown to be comparable with those at the lower end of naturally occurring enzymes, and they may improve further as our understanding of how to design transition-state analogues develops (17). The selection of catalysts from a library of designed amino acid sequences was shown to provide enzyme-like proteins with moderate catalytic power in the hydrolysis of active esters (18). Tremendous progress in computational methodology has been demonstrated in the construction of an enzyme-like catalyst for p-nitrophenyl-ester hydrolysis (19) and in the construction of an enzyme with triose phosphate isomerase activity (20) by using folded protein scaffolds as the starting points for design.

Here, we report on an approach to enzyme design that is independent of the roles of the original active-site residues in chemical transformations. It exploits the binding affinity of enzymes for their natural substrates only to position nonnatural substrates precisely in reactive sites tailored to provide efficient catalysis by a minimum of modifications. Catalytic efficiency in protein scaffolds arises from cooperation between residues that are ubiquitous in every protein scaffold. Incomplete reactive sites with latent catalytic potential are likely to exist by coincidence or as parts of active sites for other reactions into which minimal catalytic units, selected for maximum impact, could be introduced to activate unprecedented functions. General-acid, general-base, or nucleophilic catalysis by His residues in their reactions with active esters are likely to exist by coincidence or as parts of active sites for other reactions into which minimal catalytic units, selected for maximum impact, could be introduced to activate unprecedent functions. General-acid, general-base, or nucleophilic catalysis by His residues in their reactions with active esters. The incorporation of a His residue into a protein scaffold in which its mobility was restrained was expected to raise its catalytic power. The combination of effects on pKₐ values by the hydrophobic pocket and proximity effects due to substrate binding could give rise to a functional catalyst with enzyme-like properties. Human GST A1-1 is an enzyme that rids the body of electrophilic toxic compounds by conjugating them to glutathione (GSH; γ-Glu-Cys-Gly) (22). The hydrophobic substrate-binding site (H site) binds the electrophilic substrate, which is shielded from solvent by a flexible helical segment that is stabilized when GSH is bound in the GSH-binding site (G site) (23). We found evidence that GST A1-1 would readily accommodate modifications and, thus, be a

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Abbreviations: GSB, S-glutathionyl benzoate; GSH, glutathione.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB code 1USB for A216H S-benzylglutathione complex).

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carried out by using a Cary-100 UV-visible spectrophotometer. All UV spectroscopic measurements, with one exception, were performed at pH 7 and 298 K. Saturation kinetics were run with a concentration of A216H of 5 μM and GSB concentrations in the range of 5–80 μM. The results were analyzed by using the Michaelis–Menten equation in the form in which no assumption has been made that $[E] << [S]$. The buffers used to record the pH profile were 100 mM sodium acetate (pH 5–5.6) and 100 mM sodium phosphate (pH 5.7–8). Reaction rates were determined by monitoring the concentration of GSB spectrophotometrically at 266 nm. In the trapping of the acyl intermediate, various concentrations of MeOH (0–1 M) were mixed with A216H or wild type (final concentration 5 μM) in 100 mM sodium phosphate (pH 7), and GSB was added to a final concentration of 75 μM. The reactions were followed spectrophotometrically at 266 nm and 298 K. The reaction product methylbenzoate was identified by reverse-phase HPLC. Typical measurements for the saturation-kinetics plot and the pH-dependence determination were carried out in duplicate, with maximum deviations from the mean value of <10%. The rate constants in the presence of MeOH were estimated from single measurements only. Liberated GSH eventually reacts with a solvent exposed Cys residue to form a GSH–GST conjugate by disulfide formation. Such modifications have been shown not to affect the catalytic activity of human GST A1-1 (30).

**Synthesis of GSB.** GSH was prepared by solid-phase peptide synthesis using standard 9-fluorenylmethoxycarbonyl (Fmoc) protocols and Fmoc-Gly-Wang resin (0.7-mmol scale) as described (29). Cys was orthogonally protected with a 4-methoxytrityl group, and the thiol was selectively deprotected with 1% trifluoroacetic acid in dichloromethane to enable thiol-ester coupling with benzoic acid. The coupling was performed in dimethylformamide using benzoic acid/1-hydroxybenzotriazole/\(N,N'\)-disopropylcarbodiimide/disopropylethylamine (2.3:3:5 dilution). Global deprotection and cleavage from the solid support was enabled by trifluoroacetic acid/H\(_2\)O (97.5:2.5 dilution), followed by precipitation of the peptide with diethyl ether. GSB was purified by reverse-phase HPLC (29). GSB concentrations were determined spectrophotometrically by using an extinction coefficient of \(\varepsilon_{266} = 7,889\ M^{-1}cm^{-1}\).

**Kinetic Measurements.** Kinetic measurements with the exception of measurements used to determine saturation-kinetics parameters were performed at protein concentrations of 5 or 25 μM and a GSB concentration of 75 μM in 100 mM sodium phosphate at pH 7 and 298 K. Reaction rates were determined by monitoring the concentration of GSB spectrophotometrically at 266 nm. The chemical shifts of the ring protons of benzoic acid (7.47, 7.54, and 7.86 ppm) and of those of GSB (7.56, 7.71, and 7.99 ppm) were determined in independent experiments.

**Product Identification.** The benzoic acid produced in the hydrolysis reaction was identified under turnover conditions by reverse-phase HPLC and by NMR spectroscopy (Inova-600, Varian). The NMR experiments were carried out at 600 MHz and 298 K by using 10% D\(_2\)O in 90% H\(_2\)O as the solvent. The chemical shifts of the ring protons of benzoic acid (7.47, 7.54, and 7.86 ppm) and of those of GSB (7.56, 7.71, and 7.99 ppm) were determined in independent experiments.

**Background Hydrolysis.** N-acetylated GSB was synthesized by mixing 75 μM GSB and excess N-hydroxysuccinimide acetate in 100 mM sodium phosphate (pH 7). The mixture was incubated at 298 K,
and aliquots were collected for 14 days. The concentrations of remaining substrate and hydrolysis products were analyzed by reverse-phase HPLC.

The Determination of the \( pK_a \) of Y9. The \( pK_a \) of Y9 in the A216H and F220H mutants was determined by difference UV spectroscopy at 298 K using buffers in the range of pH 5–9 as described (31). The protein concentration was 15 \( \mu M \), and spectra were recorded at 220–360 nm. The spectrum at pH 5.5 was subtracted from the other spectra, and the \( pK_a \) of Y9 was calculated by plotting the values of the peak arising at 250 nm against pH.

Digestion Experiments. GST variants from reaction mixtures were digested by trypsin or by Staphylococcus aureus protease V8 (Pierce). We digested 20 \( \mu M \) wild type or A216H mixed with 40 \( \mu M \) GSB by trypsin for 3 h in 100 mM \( \text{NH}_2\text{HCO}_3 \) (pH 8) at ambient temperature with a protein/trypsin ratio of 100:1. In the case of protease V8, 10 \( \mu M \) wild type or A216H was mixed with 20 \( \mu M \) GSB and digested in 50 mM \( \text{NH}_2\text{H}_2\text{COO} \) (pH 4) at 310 K for 3 h with a protein/protease ratio of 40:1. The digests were desalted with ZipTips (C-18, Millipore) and analyzed by matrix-assisted laser desorption ionization–time of flight MS (Voyager 4212, Applied Biosystems) by using \( \alpha \)-cyano-4-hydroxycinnamic acid matrix with detection in the positive mode.

Results and Discussion

The Single Mutant A216H Is an Enzyme. The GST A1-1 mutant A216H catalyzed the hydrolysis of GSB (5 \( \mu M \) A216H/75 \( \mu M \) GSB/100 mM sodium phosphate, pH 7, 298 K) to form GSH and benzoic acid, as demonstrated by HPLC and NMR spectroscopy under turnover conditions. The transformation of GSB into benzoic acid and GSH was readily followed by monitoring the gradual appearance of the resonances of the aromatic-ring protons of benzoic acid and the gradual disappearance of the ring protons of GSB, as well as by HPLC. The reaction followed saturation kinetics with a \( k_{cat}/K_M \) of 156 M\(^{-1}\)min\(^{-1}\) (\( k_{cat} = 7.8 \times 10^{-4}\)min\(^{-1}\), \( K_M = 5 \mu M \), Fig. 2). In contrast, the wild-type protein did not catalyze the hydrolysis of GSB but consumed one equivalent of GSB, without release of benzoic acid, as demonstrated by HPLC under reaction conditions. The incorporation of a single amino acid residue by rational design had, thus, converted the wild-type protein into a turnover system for thiol-ester hydrolysis.

The first-order rate constant of the uncatalyzed reaction was 1.1 \( \times 10^{-5}\)min\(^{-1}\) (100 mM phosphate, pH 7, 298 K) and the observed catalytic efficiency (32) (\( k_{cat}/K_M \)) of the A216H-catalyzed reaction was, thus, >10\(^7\) M\(^{-1}\)\text{min}^{-1}. A catalytic efficiency \( k_{cat}/K_M \) of almost two orders of magnitude, in addition to the proximity effect provided by the enzyme substrate binding, accounted for the observed catalysis. The mutant A216H provides a rare example of a successful incorporation of a chemical function based on the common amino acid residues into a protein scaffold, resulting in a new enzyme with non-natural activity. Because of the competing intramolecular acyl transfer reaction of GSB to form N-acylated GSH, N-acylated GSB was used as the substrate to determine the rate of the uncatalyzed background reaction, but N-acylation was not expected to affect the rate of thiol-ester hydrolysis. Although evolutionary pressure has made it unlikely that single-point mutations can lead to enhanced efficiencies in natural enzymes catalyzing their natural reactions, these results show that single mutations can lead to altered chemical functions, suggesting that searching for new chemistries, rather than new specificities, may be a viable route to new enzymes.

The Benzoate Ester Formed at the Side Chain of Y9 Is an Intermediate on the Reaction Pathway. The fate of the benzoyl group in the wild-type protein was determined by matrix-assisted laser desorption ionization–time of flight MS by subjecting the reaction mixture to cleavage by trypsin or S. aureus protease V8 and comparing the molecular masses of the peptide fragments to those obtained in the absence of substrate. The fragment containing Y9 (residues 7–13 for trypsin) was readily observed in the wild-type enzyme and was acylated to a large degree by benzoic acid under reaction conditions (29). Under the same conditions, A216H showed only minor acylation at the side chain of Y9. It appeared that, in the A216H-catalyzed reaction, the acylated form of Y9 was an intermediate that reacted further to form a reaction product. As a by-product, the released GSH was found by matrix-assisted laser desorption ionization–time of flight MS to form a disulfide bridge with C112, the only Cys residue in the GST A1-1 protein scaffold. Conjugation with GSH does not affect the enzymatic activity of human GSH transferase A1-1 (30).

The Mechanism of Hydrolysis of GSB. Key residues for thiol-esterase activity were identified by site-directed mutagenesis. F220H showed almost identical behavior to that of the wild type and consumed one equivalent of substrate but did not turnover. The double mutant A216HF220H had a catalytic efficiency that was approximately equal to that of A216H and demonstrated turnover kinetics, suggesting that H216 was the critical residue in both mutants. We concluded that H216 is essential for catalyzing the hydrolysis of the acyl intermediate and that H220 is inactive. The mutants Y9H and Y9F/A216H/F220H were inactive and remained unacylated, showing that Y9 also was essential for the catalytic function. Therefore, the roles of H216 and Y9 were further elucidated by determining their dependence on pH. Attempts to determine the \( pK_a \) values of H216 by NMR spectroscopy failed because of the lack of spectral assignments. The \( pK_a \) values of Y9 in the wild type (8.2 ± 0.1) had been determined in ref. 31, and the \( pK_a \) values of Y9 in A216H (7.8 ± 0.3) and F220H (7.6 ± 0.2) were determined by UV spectroscopy in a similar way and were found to be significantly lower than the \( pK_a \) of Tyr residues in small peptides (9.7) (33). The pH dependence of \( k_{cat}/K_M \) was determined for the A216H-catalyzed reaction (Fig. 3), and it suggested that the catalysis depended on a residue in its unprotonated form with a \( pK_a \) of ~6. Clearly, this
residue could not be Y9, but the observed pH dependence was instead in agreement with the expected pKₐ value of a His residue, although the identity of the catalytically active residue responsible for the observed pH profile has not been unequivocally established. The data suggest that the His residue is involved in the rate-limiting step.

Further mechanistic information about the His residue and the identity of the rate-limiting step was obtained from experiments that were designed to trap the reaction intermediate by MeOH (Fig. 4). The rate of the hydrolysis reaction catalyzed by A216H was increased by the addition of MeOH under conditions in which the structure of the enzyme was not adversely affected, as evidenced by tests of activity toward the standard GST substrate 1-chloro-2,4-dinitrobenzene. Methylbenzoate, the reaction product obtained upon MeOH trapping, was identified by HPLC in the A216H-catalyzed reaction, whereas no methylbenzoate was observed with the wild-type enzyme. Therefore, the acyl intermediate is on the reaction pathway and the breakdown is the rate-limiting step.

The most likely mechanism of the A216H-catalyzed reaction enabled by the introduction of H216 included the formation of a Michaelis complex between GSB and the enzyme, acylation of Y9, and hydrolysis of the acyl intermediate. In the wild-type reaction, Y9 was terminally acylated because the leaving group was not reactive enough to ensure turnover by spontaneous hydrolysis. In contrast, in the A216H-catalyzed reaction, the His residue catalyzed the deacylation of Y9 to form benzoic acid with regeneration of the free enzyme. The pH dependence excluded the possibility that His is a general-acid catalyst, but whether it catalyzes the breakdown of the intermediate by nucleophilic or general-base catalysis could not be determined based on the kinetic data. A crystal structure was determined of the complex between A216H and S-benzylglutathione to 2.1 Å resolution (see supporting information, which is published on the PNAS web site), to estimate whether the His residue was close enough to the carbonyl carbon of the benzoyl residue of the acyl intermediate to form a covalent bond, or whether a water molecule would have to be inserted in between the His nitrogen atom and the carbonyl carbon (Fig. 5). The observed distance in the complex (7 Å between the Tyr oxygen and the His nitrogen, N/H₉₂₅₅) suggests general-base catalysis, but because it is not a representation of the structure of the true enzyme–substrate complex, it does not constitute rigorous proof. Nucleophilic catalysis by His residues is uncommon in native enzymes, probably because the efficiency in nucleophilic catalysis decreases with the difference in pKₐ between the leaving group and the nucleophile, and realistic substrates have poorly reactive leaving groups in comparison with His. However, in the present case, the pKₐ of Y9 has been reduced in the binding pocket to a level at which it most likely is comparable with that of H216, and therefore, Y9 can, in principle, be efficiently expelled by...
Thiol-ester hydrolytic activity was achieved by the introduction of MeOH enhances the reaction rate but only if the His benzoate is higher in free energy than the Tyr benzoate, which is improbable because the pKₐ of H216 is most likely <7.8 (the pKₐ of Y9). Although no definitive proof has been obtained, the available evidence supports a reaction mechanism in which H216 acts as a general-base catalyst. It is noteworthy that the attack by MeOH appears also to be catalyzed by general base because no methylbenzoate is formed in the reaction with the wild-type enzyme. The crystal structure reveals further that the distance between the phenolic oxygen of Y9 and the H216 acts as a general-base catalyst. It is noteworthy that the His benzoate is higher in free energy than the Tyr benzoate, although it was required for catalysis, and it was concluded that the most likely function of Y9 in rat GST was to act as a general acid or general base in the hydrolysis of glutathionyl ethacrynate but by a different reaction mechanism from the one introduced here (34). There was no detectable acylation of Y9, although it was required for catalysis, and it was concluded that the most likely function of Y9 in rat GST was to act as a general acid or general base in the hydrolysis of the bound substrate. The GSH thiol ester of ethacrynic acid is the case for A216H, Y9 does not appear to function as a nucleophilic catalyst in any of them.

Conclusion
Thiol-ester hydrolytic activity was achieved by the introduction of a single His residue in the active site of GST A1-1 that enabled a new reaction pathway from the irreversibly acylated Y9. It is likely that dead-end reaction pathways similar to that of the wild-type GST A 1–1 exist in other natural proteins and can be accessed by minimal modifications to the protein scaffold to engineer enzymes with nonnatural functions. The limited invasion of the folded protein is expected to minimize the risk for structural rearrangements and to increase the probability that rational incorporation of new catalytic functions will be successful. The affinity of GST A1-1 for GSH, the natural substrate of GST A1-1, was exploited in binding GSB in a position in which Y9 could attack the carbonyl carbon to form the acyl intermediate that was subsequently hydrolyzed by H216. The covalent binding of the acyl intermediate in a promiscuous enzyme such as GST suggests that various nucleophiles can be used to trap a multiplicity of acyl groups and form a catalytic machinery for alternative chemical transformations.

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