Directed Evolution of the Promiscuous Esterase Activity of Carbonic Anhydrase II[†]

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ABSTRACT: A promiscuous activity of an existing enzyme can confer an evolutionary advantage by providing an immediate response to a new selection pressure and a starting point for the divergence of a new enzyme. This work seeks to examine how this process might take place. Human carbonic anhydrase II (hCAII) is an enzyme that evolved to catalyze the reversible hydration of CO_2 and performs this task at a remarkable rate ($k_{cat} \approx 10^6$ s^{-1}). hCAII also exhibits promiscuous activity toward highly activated esters such as 4-nitrophenyl acetate. We describe a much weaker esterase activity of hCAII toward the bulkier and much less activated ester substrate 2-naphthyl acetate (2NA). Directed evolution of hCAII produced a variant with 40-fold higher rates toward 2NA, owing to two mutations: one within the active site (Ala65Val) and one at its mouth (Thr200Ala). Structure−activity studies suggest that these mutations led to adaptation of the active site for bulkier substrates and for the catalysis of nonactivated esters. The mutations did not, however, significantly alter the native activity of hCAII. Our results support the notion that the evolution of a new function can be driven by mutations that increase a promiscuous function (which serves as the starting point for the evolutionary process) but do not harm the native function.

There is increasing evidence that the ability of proteins to act upon targets additional to those involved in their physiological role is a common feature (*1*, *2*, *3*). Such behavior has been described as "promiscuous", although a variety of other terms are being used [cross-reactivity, multispecificity, substrate ambiguity, or moonlighting (*4*)]. There is no doubt that certain breaches of specificity can exert a deleterious effect on function and would be therefore ultimately selected against. Nevertheless, there is a clear evolutionary advantage to a degree of promiscuity; by conferring upon the individual, at the *phenotypic* level, the advantage of adaptability enjoyed by the population at the *genotypic* level. However, while it is generally accepted that genotypic diversity confers adaptability upon a population, it remains controversial that the functional diversity of a single protein may play a similar role. It is our belief that protein promiscuity is a crucial phenotypic starting point for evolution because it allows organisms to respond rapidly to new selective pressures. This gives time for the longer process of gene duplication and divergence to bring a vestigial promiscuous activity to a stage where it becomes the native activity of a new protein.

For a new protein to evolve, each intermediate along the evolutionary pathway must be active. Of equal importance is the fate of the original activity, because this activity may be as essential for the survival of the organism as the evolving promiscuous function. One possibility is to protect the original activity via a gene duplication event, allowing mutation and selection to apply to only one copy of the gene. Indeed, gene duplication is known to occur under selection pressures for higher enzymatic activity, for example, in the presence of inhibitors (*5*). However, the short-term mutational events that lead to the preservation and divergence of duplicated genes are as yet unclear. Ohno's original model of gene duplication (*6*) assumed a completely asymmetric evolutionary trajectory where the ancestral gene maintains the original function, while its copy diverges toward a new function. However, challenges to this view have emerged, and alternative models have been suggested (*7*). A theory informed by work carried out in this lab is that the original activity is resistant to mutations that, under selection, dramatically increase promiscuous activity (*8*). This model allows an apparent multiplication of function to occur and for gene duplication and diversification of function to take place in parallel or even at a later stage (*9*).

We have sought to support the above theories by laboratory evolution of a variety of promiscuous activities exhibited by several enzymes, including the esterase activity of human carbonic anhydrase II (hCAII)[1] (preliminary results from this study were described in ref *8*). hCAII is one member of a large family of ubiquitous zinc metalloenzymes whose physiological role is to catalyze the reversible hydration of carbon dioxide. The structure of hCAII has been solved in isolation and complexed with a variety of inhibitors and activators (*10*, *11*, *12*), allowing a good deal of rational design-based investigations (*13*, *14*).

hCAII is one of the most proficient enzymes known, exhibiting a k_{cat} value of around 10^6 turnovers per second and a k_{cat}/K_M value close to the diffusion limit ($\sim 10^8$ s^{-1} M^{-1}; *14*). Catalysis takes place in a 15-Å wide by 15-Å deep

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[1] Abbreviations: 2NA, 2-naphthyl acetate; hCAII, human carbonic anhydrase II; PNPA, 4-nitrophenyl acetate; LFER, linear free-energy relationship; WT, wild type; NEB, New England Biolabs; DANS, dansylamide.

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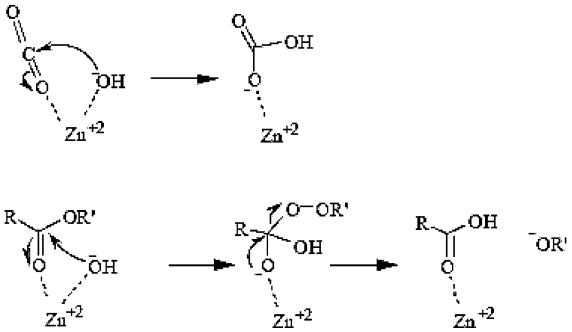
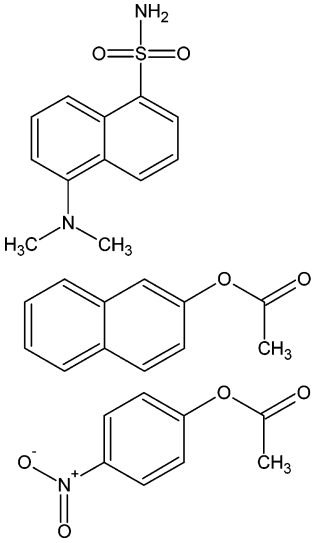
Scheme 1: Mechanistic Similarity between the Hydration of CO_2 (Native Activity of CAII) and the Promiscuous Esterase Activity[a]



[a] Both reactions proceed by OH^- nucleophilic attack and an oxyanionic product or intermediate.

Chart 1: Substrates and Inhibitors Used in the Study



Dansylamide (DANS)

2-naphthylacetate (2-NA)

p-nitrophenylacetate (PNPA)

hydrophobic pocket that can accommodate ligands much larger than CO_2, including a range of aromatic inhibitors. hCAII also has the ability to catalyze the hydrolysis of activated esters such as 4-nitrophenyl acetate (PNPA) (*16*). This promiscuous esterase activity probably stems from the mechanistic similarity between hydration of the carbonyl of CO_2 and that of an ester (i.e., nucleophilic attack by a zinc-coordinated OH^- ion and the stabilization of the resulting oxyanionic intermediate, Scheme 1). Indeed, a wealth of zinc metallohydrolases with catalytic centers similar to CAII are known and include amidases and peptidases (*17* and references therein). Nevertheless, the steric and electronic natures of CO_2 and PNPA do differ significantly (Chart 1), as well as the respective intermediates and transition states that lead to and from them. This gives rise to a difference of ca. 10^5-fold between the rate of hydration of the native substrate (CO_2; $k_{cat}/K_M \approx 10^8\ s^{-1}\ M^{-1}$) and the promiscuous hydrolysis of PNPA ($k_{cat}/K_M \approx 10^3\ s^{-1}\ M^{-1}$) (*16*). Moreover, catalysis of the hydrolysis of activated esters such as PNPA, with leaving groups of pK_a around 7, is not a demanding task because the rate of base-catalyzed hydrolysis is inherently fast and limited by the rate of hydroxide attack and not by leaving-group departure (*18*). Whether esters that are non-activated or less activated than PNPA are substrates for CAII has not been explored previously. Here, we describe the linear free-energy relationship (LFER) of hCAII with a series of ester substrates whose reactivities fall as leaving-group pK_a values (pK_a^{LG}) increase from 7.14 (PNPA) to 10.3. We found that the wild-type (WT) enzyme's active-site microenvironment is in fact significantly less tolerant to the negative charge that accompanies the transition states of the hydroxide-catalyzed hydrolysis of substrates with high pK_a leaving groups ($\beta^{LG} = -0.68$) than bulk aqueous solvent ($\beta^{LG} \approx -0.3$; *19*).

We then used directed evolution to increase the reactivity of hCAII toward the nonactivated ester substrate 2NA, rather than the rational design approach that has been previously applied with this enzyme (e.g., refs *13*, *14*, *20*, and *21*). We selected libraries of the hCAII gene for increased hydrolytic activity with 2NA, a bulky substrate with a pK_a^{LG} of 9.51, which produces a k_{cat}/K_M (ca. 25 s^{-1} M^{-1}) that is approximately 80 times lower than that with PNPA. Three rounds of mutation, selection, and recombination led to a 40-fold improvement in esterase activity toward 2NA, while the effect on the native carbonic anhydrase activity of the enzyme was minimal. The improved variant exhibited an increased affinity for dansylamide (DANS, an inhibitor containing a naphthyl moiety; Chart 1) compared to WT (WT $K_d^{DANS} = 0.8$ versus 0.03 μM for the 40-fold evolved variant), suggesting that the source of the improvement was primarily due to an enhanced ability to accommodate bulkier ligands.

MATERIALS AND METHODS

Libraries Construction: (i) Round 1 Libraries. The hCAII gene was amplified from human foetal cDNA (Clontech) using primers CAIIf and CAIIr (appending *Nde*I and *Not*I restriction sites, respectively; Table 1). The product of the amplification was digested with *Not*I and *Nde*I (NEB) and ligated into the similarly digested cloning vector pET20b (Novagen) by incubating overnight with T4 DNA ligase (NEB) at 16 °C. This plasmid was used as a template for amplifications by biased error-prone PCR (*22*) using primers T7f and T7r (Table 1). Random mutagenesis was obtained by amplification in the presence of dNTP biases of 4:1, 10:1, and 20:1 TG/CA (C and A concentrations were each maintained at 0.2 mM) and manganese chloride (0.05 mM). The original plasmid template was subsequently removed by *Dpn*I digestion (NEB). The amplified fragments were diluted and re-amplified by nested PCR using the primers CAIIf and CAIIr, digested with *Not*I and *Nde*I, and cloned into precut and dephosphorylated pET20b(+) (Novagen). The ligated plasmids were transformed into DH5α cells. Plasmid DNA was extracted from the pooled colonies ($\geq 10^4$ transformants were obtained for each library). The PCR conditions, mutation rate, percent transitions, and percent nonsynonymous mutations are presented in Table 2.

(ii) Round 2 Libraries. The variants isolated from screening of round 1 libraries were subjected to DNA shuffling (*23*). In brief, the genes were PCR-amplified and a total of 10 μg was digested with DNAseI (NEB). DNA fragments of 50 ± 25 bp were gel-purified (Qiagen) and assembled by PCR without any primers, employing 35 cycles of melting at 95 °C for 30 s, annealing at 65 °C falling 3 °C every 90 s until 41 °C was reached, and then extension at 72 °C for 90 s. The assembled genes were amplified by nested PCR, cloned, and transformed, and the resulting plasmids were extracted as described above.

Table 1: Oligonucleotides Used in the Study

primer name	sequence (5′−3′)[a]
T7f	AACCCCGCCAGCCTAG
T7r	AAGAAAGCGAAAGGAGCGGG
CAIIf	GAGATATA**CATATG**ATGTCCCATCACTGGGGGTAC
CAIIr	CGAGCTCA**CGCCGGCG**AATAATAAACTTCCTTCGAAACTAAACG
A65F	G**T**TTTCAACGTGGAGTTTG
A65R	ATGACCATTGTTGAGGATCC

[a] Noted in bold are the appended restriction and mutation sites.

Table 2: Gene Libraries Used in This Study[a]

dNTP bias in PCR (TG/CA)	mutation rate (mutations/kbp)	transitions (%)	synonymous mutations (%)
4:1	1.9	83.3	30.2
10:1	5.1	85.7	25.0
20:1	9.8	87.5	28.0

[a] Library analysis was based on sequencing data from five randomly selected clones.

(iii) Round 3 Libraries. Clone M2, isolated from screening of the round 2 library, was subjected to a further round of diversification by error-prone PCR under the same conditions as round 1, producing two libraries, one because of a 20:1 bias and the other because of a 10:1 bias. The mutated genes were amplified and cloned, and the resulting plasmids were extracted as described above.

Screening for Esterase Activity. Escherichia coli "Origami" (DE3, Novagen) cells were transformed with the libraries, grown overnight at 37 °C, and duplicated with velvet cloth. One replica plate was overlaid with soft agar (0.5%) in an activity buffer (50 mM Tris at pH 8.6 and 0.5 mM $ZnSO_4$) supplemented with 2NA (0.3 mM) and fast red (1.3 mg mL^{-1}, Sigma). Duplicated colonies corresponding to those exhibiting a red color were picked from the replica plate into 500 μL of LB/Amp (100 μg mL^{-1})/$ZnSO_4$ (0.5 mM) in a 96-deep-well plate (Nunc) and grown overnight at 37 °C. Aliquots (20 μL) were removed from the plates and stored at 4 °C, and the remaining cells were lysed with BugBuster (200 μL, Novagen). The lysates were clarified by centrifugation and assayed for 2NA (50 μL of lysate and 0.4 mM 2NA) and PNPA (10 μL of lysate and 0.4 mM PNPA) hydrolysis in 96-well plates (Nunc). Variants exhibiting higher activity were subcloned, grown in 1.5 mL LB/Amp (100 μg mL^{-1})/$ZnSO_4$ (0.5 mM), and their lysates were re-assayed for esterase activity. Plasmids were extracted from the improved variants and used as templates for further rounds of library construction and screening.

Expression and Purification of WT and Mutant CAII. A single colony was inoculated into LB (5 mL) and grown overnight at 37 °C. An aliquot (1 mL) of this culture was added to LB/Amp/$ZnSO_4$ (1000 mL) and grown overnight at 37 °C. Cells were harvested by centrifugation, resuspended in activity buffer supplemented with benzonase (Novagen), and lysed by sonication. Cell debris was removed by centrifugation, and the supernatant was incubated at 4 °C for 1 h with 10% (v/v) agarose-coupled 4-aminomethylbenzene sulfonamide (Sigma), producing agarose-bound enzyme. The slurry was loaded onto an econo-column (BioRad) and washed first with activity buffer and then with wash buffer (50 mM Na_2SO_4, 50 mM $NaClO_4$, and 25 mM Tris at pH 8.8). CAII was eluted from the column by the addition of the elution buffer (200 mM $NaClO_4$ and 100 mM NaAc at pH 5.6). The eluted fractions were analyzed for esterase activity, dialyzed extensively against activity buffer, and then concentrated where necessary.

Enzyme Kinetics. 2NA and PNPA were purchased from Sigma. The synthesis and characterization of the other aryl esters will be described elsewhere (*24* and references therein). Assays for esterase activity were carried out in 0.2 mL volumes in 96-well plates (Nunc) using a variety of enzyme (70 nM−30 μM) and substrate (0.05−5 mM) concentrations. Initial rates of product release were monitored at 405 nm (PNPA) or 320 nm (2NA) (extinction coefficients at pH 8.8 were 10 510 and 900 OD M^{-1}, respectively). Where substrate solubility was allowed (solubility range was 0.3 → 5 mM), kinetic parameters were determined by fitting data to the Michaelis−Menten model (eq 1)

$$V_o = \frac{k_{cat}[E]_0[S]_0}{[S]_0 + K_M} \quad (1)$$

Otherwise, k_{cat}/K_M values were derived from a linear fit (eq 2)

$$V_o = [E]_0[S]_0(k_{cat}/K_M) \quad (2)$$

The rates of spontaneous hydrolysis (with no enzyme) were subtracted from the enzymatic initial rates. The rate of bicarbonate dehydration was measured in an Applied Photophysics stopped-flow apparatus by the changing indicator method as described by Khalifah (*25*). The reactions were followed at 400 nm and carried out in MOPS buffer (50 mM) using 4-nitrophenol (0.2 mM) to indicate enzymatic (25 nM) dehydration of $NaHCO_3$ (1.25−200 mM). Catalytic parameters were derived from data fit to eq 1.

Affinity Constants (K_d) for DANS. The measurement of the affinity constants (K_d) for DANS was carried out by adding an increasing amount of hCAII (approximately 0.07 μM per aliquot) to a solution of DANS (0.25 μM). The data were fit directly to an equilibrium model (K_d = [DANS]-[hCAII]/[hCAII−DANS], where [DANS] and [hCAII] are the concentration of the free (unbound) ligand and enzyme, respectively, and [hCAII−DANS] is the concentration of the complex) using eq 3 (adapted from ref *26*)

$$F = 1 - \frac{F_a - F_{min}}{F_{max} - F_{min}} = F_{min} - F_{max}\left((0.25 + [E]_o + K_d) - \sqrt{((0.25 + [E]_o + K_d)^2) - (4 \times 0.25[E]_o)}\right) / 2 \quad (3)$$

where 0.25 = DANS concentration in micromolars, $[E]_o$ = hCAII concentration in micromolars, K_d = the dissociation constant for DANS in micromolars, F is the observed fluor-

Table 3: Kinetic Parameters of WT hCAII and Variants M2, M3, MD, and MT

variant	2NA k_{cat}/K_M (M^{-1} s^{-1})	2NA × WT	PNPA k_{cat}/K_M (M^{-1} s^{-1})	PNPA/2NA	HCO_3^- k_{cat}/K_M (M^{-1} s^{-1})	HCO_3^- K_M (mM)	K_d^{DANS} (μM)	mutations
WT	25 ± 5	1	2050 ± 160	82	2.8×10^7	28.6 ± 3.5	0.82 ± 0.072	
M2	250 ± 20	10	7080 ± 250	28.3	2.06×10^7	50.2 ± 3.4	0.27 ± 0.045	Asp110Asn, Thr200Ala
M3	975 ± 120	39	8610 ± 370	8.8	1.33×10^7	50 ± 4.2	0.032 ± 0.01	Ala65Val, Asp110Asn, Thr200Ala
MD	20 ± 4	0.8	1690 ± 180	102			0.45 ± 0.066	Asp110Asn
MT	250 ± 25	10	6920 ± 320	27.7			0.072 ± 0.012	Thr200Ala
MAV	70 ± 6	2.8	9000 ± 440	128.6			0.12 ± 0.03	Ala65Val

escence, F_a is the fluorescence at a given enzyme concentration, and F_{max} and F_{min} are, respectively, the fluorescence values at $[E]_o = 0$ and at saturating $[E]_o$ concentrations.

Creation of Single Mutants. The single mutants (Asp110Asn and Thr200Ala) were constructed by crossing the newly evolved variant M2 with WT hCAII. Two *Pst*I sites were identified, one at position 349 of the hCAII gene and the other at position 2526 of the pET20b plasmid (bisecting the ampicillin resistance gene). *Pst*I digestion of pET20b::M2 produced two DNA fragments (self-ligation of which would produce ampicillin-sensitive clones), one containing amino acid 110 of hCAII and the other containing amino acid 200. Each fragment was ligated with its WT partner, cloned, and sequenced and the protein was purified as described above.

The single mutant Ala65Val was produced using primers A65F and A65R (Table 1) in a "whole plasmid" PCR reaction. The primers (40 pmol μL^{-1}) were prephosphorylated in a 20 μL reaction by incubating at 37 °C for 1 h with ATP (1.25 mM), T4 PNK enzyme (NEB, 1 μL) and 10× T4 ligase buffer (NEB, 2 μL). Phoshphorylation was stopped by heat denaturation at 65 °C for 20 min. WT plasmid template was amplified with the appropriate primers using pfu Turbo DNA polymerase (Stratagene) according to the protocol of the manufacturer. Template DNA was removed by digestion with *Dpn*I (NEB) at 37 °C for 1 h. The amplified fragment was self-ligated, cloned, and purified as described above.

RESULTS

Directed Evolution. Random genetic diversity was created by error-prone PCR amplification of the hCAII gene under varying conditions, producing the *round 1* libraries (Table 2). Sequencing of randomly chosen clones from these libraries indicated an average mutation rate between one and seven mutations per gene. Between 10^3 and 10^4 colonies from each library were screened for 2NAase activity. Screening was based on the conversion of one of the products (2-naphthol) into an insoluble red dye (*27*). The activity of WT hCAII ($k_{cat}/K_M^{2NA} = 25$ M^{-1} s^{-1}) was below the detection limit of this assay. Colonies that produced a red color (approximately 5% of the total) were picked from a replica plate and grown in liquid medium in 96-deep-well plates, and their activity was confirmed by assaying clarified lysates for hydrolysis of PNPA and 2NA. The 4:1 library (Table 2) produced no clones with a detectable improvement over the WT, whereas the 10:1 and 20:1 libraries gave variants exhibiting 2−4-fold improvements in 2NAase activity. Changes in the ratio of activities between PNPA and 2NA indicated that the improvements in activity were more likely to have arisen from changes in specific activity rather than increases in expression (in any case, the WT hCAII expressed to high levels under the assay conditions). Sequencing of a sample of these clones showed that a range of mutations had provided the improvements observed (Supplementary Table 1 in the Supporting Information). At this stage, the Thr200Ala mutation was present in two of the sequenced clones, most probably accompanied by deleterious companion mutations (Thr200 alone was later found to improve activity by 10-fold). Plasmids were extracted from 20 of the most active mutants, and the hCAII mutant genes therein were subjected to DNA shuffling, giving the *round 2* library. This library was screened as indicated above. On this occasion, approximately 50% of the colonies produced a red color with 2NA and fast red. To keep the number of clones tested to a manageable number, positives were denoted by both the depth of red color and the speed that it arose. This approach produced a variant (M2) which, when purified was found to be 10-fold more active than WT at 2NA hydrolysis. After DNA sequencing, the mutations Asp110Asn and Thr200Ala were identified.

Variant M2 was rediversified by error-prone PCR to give the *round 3* library that was screened as before. At this point, the dynamic range of the screen was exhausted, because the majority (>80%) of colonies produced a red color with 2NA and fast red. Nevertheless, selection of clones, in which color development was the fastest, and a wide screen of lysates in 96-well plates gave ultimately a mutant (M3) 39-fold more active than WT. Sequencing showed that the Thr200Ala and Asp110Asn mutations were retained alongside a new mutation of Ala65Val.

Characterization of Single Mutants. WT hCAII and its mutants, M2, M3, MD, MT, and MAV, were purified and characterized in detail (Table 3). The kinetic parameters are summarized in Table 3. The limited solubility of 2NA made it impossible to observe saturation; therefore, k_{cat}/K_M values only were determined. The k_{cat}/K_M values of 2NA, the target substrate for evolution, increased from 25 M^{-1} s^{-1} for WT to 250 M^{-1} s^{-1} for the M2 variant and 975 M^{-1} s^{-1} for the M3 variant. The rate of PNPA hydrolysis increased also but to a significantly lesser extent, improving only 3.5-fold for M2 and 4-fold for M3. The preference of PNPA over 2NA, therefore, decreased by about 10-fold, from ca. 80-fold in the WT enzyme to 8.8-fold in the M3 variant. Interestingly, assaying the rates of carbonate dehydration indicated that the native activity of the enzyme was comparatively unchanged, with there being little difference between WT and M2 and only a 2-fold reduction from WT to M3 (Table 3). Ultimately, a 40-fold increase in activity with the promiscuous substrate under selection (2NA) was accompanied by a modest improvement in a nonselected promiscuous activity and a minimal disruption of the physiological activity.

Table 4: Rates of Catalysis with Aryl Esters with Leaving Groups of Varying pK_a Values (Brønsted Analysis)

pK_a	substrate[a]	WT k_{cat}/K_M (M^{-1} s^{-1})	M3 k_{cat}/K_M (M^{-1} s^{-1})	M3/WT
10.29	4-methoxyphenyl acetate	5.3 ± 1	100 ± 8	19
10	phenylacetate	40 ± 4	620 ± 44	16
9.51	2-naphthyl acetate	25 ± 4	975 ± 120	40
9.38	4-chlorophenyl acetate	53 ± 3	1570 ± 70	30
9.28	3-fluorophenyl acetate	84 ± 5	1940 ± 100	23
8.61	3-cyanophenyl acetate	72 ± 4	2820 ± 150	39
8.47	4-methyl benzoate phenylacetate	130 ± 7	1690 ± 72	13
8.43	2,4-difluorophenyl acetate	133 ± 8	7970 ± 400	60
8.05	4-acetoxyphenone acetate	324 ± 16	7320 ± 375	23
7.95	4-cyanophenylacetate	210 ± 15	12 000 ± 560	57
7.81	2,3-difluorophenyl acetate	1140 ± 62	23 930 ± 1300	21
7.66	4-acetate hydroxybenzaldehyde	660 ± 30		
7.3	2,6-difluorophenyl acetate	770 ± 45	10 470 ± 480	14
7.14	4-nitrophenyl acetate	2050 ± 162	8610 ± 370	4

[a] The synthesis, characterization, and spectral properties of the above substrates and their respective phenol products will be described elsewhere (*24*).

Crossovers of the newly evolved variants with the WT gene revealed that the contribution of the Asp110Asn mutation to the improved variants M2 and M3 was apparently negligible, with the Asp110Asn mutation on its own (the MD variant) producing k_{cat}/K_M values for PNPA and 2NA that were indistinguishable from those of the WT. Furthermore, the Thr200Ala mutation in isolation (variant MT) exhibited the same improvements detected in the double mutant M2 (Table 3). Interestingly, the Ala65Val mutation alone produced a similar k_{cat}/K_M PNPA value to that of M3 or indeed MT (Thr200Ala), suggesting that the effect of these mutations is not additive for PNPA. In contrast, a 3-fold increase in the 2NAase activity was observed for Ala65Val, implying this was additive to the 10-fold increase seen with Thr200A alone.

Affinity Constants for DANS. In view of the fact that K_M values for 2NA could not be obtained, DANS was used to probe the complementarity of the WT and variants for two-ringed substances similar to 2NA. To this end, we used a fluorescence assay to determine the K_d^{DANS}. The K_d of the WT (0.82 μM) was found to be essentially identical to previously reported values (*28*, *29*, *30*). The affinity of M2 (K_d = 0.23 μM) is ca. 4-fold higher than that of the WT, while the Thr200Ala mutation on its own (MT variant) exhibits ca. 10-times higher affinity for DANS [K_d = 0.07 μM; a very similar value was reported for this mutant by Krebs and Fierke (*31*)]. In contrast to the initial kinetic findings (Table 3), it appears that the Asp110Asn mutation may actually have a subtle effect in decreasing the affinity of the mutants toward DANS. Finally, the affinity of M3 (K_d = 0.03 μM), which shows the highest 2NAase activity, was improved by about 7-fold relative to the M2 variant and almost 30-fold relative to the WT, suggesting that the Ala65Val mutation, which is unique to this mutant, has a significant impact on DANS binding. An assay of the Ala65Val mutant (K_d = 0.12 μM, almost 7-fold lower than that of the WT) supported this view.

LFER. We were interested in exploring CAII's mechanism of ester hydrolysis and the consequences of selection for hydrolysis of a substrate with a leaving group of high pK_a and little electronic or structural similarity to CAII's physiological substrates (CO_2 and HCO_3^-). To this end, we examined the rates of WT hCAII and the newly evolved variant M3 with a series of aromatic ester substrates of varying leaving-group pK_a values (pK_a^{LG}, Table 4). The results are presented in a Brønsted plot (Figure 1).

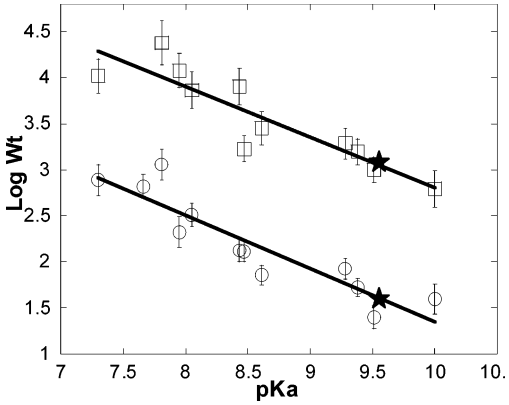


FIGURE 1: LFER for hCAII-catalyzed ester hydrolysis (the respective data are provided in Table 4). Shown are log(k_{cat}/K_M) values observed with WT hCAII (○) and the M3 variant (□), with a series of phenyl esters having different leaving-group pK_a values (pK_a^{LG}). A linear fit of the data points (log(k_{cat}/K_M) = βpK_a^{LG}) gave β or Brønsted, values of −0.58 ± 0.03 and −0.55 ± 0.03, for the WT and M3 variant, respectively (pK_a^{LG} points 10.29 and 7.14 were excluded; see the text). Asterisks denote 2NA data points.

The relationships between log(k_{cat}/K_M) and pK_a^{LG} are largely linear for both enzyme variants, indicating no change in the rate-determining step between pK_a = 7.1 and 10. Furthermore, the rates with 2NA (pK_a^{LG} = 9.51) fit the same line despite the steric difference between the naphthyl leaving group and the phenyl groups of all other substrates. This suggests that all of these substrates bind the same active site and react in a similar mode. The rates of both WT and M3 seem to fall at pK_a > 10, but because we could test only one phenyl substrate in that range (4-methoxyphenyl acetate; pK_a^{LG} = 10.29), it is impossible to determine whether this is a continuous trend or merely a local deviation. As such, these points were excluded from linear fits. The other extreme point, PNPA, was also removed from the fit because of its strong deviation from the general trend. This produced essentially identical β^{LG} values for WT (−0.58 ± 0.03) and M3 (0.55 ± 0.03), suggesting that the mutations leading to higher esterase activity in CAII did not significantly alter the charge distribution of the transition states of the esters tested.

DISCUSSION

In this study, we used the tools of directed evolution to select variants with increased 2NAase activity from libraries

of hCAII, in which random mutations were incorporated at different rates (Table 2). This resulted in two variants, M2 and M3, that exhibit ca. 10- and 40-fold increases in 2NAase rates relative to that of WT hCAII (Table 3). These improvements took place in the context of modest (around 2-fold) decreases in WT anhydrase activity indicating that, at this early stage of evolution, the enzyme remains principally an anhydrase but with an enhanced promiscuous esterase activity. LFER analysis and inhibitor-binding measurements indicated that the improvements in esterase activity arose principally from steric adaptation of the active sites of the newly evolved hCAII variants, as discussed below. The evolved variants exhibit a modest degree of selectivity toward 2NA for which they were selected. The improvements toward PNPA are consistently lower than those observed with 2NA, and M3 shows only 4-fold higher rates than the WT with PNPA (Table 3). The latter is primarily due to the fact that the effect of Ala65Val and Thr200Ala is additive with respect to 2NA but not PNPA.

Electronic Active-Site Characteristics. The interpretation of Brønsted plots for enzymes is complicated by enzyme−substrate binding, but in many cases, it is possible to derive useful information regarding an enzyme's mechanism and the nature of transition states that may form (*33*), particularly in the case of WTCAII, where such analysis has not been reported before. Our conclusion is also restricted by the limited solubility of the substrates tested, which meant that K_M and k_{cat} values could not be independently determined, restricting the leaving-group analysis to k_{cat}/K_M values only. Nevertheless, the steric difference between the naphthyl leaving group of 2NA and the phenyl groups of all other substrates seems to have a minimal effect, because the rates with 2NA ($pK_a^{LG} = 9.51$) largely fit the same line. With the exclusion of the most activated substrate (PNPA), we found both WT hCAII and its M3 variant to exhibit significantly lower β^{LG} values (-0.58 ± 0.03 for WT and -0.55 ± 0.03 for M3) than the hydroxide-driven hydrolysis of aryl esters in bulk water ($\beta^{LG} \approx -0.3$; *19*). This suggests that, as expected, the active-site microenvironment of hCAII is more hydrophobic and aprotic than bulk water, in particular, around the ester's leaving-group oxygen. This is expected because the native activity of hCAII (the hydration of CO_2; Scheme 1) does not require protonation and the HCO_3^- product has a pK_a (6.35; *34*) that is lower than that of PNPA's leaving group ($pK_a^{LG} = 7.14$), not to mention 2NA's leaving group ($pK_a^{LG} = 9.51$). Because the β^{LG} value for ester aminolysis by imidazole approaches -1.0, indicating a transition state in which the leaving group carries a high degree of negative charge (*19*), it could follow that the highly activated catalytic hydroxide at the active site of hCAII leads to a similar type of transition state. If this were the case, then leaving-group departure rather than nucleophilic attack would be rate-determining. The similarity between β^{LG} values for WT and M3 indicate that, at this stage, the evolution toward an improved 2NAase activity did not alter the mechanism of catalysis. It is possible, however, that further along the evolutionary path more significant changes in the electronic characteristics of the active site would be necessary to obtain higher activity toward esters with high pK_a leaving groups.

Steric Active-Site Characteristics. It appears that the active sites of the evolved variants exhibit some steric changes that may affect substrate binding. The solubility of 2NA did not allow K_M values to be determined; therefore, as mentioned above, it remained unclear whether the higher rates exhibited by M2 and M3 are the result of higher k_{cat} values or lower K_M values (i.e., improved substrate binding). To assess whether steric changes had taken place, we measured the WT and variants affinity toward DANS, a well-known inhibitor of hCAII that exhibits a structural resemblance to 2NA. Both 2NA and DANS possess a naphthyl group (Chart 1), while the zinc-ligating sulfonamide group of DANS (*35*) presents as a partial mimic of the reaction intermediate for ester hydrolysis. We found that increases in catalytic efficiency (k_{cat}/K_M) correlated with increases in affinity (or decreases in K_d) for DANS. The K_d value of WT hCAII was around 0.8 μM (Table 3). The affinity of the M2 and MT mutants that exhibit ca. 10-fold higher 2NAase activity is 3−10-fold higher ($K_d = 0.23$ and 0.07 μM), and that of M3 (which shows 40-fold higher 2NAase activity) is almost 30-fold higher than that of the WT. It appears, therefore, that the improved ability of the active site of the newly evolved variants to accommodate the bulky naphthyl group may be one of the reasons for their higher catalytic efficiency. It is, however, unlikely that the 40-fold improvement in k_{cat}/K_M is solely due to a decrease in K_M. The K_M value of WT hCAII for PNPA has been reported to be in the millimolar range (*16*). Assuming a similar or even higher K_M for 2NA, a 40-fold decrease should have yielded a K_M that is significantly lower than 1 mM. The fact that we observed no deviations from linearity of the V_o versus $[S]_0$ plot up to 0.6 mM 2NA suggests that the K_M for M3 is well above 1 mM, implying the improvements in catalysis are not entirely due to K_M.

Role of Individual Mutations. Sequencing of variant M3 showed the accumulation of three mutations (Asp110Asn, Thr200Ala, and Ala65Val), two of which (Asp110Asn and Thr200Ala) appeared in variant M2, from which M3 evolved. Consultation of crystal structures and literature describing hCAII engineering (e.g., refs *31* and *36*) suggested that only the latter two mutations, Thr200Ala and Ala65Val, were significant. Generation of the Asp110Asn, Ala65Val, and Thr200Ala mutations in isolation tended to support this hypothesis, with Asp110Asn (variant MD) having no apparent influence on 2NAase or PNPase activity, Ala65Val approximately a 3-fold improvement in 2NAase activity, and Thr200Ala on its own (variant MT) showing similar behavior to M2 (Table 3). Interestingly, amino acids Thr200 and Ala65, which we found to be modified through random mutagenesis and selection, have been explored by other workers in protein engineering experiments, where various active-site amino acids were targeted for mutation (e.g., refs *31* and *36*) and will be discussed later.

The structure of hCAII in complex with DANS (*35*, Figure 2) suggests that the methyl side chain of Ala65 is comparatively distant from the catalytic zinc (7.1 Å) and DANS (7.5 Å from the nearest DANS atom). However, Ala65 is adjacent to His64, the putative CAII proton shuttle (*37*). Proton-shuttling via His64 is thought to be rate-limiting in the high catalytic rates of CAII, and adjacent side chains have been shown to have an influence upon this function (*38*). Jackman (*36*) diversified Ala65, although valine was not one of the substitutions reported. Mutations into bulky side chains reduced CO_2 hydrase activity to a degree (e.g., Ala65Phe gives a 26-fold reduction). On the other hand, these same bulky and more hydrophobic residues were found to improve

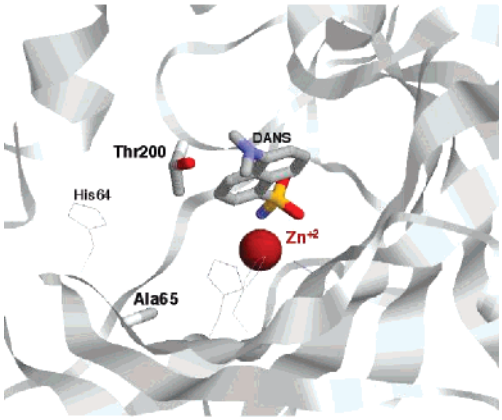


FIGURE 2: View of hCAII active site (adapted from the structure of hCAII in complex with DANS; PDB accession code 1OKL; *34*). Shown in sticks are the DANS ligand and the side chains of residues Thr200 and Ala65 found to be mutated in the newly evolved variants. Also shown are the active-site zinc atom and the three histidine side chains that ligate it (His94, 96, and 119; in wire frame), and the side chain of His64 that is presumed to act as a proton shuttle. [NB, the numbering of hCAII crystal structures has varied between crystal structures. It is therefore important to note that our Thr200Ala mutation occurs on the second of two adjacent threonines in this region of the protein].

PNPA hydrolysis (e.g., Ala65Phe gave a 2-fold increase) through, as suggested by the authors, "increasing affinity" [although the K_M for PNPA was not reported (*36*)]. This indeed may be the case for the Ala65Val substitution seen here (Ala65Val gives a 4-fold increase in PNPAase), because the mutation to valine probably represents a compromise between size constraints (large side chains interfere with His64 and proton shuttling) and an increase in complementarity to large aromatic ligands. In other words, the substitution to valine caused a mild decrease in the CO_2 hydratase activity (~2-fold decrease in k_{cat}/K_M; Table 3), while increasing the 2NAase activity and affinity toward DANS by 3- and 7-fold, respectively (Table 3).

The side chain of Thr200 lies over the opening of the hCAII active site (Figure 2), with its oxygen apparently in direct contact with the naphthyl ring of DANS (3.2 Å to the nearest atom). It is therefore likely that the mutation to alanine enhances the availability of the active site to bulkier and more polar compounds such as PNPA, DANS, and 2NA, resulting in improved affinity and rates (Table 3). Indeed, Thr200 has been diversified by a number of workers including Krebs and Fierke (*31*) who found that, when compared with WT, Thr200Ala produced a 2.5-fold improvement in PNPAase activity and a 30% increase in CO_2 hydration. In addition, reductions in K_d were seen for both DANS {16-fold [0.8 (WT) to 0.05 μM]) and acetazolamide (2.7-fold [8.2−3 nM]}. These results are in broad agreement with our observations (Table 3).

Implications to Protein Evolution. This work provides further support for the possible role of functional promiscuity as a source of starting points for evolution of new proteins. The functional range of our selection assay was rapidly exhausted, allowing only a few rounds of mutation and selection to take place. The improved variants evolved here exhibit low k_{cat}/K_M 2NAase values (250−975 M^{-1} s^{-1}). These rates are more than 10^5-fold lower than the remaining native function; therefore, the enzyme remains principally an anhydrase that benefits from increased promiscuous esterase activity. Nevertheless, improvements of 10- and 40-fold can provide a distinct selective advantage, especially in the context of a limited change to the physiological activity, provided that the cellular levels of the protein are in the micromolar range (*39*). This was demonstrated recently by genetic screens performed *in vivo*, in which the promiscuous activities of enzymes were shown to complement *E. coli* strains deficient in the enzymes that normally mediate these functions (*40*, *41*). These selectable promiscuous activities were as low as 200 M^{-1} s^{-1} (*41*). Our findings suggest that improvements in promiscuous activities can be achieved by relatively minor genotypic changes (a single or a double mutation, as shown here), to provide an immediate selective advantage.

What makes these improvements particularly significant from an evolutionary point of view is the fact that the toll of these mutations on the native activity of the enzyme was minimal. Physiologically, where the enzyme could be exposed to both substrates simultaneously, this may be enhanced by the native activity being due to a small substrate (CO_2), while the improved promiscuous activity is with the much larger 2NA. Under these conditions, it is likely that there would be little interference by 2NA with the native anhydrase activity. If the opposite were the case, however, a small promiscuous substrate could have a significant deleterious effect on a native activity with a larger substrate. This was not the case here, however, and it was observed that both improved mutants retained a good deal of their native function despite 10- and 40-fold improvements in esterase activity. Mutant M2 with 10-fold higher esterase activity exhibits ca. 70% of the native activity of the WT, and the most active 2NAase mutant M3 shows a 40-fold increase in the 2NAase activity yet only a 2-fold decrease in the carbonic anhydrase activity [a preliminary description of these findings is provided elsewhere (*8*)]. The physiological (or native) activity of CAII, therefore, showed an ability to withstand mutations in two conserved regions of the protein, despite the absence of any positive selection for bicarbonate dehydration (the only selection criterion applied was increased 2NAase activity). The robustness of the native activity against mutation may be the result of many millions of years of evolution, during which mutational drift and purifying selection for robustness took place (*42*). Alternatively, this robustness could be simply a consequence of the native activity being highly optimized. In any case, our results indicate that the promiscuous activities of an enzyme exhibit much greater plasticity and evolvability than its native activity.

Because the native anhydrase activity remained largely unchanged, what the few rounds of evolution applied here seems to have created is the start of a generalization of the function of CAII, rather than a respecialisation (which might be expected from a directed evolution experiment where a positive selection is applied). It is difficult to make grand postulations based on a single experiment, but other observations in our lab and elsewhere (*8* and references therein) suggest that this type of diversification is typical to the early stages of evolution, where a promiscuous activity of a protein is dramatically enhanced, while the native activity remains almost unchanged. Mutations of the type described here provide the benefit of parsimony because the mutation and selection act upon the same gene and gene product that are

responsible for the original activity. The contribution of the mutations observed here (Thr200Ala and Ala65Val) to the evolvability of hCAII is probably due to the fact that, despite their high conservation, the mutated residues are not part of the scaffold of hCAII nor of the actual catalytic machinery of the enzyme (e.g., zinc-ligating residues, His64, etc.). Both residues are part of the walls and perimeter of the enzyme's active site. Thr200 is also in a surface loop that exhibits no defined secondary structure (Figure 2) and therefore probably confers some conformational flexibility. Additional rounds of mutation and selection would have led to a further increase in 2NAase activity and, inevitably, to a significant decrease in the carbonic anhydrase activity. These could not, however, be performed with the screening methodology applied here, because its dynamic range had been largely exhausted.

In summary, we have identified two mutations in conserved regions of carbonic anhydrase that led to a 40-fold improvement in the hydrolytic activity of hCAII toward ester substrates with high pK_a leaving groups. We have shown that the promiscuous esterase activity of hCAII can act as a starting point for the evolution of a more proficient esterase and that application of selection pressure quickly produces significant improvements in that activity.

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SUPPORTING INFORMATION AVAILABLE

Sequencing data from a representative sample of positive mutants from round 1 of the selection are presented in Supplementary Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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