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Redesign of human carbonic anhydrase II for increased esterase activity and specificity towards esters with long acyl chains

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Abstract

The effect, of modulating the shape and the size of the hydrophobic pocket on the esterase activity and specificity of human carbonic anhydrase II (HCAII) for esters with different acyl chain lengths was investigated. Following an initial screen of 7 HCAII variants with alanine substitutions in positions 121, 143 and 198, detailed kinetic measurements were performed on HCAII and the variants V121A, V143A and V121A/V143A. For some variants, an increased size of the hydrophobic pocket resulted in increased activities and specificities for longer substrates. For V121A/V143A, the rate of hydrolysis for paranitrophenyl valerate was increased by a factor of approximately 3000. The specificities also changed dramatically, for example V121A/V143A is 6.3 times more efficient with paranitrophenyl valerate than paranitrophenyl acetate, while HCAII is >500 times more efficient with paranitrophenyl acetate than paranitrophenyl valerate. An automated docking procedure was performed on these variants with transition state analogues (TSAs) for the hydrolysis reaction. It was possible to correlate the catalytic rate constants to the docking results, i.e. for each variant, efficient hydrolysis was generally correlated to successful TSA-docking. The observations in this paper show that the redesign increased the catalytic rates for substrates with long acyl chains by removal of steric hinders and addition of new favourable binding interactions.

Introduction

One of the important aspects of enzymes is their capacity for substrate recognition, allowing for highly specific reactions. In recent years, much research has been devoted to developing new enzymes for particular reactions.[1] In our work concerning the engineering of enzyme reaction specificities, we have focused on a well characterised system, human carbonic anhydrase II (HCAII). This zinc containing enzyme (CA; carbonate hydro-lyase, EC 4.2.1.1) catalyses the reversible hydration of carbon dioxide: $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. The catalytic process occurs through two steps. First, the carbon dioxide is attacked by a zinc-coordinated hydroxide ion. The resulting metal-bound HCO_3^- ion is displaced by a water molecule. Second, a proton from the water molecule is transferred to the surrounding medium via a nearby histidine residue.[2]

In addition to the physiologically relevant reaction, carbonic anhydrase catalyses a few other reactions involving carbonyl systems,[3] such as the reversible hydration of acetaldehyde[4, 5] and the hydrolysis of esters[6], including different forms of substituted phenyl esters.[7-10] Bovine carbonic anhydrase displays a specificity pattern with respect to the size of the aliphatic tail of the ester, with a smaller activity for substrates with large and bulky tails.[9, 10] It has been suggested that the ester substrates bind to a hydrophobic portion of the active site, close to the zinc ion,[7] a site now identified as the so-called hydrophobic pocket. While the exact positioning of the ester substrate is unknown, a hypothetical model for the binding of the ester substrate to HCAII has been presented.[11] It has been experimentally shown that the esterase activity is related to the identity of some of the residues that constitute the hydrophobic pocket, and that the V143G and V143A mutants of HCAII

have significantly increased specificity towards paranitrophenyl propionate (pNPP) compared to paranitrophenyl acetate (pNPA).[11]

The catalytic versatility of HCAII has been the subject of several recent publications. In one study, directed evolution resulted in a 40-fold increase in the hydrolysis rate for the non-activated substrate 2-naphtylacetate for the most active variant, which had mutations in other parts of the active site than the hydrophobic pocket.[12] In another interesting study, carbonic anhydrase was turned into a peroxidase, capable of stereoselective epoxidation of p-chlorostyrene, by substituting the active site zinc with manganese.[13]

The aim of the present study was to redesign HCAII by mutations that modulate the shape and increase the size of the hydrophobic pocket, to allow for specific binding and efficient catalysis of substrates with increasing acyl chain lengths. Therefore we used a repertoire of substrates that differ in acyl chain length (from one to five carbon atoms); paranitrophenyl acetate (pNPA), paranitrophenyl propionate (pNPP), paranitrophenyl butyrate (pNPB), paranitrophenyl valerate (pNPV) and paranitrophenyl caproate (pNPC). Alanine substitutions were done for valine in positions 121 and 143 and for leucine in position 198, which all delimit the hydrophobic pocket, and are therefore expected to be involved in substrate binding. We also investigated the effects of all combinations of these mutations. Enzymes with increased specificities for larger substrates were selected from *E. coli* lysates using a plate screening assay. After production and purification the kinetic properties of the selected variants were studied in greater detail. We observed large increases in catalytic efficiencies and changed specificities as a result of the redesign. Further, we performed automated docking of transition state analogues (TSA) with HCAII and the variants V121A, V143A and V121A/V143A, and found that the results from kinetic

measurements correlated well with the incidence of apparent productive binding of TSA in the automated docking.

Experimental section

Mutagenesis

Mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA) according to the instructions, unless otherwise stated. Primers were purchased from DNA technology A/S (Denmark), and the sequencing was performed by GATC (Germany) or Dynamic Code AB (Sweden). Mutant plasmids were purified and the sequences of the entire coding region were determined to verify correct sequences. The plasmid pACA,[14] with a C206S substitution[15] was used as the original mutagenesis template. It contains the HCAII gene, except that a base substitution has been made so that the wild type cysteine in position 206 has been replaced by a serine. The protein is therefore referred to as pseudo wild type HCAII ($HCAII_{pwt}$). The stability, the CO_2 hydration activity and the esterase activity (hydrolysis of pNPA) of $HCAII_{pwt}$ is virtually identical to that of wild type HCAII.[15, 16] The $HCAII_{pwt}$ variant was chosen for two reasons: First, it reactivates much more efficiently than wild type HCAII after denaturation, which indicate that variants which are based on this scaffold may give higher yield than those based on the wild type scaffold. Second, removal of an SH-group probably makes the protein less sensitive to oxidizing conditions at high pH, allowing the use of enzyme variants under such conditions.

Preparation of enzyme variants

Enzyme variants for the kinetic measurements were produced in *E. coli* BL21(DE3), containing the appropriate plasmid. Cultures were grown in LB medium containing 75 μg/ml ampicillin at 37°C to OD_{600} = 0.8. Protein expression was induced by adding 0.5 mM IPTG and 0.5 mM $ZnSO_4$, followed by incubation at room temperature for 12-14 h. The bacteria were lysed by use of a sonicator (Misonix), or a beadbeater (Biospec products). After cell debris had been removed by centrifugation, enzyme was purified essentially according to the affinity chromatographic procedure used by Khalifah et al.[17] This method utilises the strong affinity between carbonic anhydrase and its inhibitor bensenesulfonamide, which is covalently linked to an agarose resin. The high purity was verified by SDS-PAGE. The concentrations were determined by measuring the absorbance at 280 nm and using a molecular extinction coefficient of 55400 $M^{-1}cm^{-1}$.[18]

Measurement of esterase kinetics

Ester hydrolysis, by purified enzyme variants, was monitored by measuring the absorbance at the isobestic point (348 nm) of the chromogenic group paranitrophenol, which is released upon substrate hydrolysis. Reactions were performed at 25 ˚C in a quartz cuvette with 1 cm lightpath, using a Cary 100 Bio spectrophotometer (Varian). Enzyme concentrations were between 0.11-7.45 μM and substrate concentrations were between 0.08-0.37 mM. The substrates were dissolved in acetone, giving a final acetone concentration of 5 %. All measurements were done at pH 8.5 using a 50 mM Tris-SO_4 buffer, with the ionic strength kept at 0.1 by adding Na_2SO_4. Control experiments were performed in which 10 μM acetazolamide was added to samples with the most efficient enzyme-substrate pairs (i.e.V121A with

pNPA, V143A with pNPP and V121A/V143A with pNPV. The solubility of the esters used is well below K_M for pNPA, pNPP and pNPB for wild type carbonic anhydrase II, making it difficult to determine k_{cat} and K_M.[10] Instead, the apparent second order rate constants, k' (= k_{cat}/K_M when $[S] << K_M$), were calculated from the initial slopes corrected for background hydrolysis, using the equation $v = k' \cdot [E]_0 \cdot [S]_0$.[19] In attempts to determine K_M and k_{cat} separately, the rates were also measured at several substrate concentrations for the variants that showed increased esterase activity (see supplementary material). $\Delta\varepsilon_{348}$ values used were 5.15 $mM^{-1}cm^{-1}$ (pNPA), 5.26 $mM^{-1}cm^{-1}$ (pNPP), 5.26 $mM^{-1}cm^{-1}$ (pNPB) and 5.23 $mM^{-1}cm^{-1}$ (pNPC).[10] For pNPV a $\Delta\varepsilon_{348}$ value of 5.26 $mM^{-1}cm^{-1}$ was used.

Automated docking

Automated docking of the transition state analogues (TSA) to the active site of HCAII variants was done using Autodock 3.0.[20-22] The TSAs were built using WebLabViewer Pro (Molecular simulations). Structures for the mutant variants were constructed by deleting methyl groups from the wild type HCAII pdb file (pdb accession code 2cba).[23] All water molecules were deleted except four, which has previously been found to be important for the docking performance of sulfonamides to HCAII.[24] The zinc bound hydroxide was not included in the docking (because the oxygen atom of the Zn-OH corresponds to one of the oxygen atoms in the TSA phosphonate group). To validate the parameters used for docking, the sulfonamide inhibitor dorzolamide was docked to the wild type HCAII structure, resulting in properly docked structures as determined by comparison to the crystal structure of the dorzolamide and HCAII complex (pdb accession code 1cil).[25] The following Zn parameters were used: r = 1.1 Å, ε = 0.25 kcal/mol, q = +2.0 e. A grid consisting of

60*60*60 points, separated by 0.375 Å and centered on the macromolecule, was used. 100 runs of a Lamarckian genetic algorithm were performed for each combination of ligand and enzyme variant, using a population size of 100 and a maximum number of energy evaluations of 2000000. 50 local search runs were permitted with a maximum number of iterations of 300. Default values were used for the other parameters. Initial positions for the ligands were randomized. All flexible bonds in the ligands were set as active. From each run, the conformation and position of the best docked molecule was retrieved, resulting in 100 docked molecules for each combination of ligand and enzyme variant.

Results

Kinetic measurements on purified enzyme variants

Variants were selected for detailed study from 7 different variants with combinations of alanine substitutions in positions 121, 143 and 198, using a plate screening assay (see supplementary material). For high precision kinetic measurements, $HCAII_{pwt}$ and the variants V121A, V143A, and V121A/V143 were produced in *E. coli* BL21(DE3) and purified using affinity chromatography.[17]

Calculated activities ($k' = k_{cat}/K_M$) and specificities from measurements of esterase kinetics on purified enzyme variants are shown in table 1. In table 2, the relative efficiencies compared to $HCAII_{pwt}$ are shown for each substrate, for variants with alanine mutations in positions 121 and 143.

Table 1

Activities[a] and specificities[b] for enzyme variants

	pNPA[a]	pNPP[a]	pNPB[a]	pNPV[a]	pNPC[a]
		pNPP/pNPA[b]	pNPB/pNPA[b]	pNPV/pNPA[b]	pNPC/pNPA[b]
$HCAII_{pwt}$	2080 +/- 61	516 +/- 9	47 +/- 2	3.2 +/- 0.6	1.7 +/- 1.4
		(0.25)	(0.02)	(<0.002)	(<0.001)
V121A	472 +/- 5	88 +/- 1	14.6 +/- 0.4	13.6 +/- 0.3	10.4 +/- 1.7
		(0.19)	(0.03)	(0.03)	(0.02)
V143A	645 +/- 16	13882 +/- 531	2713 +/- 138	803 +/- 28	41 +/- 6
		(21.5)	(4.2)	(1.2)	(0.06)
V121A/V143A	1554 +/- 27	1820 +/- 55	2491 +/- 38	9810 +/- 207	2178 +/- 144
		(1.2)	(1.6)	(6.3)	(1.4)

[a] The measurements were carried out at 25 ˚C, pH 8.5. The apparent second order rate constants ($k' = k_{cat}/K_M$), expressed in $M^{-1}s^{-1}$, are presented with a 95 % confidence interval based on triplicate measurements.

[b] The specificity (values in parenthesis) is expressed as the ratio between the rate constants for each variant with the indicated substrates.

Table 2

Relative efficiencies[a] of mutants compared to $HCAII_{pwt}$

	V121A	V143A	V121A/V143A
pNPA	0.227 +/- 0.004	0.310 +/- 0.006	0.747 +/- 0.013
pNPP	0.170 +/- 0.002	27.0 +/- 0.5	3.54 +/- 0.06
pNPB	0.312 +/- 0.009	58 +/- 2	53 +/- 1
pNPV	4.4 +/- 0.4	258 +/- 24	3150 +/- 290
pNPC	13 +/- 9	52 +/- 35	2800 +/- 1800

[a] For each substrate, the ratio of the apparent second order rate constant for each mutant and $HCAII_{pwt}$ is shown. Mean values and a 95 % confidence interval is given, based on the range of possible ratios that can be calculated using the three individual samples measured for each substrate.

In order to find possible differences between the most efficient variants in substrate binding, attempts were made to determine K_M and k_{cat} from Michaelis-Menten analysis. However, it was only possible to determine K_M and k_{cat} for the V121A/V143A variant and only with the substrates pNPB (K_M = 2.0 +/- 0.9 mM) and pNPV (K_M = 0.65 +/- 0.09 mM). The low solubility of the substrates precluded determination of K_M and k_{cat} from measurements on other substrates and other high activity variants (see supplementary material).

The catalytic activity (both as esterase and as CO_2 hydrolase) of wild type carbonic anhydrase is specifically inhibited by sulfonamide inhibitors, binding directly to the active site Zn^{2+}.[2, 10, 25, 26] For all mutant variants in this study, essentially complete inhibition (more than 90 %) of the activity was achieved by

adding 10 μM acetazolamide. This clearly shows that the reactions involves the zinc bound hydroxide.

Automated docking experiments

A series of automated docking experiments were performed for enzyme variants with alanine mutations in positions 121 and 143. The transition state analogues related to the various substrates were docked to wild type and mutant HCAII variants using Autodock 3.0.[20-22] The structures of pNPB and $pNPB_{TSA}$ are shown in figure 1.

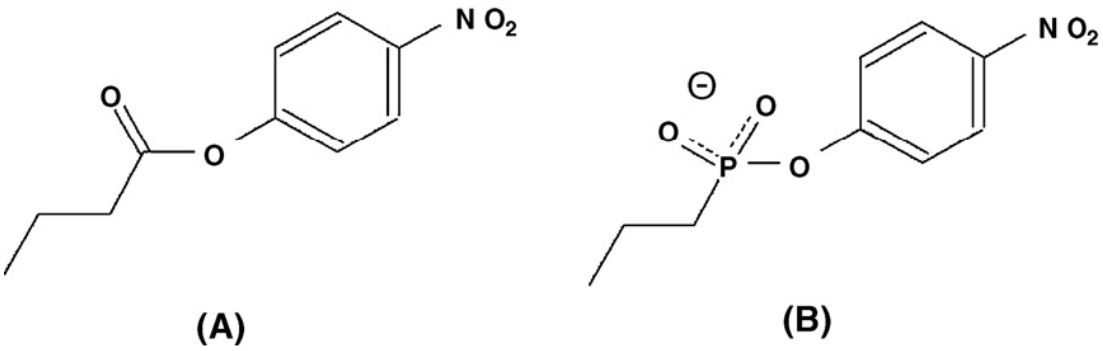

Figure 1. Structure of substrates and transition state analogues (TSAs) used, exemplified by pNPB (A) and $pNPB_{TSA}$ (B).

The transition state analogues for the ester substrates (e.g. figure 1B) were docked to the enzyme variants. Table 3 shows the number of docked TSAs that had an apparent productive interaction with the zinc ion, with at least one of the phosphonate oxygens positioned close to the zinc ion and the acyl chain pointing in the general direction of the hydrophobic pocket. The distance between the phosphonate oxygens and the zinc ion was generally between 1.5 and 3.0 Å, with at least one oxygen atom

closer than 1.7 Å. In most of the docking runs, this was the binding mode found for the strongest binders. The only exceptions were found for the docking of $pNPA_{TSA}$, $pNPP_{TSA}$ and $pNPB_{TSA}$ to V121A/V143A, for which a few among the strongest binders had the paranitrophenyl group positioned into the hydrophobic pocket, and the docking of $pNPV_{TSA}$ and $pNPC_{TSA}$ to wild type, for which some strong binders had the nitro group pointed towards Zn^{2+}.

Table 3

Fraction of Transition State Analogues (TSAs) with productive interactions[a]

Mutant	$pNPA_{TSA}$	$pNPP_{TSA}$	$pNPB_{TSA}$	$pNPV_{TSA}$	$pNPC_{TSA}$
$HCAII_{wt}$	65	64	47	18	8
V121A	58	61	45	26	16
V143A	44	69	64	66	31
V121A/V143A	42	58	60	71	46

[a] The number of docked TSAs out of 100 for which at least one of the two phosphonate-oxygens is positioned close to the zinc ion and the acyl chain points in the general direction of the hydrophobic pocket.

It was observed that the phosphorous atom of the TSA was positioned close to the position (generally within 0.7 Å) of the HCO_3^- carbon of the natural HCAII reaction product. The position of the bicarbonate ion was taken from the crystal structure of the complex between a bicarbonate ion and a HCAII variant.[27]

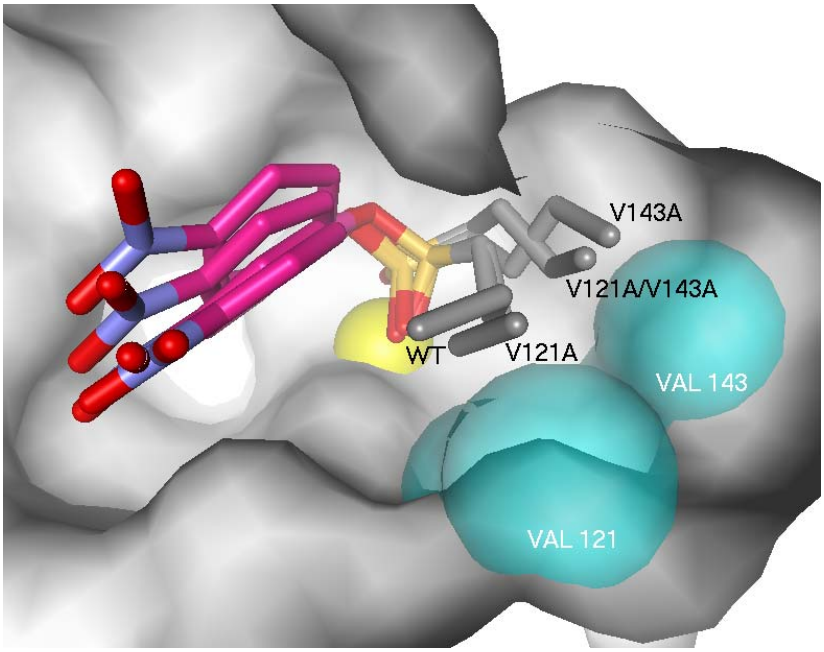

Figure 2. Representative docked $pNPV_{TSA}$ molecules, resulting from automated docking to $HCAII_{wt}$, V121A, V143A and V121A/V143A, superpositioned in the active site of wild type carbonic anhydrase. The active site zinc atom is shown in yellow. For each molecule, black labels indicate the variant used for docking. The CG1 and CG2 atoms of Val-121 and Val-143 are highlighted as green spheres, to indicate the free volume created when the valines in these positions are mutated into alanines. All the molecules are docked with the acyl chains positioned in the hydrophobic pocket. Note that the intact side chain of Val-143 forces the $pNPV_{TSA}$ to adopt a strained conformation when docked to $HCAII_{wt}$ and V121A, while the removal of valine (CG1 and CG2 atoms) in V143A and V121A/V143A allows docking with a less bent acyl chain.

For some of the TSAs we observed differences in binding mode for the different variants. For example, the acyl chain of $pNPV_{TSA}$ (figure 2) was bent for wild type and V121A, so that it pointed out of the hydrophobic pocket. For V143A and V121A/V143A it pointed into the hydrophobic pocket, close to the residues in positions 121 and 143. Docking experiments with cis and trans unsaturated $pNPV_{TSA}$ indicate that the observed differences in binding mode are due to a preference in wild type and V121A for bent $pNPV_{TSA}$ (see supplementary material). V143A seems to prefer molecules in a straight conformation, while V121A/V143A can bind well to both bent and straight $pNPV_{TSA}$. In a future expansion of this study, it would be interesting to experimentally test the variants for hydrolysis of the two forms of unsaturated pNPV, to see if the entropic cost of bending the saturated substrates into the preferred conformation is important for the efficiency of catalysis. It would be expected that wild type and V121A discriminate well between cis and trans unsaturated pNPV.

Discussion

Activity and specificity differences among variants with enlarged hydrophobic pockets

The hydrophobic pocket of HCA II, close to the zinc-bound hydroxide, is defined by the residues Val-121, Val-143, Leu-198 and Trp-209.[28] Results from earlier studies show that these residues are important for the interaction between the enzyme and pNPA, for which most of the studied variants has a decreased esterase activity.[14, 29-31] The specificity of HCAII towards pNPA and pNPP has been investigated for variants with single alanine point mutations of Val-121, Val-143 and

Leu-198.[11] It was shown that mutation of Val-121 and Leu-198 to Ala lowered the specificity toward pNPP compared to pNPA. In addition, the activities for both substrates were lowered. Mutation of Val-143 to Ala and Gly, on the other hand, was found to increase the rate of pNPP hydrolysis, while lowering the rate for pNPA. The result was an inverted specificity in favor of the larger substrate. We have further explored this topic by combining alanine substitutions for residues Val-121, Val-143 and Leu-198. Alanine mutations were chosen, since glycine might result in structural reorganization and lowered protein stability.[19] To give a more complete picture of the esterase specificity we have used a range of substrates, differing in the acyl chain length.

From the initial screen (see supplementary material), the variants V143A and V121A/V143A were found to have a higher specificity for larger substrates than $HCAII_{pwt}$. Therefore, we decided to examine these two variants in more detail, together with $HCAII_{pwt}$ and V121A (which would make it possible to separate the contributions from V121A and V143A to the properties of V121A/V143A).

Notably, the affinity between HCAII and the ester substrates used is not high. In fact, efficient enzymes are not expected to bind the substrate of the reaction with exceptionally high affinities, but rather the transition state. If an enzyme conforms to Michaelis-Menten kinetics, the specificity factor (k_{cat}/K_M) corresponds to the apparent second order rate constant for the reaction between substrate and free enzyme.[19] Thus, it is related to the difference in free energy between the ground state of the reactants in solution and the transition state of the reaction and therefore the value of k_{cat}/K_M is correlated to the binding of the transition state.[19]

Measurement of K_M is hindered (with two exceptions) by the low solubility of the esters used. Thus the substrate concentrations used in our measurements are well

below K_M, and therefore the calculated kinetic constants (k´) approximate k_{cat}/K_M for the reactions.

From the kinetic measurements (table 1), it is clear that the identities of the residues in positions 121 and 143 are important for the specificity profile of HCAII for substrates of different length. For both V121A and V143A, removal of two methyl groups results in an increased activity for some substrates, and a decreased activity for other substrates. In the case of V121A, we observe rate reductions for pNPA, pNPP and pNPB, which indicates that positive interactions are diminished between the active site and these substrates. For the longer substrates, pNPV and pNPC, the effect of removing two methyl groups is an increased activity compared to $HCAII_{pwt}$ (table 2). It seems that the valine side chain in position 121 is a steric hindrance for substrates longer than pNPB, while it contributes positively to the interaction with pNPA, pNPP and pNPB. Similarly, the V143A mutation results in a lower pNPA activity and increased rates for longer substrates, especially for pNPP. This indicates a positive interaction between the valine in position 143 with pNPA, and a negative steric effect for longer substrates.

Combining these two mutations removes the positive interaction between pNPP and Val-121, leading to a less efficient hydrolysis of pNPP by V121A/V143A compared to V143A. However, for pNPV and pNPC, the decreased steric hindrance observed for both single mutants translates into a greatly increased rate of catalysis for the V121A/V143A mutant. In fact, hydrolysis of pNPV by V121A/V143A displays the highest increase in this study, in terms of efficiency compared to $HCAII_{pwt}$. The efficiency has been increased by a factor of approximately 3000 for pNPV, and more than 1000 for pNPC (table 2). Notably the observation that V121A/V143A catalyses the hydrolysis of pNPV four times more efficiently than the

shorter substrate pNPB show that the hydrolysis of pNPV is dependent on new attractive interactions in the enlarged active site, i.e. the increased rate is not only a consequence of removed steric hinders. For these substrates the K_M values were lowered to a level that allowed their determination. The K_M value for pNPV is three times smaller than K_M for pNPB, which correlates rather well with the ratio between their hydrolysis rates (k_{cat}/K_M). Apparently the high activity towards these substrates is, at least partly, a consequence of stronger substrate binding.

Increases in specific activities similar to those obtained in this study for substrates with long acyl chains has been observed by other investigators. In an interesting study, a carboxylesterase was converted into a triacylglycerol lipase by random mutagenesis. Also, the chain length specificity was changed towards long acyl chain substrates. The specific activity towards the optimal substrate was increased by three orders of magnitude for a mutant compared to the wild type.[32]

Correlations between catalytic efficiency/specificity and results from automated docking

To probe if the observed catalytic efficiencies and specificities are correlated to binding in the transition state we performed a set of automated docking runs with transition state analogues (TSA) of the ester substrates used. Each TSA was docked to a region surrounding the active sites of HCAII, V121A, V143A and V121A/V143A. The energetically most favourably docked conformations are in most cases found to have the phosphonate oxygen atoms positioned close to the zinc ion, with acyl chains pointing in the general direction of the hydrophobic pocket. Because the initial positions and conformations of the docked molecules were random, no positioning bias was introduced in the docking calculations. The volume around the active site

which was included in the docking procedure (22.5*22.5*22.5 $Å^3$) is large compared to the size of the ligands, and therefore the probability that a significant proportion of the molecules are initially positioned close to the zinc atom is negligible. The observation that the phosphorous atom (corresponding to the ester carbonyl carbon) of the docked TSA generally is positioned within 0.7 Å from the carbon of bound bicarbonate strongly indicate productive binding. Thus the observations support the assumption that the binding mode of the docked transition state analogues resembles the position of the transition state of the hydrolysis reaction and it is reasonable to compare the TSA docking results with the experimentally observed catalytic rates.

In most cases we find that for each enzyme variant the fraction of TSAs bound with apparent productive interactions (table 3), i.e. positioned with at least one oxygen atom close to the zinc, qualitatively reproduces the pattern of the experimentally observed specificities (table 1). This is nicely illustrated by V121A/V143A, in which the fraction of productively bound TSAs steadily increases from $pNPA_{TSA}$ to $pNPV_{TSA}$, and then decreases for $pNPC_{TSA}$, which is what is observed also for the catalytic rates for the corresponding substrates.

From our assumption that TSA binding and catalytic rates are correlated it is expected that, for each enzyme variant, the substrate that is hydrolysed most efficiently should have the highest fraction of TSAs with productive interactions. With the exception of V121A, the results support this as illustrated for example by V143A that has the largest fraction of TSAs with productive interactions for $pNPP_{TSA}$ and V121A/V143A that has the largest fraction for $pNPV_{TSA}$. Further it is expected that the enzyme variant with the highest catalytic rate with a specific substrate should have the highest fraction of productively bound corresponding TSA. This is true for all substrates used in this study. In fact, the ranking of docking results in each TSA

column of table 3 for TSAs larger than $pNPP_{TSA}$ yields the same order as the ranking of catalytic activities for each substrate column of table 1. For example, for $pNPV_{TSA}$ the order is V121A/V143A, V143A, V121A and wild type, which is the same as the order of catalytic rates for these variants.

Conclusions

We have shown that the rather simple approach of enlarging the hydrophobic pocket can be used to create variants that hydrolyse substrates with acyl chains longer than pNPA more efficiently than $HCAII_{pwt}$. An increase in the catalytic rate by as much as a factor of 3000 was achieved for the hydrolysis of pNPV with V121A/V143A. The chain-length specificity is dependent on the structure of the hydrophobic pocket. Specifically, mutation of Val-143 to alanine shifted the specificity leading to the highest rate and specificity for pNPP. V121A/V143A displays a maximal rate and specificity for pNPV and has a considerable activity with pNPC. $HCAII_{pwt}$ is most efficient with pNPA. The analysis shows that the observed rate enhancements and increased specificities depend on both removal of steric hinders and introduction of additional favourable interactions between the transition states and the enzymes. It thus seems as if enlargement of a hydrophobic binding pocket can be used as a starting point for construction of esterases with specificity for esters with long aliphatic tails.

Further, we have shown that it is possible to correlate the catalytic rate constants to the results from docking of TSAs, particularly for substrates larger than pNPP. It seems as if automated docking of TSAs can be used to select appropriate mutations leading to predicted specificities. We intend to use this approach to further explore the potential of HCAII as an esterase with more demanding substrates.

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References

[1] K.E. Jaeger, T. Eggert, Enantioselective biocatalysis optimized by directed evolution, Curr. Opin. Biotechnol. 15 (2004) 305-313.

[2] A. Liljas, K. Håkansson, B.H. Jonsson, Y. Xue, Inhibition and catalysis of carbonic anhydrase, Eur. J. Biochem. 219 (1994) 1-10.

[3] S. Lindskog, L.E. Henderson, K.K. Kannan, A. Liljas, P.O. Nyman, B. Strandberg, Carbonic anhydrase, in: P.D. Boyer (Ed.), The enzymes, vol. 5, Academic press, New York, 1971, pp. 587-665.

[4] Y. Pocker, J.E. Meany, The catalytic versatility of erythrocyte carbonic anhydrase. I. Kinetic studies of the enzyme-catalyzed hydration of acetaldehyde, Biochemistry 4 (1965) 2535-2541.

[5] Y. Pocker, J.E. Meany, The catalytic versatility of carbonic anhydrase from erythrocytes. The enzyme-catalyzed hydration of acetaldehyde, J. Am. Chem. Soc. 87 (1965) 1809-1811.

[6] R.E. Tashian, C.C. Plato, T.B. Shows, Inherited variant of erythrocyte carbonic anhydrase in micronesians from Guam and Saipan, Science 140 (1963) 53-54.

[7] Y. Pocker, M.W. Beug, Kinetic studies of bovine carbonic anhydrase catalyzed hydrolyses of para-substituted phenyl esters, Biochemistry 11 (1972) 698-707.

[8] Y. Pocker, J.T. Stone, The catalytic versatility of erythrocyte carbonic anhydrase. III. Kinetic studies of the enzyme-catalyzed hydrolysis of p-nitrophenyl acetate, Biochemistry 6 (1967) 668-678.

[9] Y. Pocker, D.R. Storm, The catalytic versatility of erythrocyte carbonic anhydrase. IV. Kinetic studies of enzyme-catalysed hydrolyses of p-nitrophenyl esters, Biochemistry 7 (1968) 1202-1214.

[10] A. Thorslund, S. Lindskog, Studies of the esterase activity and the anion inhibition of bovine zinc and cobalt carbonic anhydrases, Eur. J. Biochem. 3 (1967) 117-123.

[11] B. Elleby, B. Sjöblom, S. Lindskog, Changing the efficiency and specificity of the esterase activity of human carbonic anhydrase II by site-specific mutagenesis, Eur. J. Biochem. 262 (1999) 516-521.

[12] S.M. Gould, D.S. Tawfik, Directed evolution of the promiscuous esterase activity of carbonic anhydrase II, Biochemistry 44 (2005) 5444-5452.

[13] K. Okrasa, R.J. Kazlauskas, Manganese-substituted carbonic anhydrase as a new peroxidase, Chem. Eur. J. 12 (2006) 1587-1596.

[14] S.K. Nair, T.L. Calderone, D.W. Christianson, C.A. Fierke, Altering the mouth of a hydrophobic pocket. Structure and kinetics of human carbonic anhydrase II mutants at residue Val-121, J. Biol. Chem. 266 (1991) 17320-17325.

[15] L.G. Mårtensson, B.H. Jonsson, P.O. Freskgård, A. Kihlgren, M. Svensson, U. Carlsson, Characterization of folding intermediates of human

carbonic anhydrase II: probing substructure by chemical labeling of SH groups introduced by site-directed mutagenesis, Biochemistry 32 (1993) 224-231.

[16] L.G. Mårtensson, B.H. Jonsson, M. Andersson, A. Kihlgren, N. Bergenhem, U. Carlsson, Role of an evolutionarily invariant serine for the stability of human carbonic anhydrase II, Biochim. Biophys. Acta 1118 (1992) 179-186.

[17] R.G. Khalifah, D.J. Strader, S.H. Bryant, S.M. Gibson, Carbon-13 nuclear magnetic resonance probe of active-site ionizations in human carbonic anhydrase B, Biochemistry 16 (1977) 2241-2247.

[18] P.O. Nyman, S. Lindskog, Amino acid composition of various forms of bovine and human erythrocyte carbonic anhydrase, Biochim. Biophys. Acta 85 (1964) 141-151.

[19] A. Fersht, Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding, W. H. Freeman and Company, New York, 1999.

[20] D.S. Goodsell, A.J. Olson, Automated docking of substrates to proteins by simulated annealing, Proteins: Struct., Funct., Genet. 8 (1990) 195-202.

[21] G.M. Morris, D.S. Goodsell, R. Huey, A.J. Olson, Distributed automated docking of flexible ligands to proteins: parallel applications of Autodock 2.4, J. Comput.-Aid. Mol. Des. 10 (1996) 293-304.

[22] G.M. Morris, D.S. Goodsell, R.S. Halliday, R. Huey, W.E. Hart, R.K. Belew, A.J. Olson, Automated docking using lamarckian genetic algorithm and an empirical binding free energy function, J. Comput. Chem. 19 (1998) 1639-1662.

[23] K. Håkansson, M. Carlsson, L.A. Svensson, A. Liljas, Structure of native and apo carbonic anhydrase II and structure of some of its anion-ligand complexes, J. Mol. Biol. 227 (1992) 1192-1204.

[24] S. Grüneberg, M.T. Stubbs, G. Klebe, Successful virtual screening for novel inhibitors of human carbonic anhydrase: strategy and experimental confirmation, J. Med. Chem. 45 (2002) 3588-3602.

[25] G.M. Smith, R.S. Alexander, D.W. Christianson, B.M. McKeever, G.S. Ponticello, J.P. Springer, W.C. Randall, J.J. Baldwin, C.N. Habecker, Positions of His-64 and a bound water in human carbonic anhydrase II upon binding three structurally related inhibitors, Protein Sci. 3 (1994) 118-125.

[26] D. Keilin, T. Mann, Carbonic anhydrase. Purification and nature of the enzyme, Biochem. J. 34 (1940) 1163-1176.

[27] Y. Xue, J. Vidgren, L.A. Svensson, A. Liljas, B.H. Jonsson, S. Lindskog, Crystallographic analysis of Thr200–>His human carbonic anhydrase II and its complex with the substrate, HCO_3^-, Proteins: Struct., Funct., Genet. 15 (1993) 80-87.

[28] A.E. Eriksson, T.A. Jones, A. Liljas, Refined structure of human carbonic anhydrase II at 2.0 Å resolution, Proteins 4 (1988) 274-282.

[29] C.A. Fierke, T.L. Calderone, J.F. Krebs, Functional consequences of engineering the hydrophobic pocket of carbonic anhydrase II, Biochemistry 30 (1991) 11054-11063.

[30] J.F. Krebs, F. Rana, R.A. Dluhy, C.A. Fierke, Kinetic and spectroscopic studies of hydrophilic amino acid substitutions in the hydrophobic pocket of human carbonic anhydrase II, Biochemistry 32 (1993) 4496-4505.

[31] J.F. Krebs, C.A. Fierke, Determinants of the catalytic activity and stability of carbonic anhydrase II as revealed by random mutagenesis, J. Biol. Chem. 268 (1993) 948-954.

[32] D. Reyes-Duarte, J. Polaina, N. López-Cortés, M. Alcalde, F.J. Plou, K. Elborough, A. Ballesteros, K.N. Timmis, P.N. Golyshin, M. Ferrer, Conversion of a carboxylesterase into a triacylglycerol lipase by a random mutation, Angewandte chemie international edition 44 (2005) 7553-7557.