Linköping University Postprint

Converting human carbonic anhydrase II into a benzoate ester hydrolase through rational redesign

Gunnar E. Höst and Bengt-Harald Jonsson

N.B.: When citing this work, cite the original article.

Original publication:

Gunnar E. Höst and Bengt-Harald Jonsson, Converting human carbonic anhydrase II into a benzoate ester hydrolase through rational redesign, 2008, Biochimica et Biophysica Acta, (1784), 5, 811-815.
http://dx.doi.org/10.1016/j.bbapap.2008.02.007.

Copyright: Elsevier B.V., http://www.elsevier.com/

Postprint available free at:
Linköping University E-Press: http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-11787

Converting human carbonic anhydrase II into a benzoate ester hydrolase through rational redesign

Gunnar E. Höst [a] and Bengt-Harald Jonsson [a]*

[a] Molecular Biotechnology/IFM Linköping University, SE-581 83 Linköping, Sweden

* Correspondence author

Email: nalle@ifm.liu.se

Fax: +4613122587

Keywords:

Carbonic anhydrase, hydrolysis, specificity, mutagenesis, protein engineering, rational design

Abstract

Enzymes capable of benzoate ester hydrolysis have several potential medical and industrial applications. A variant of human carbonic anhydrase II (HCAII) was constructed, by rational design, that is capable of hydrolysing para-nitrophenyl benzoate (pNPBenzo) with an efficiency comparable to some naturally occuring esterases. The design was based on a previously developed strategy, [G. Höst, L.G. Mårtensson, B.H. Jonsson, Redesign of human carbonic anhydrase II for increased esterase activity and specificity towards esters with long acyl chains, Biochim. Biophys. Acta 1764 (2006) 1601–1606.] in which docking of a transition state analogue (TSA) to the active site of HCAII was used to predict mutations that would allow the reaction. A triple mutant, V121A/V143A/T200A, was thus constructed and shown to hydrolyze pNPBenzo with k_{cat}/K_M = 625 (± 38) $M^{-1}s^{-1}$. It is highly active with other ester substrates as well, and hydrolyzes para-nitrophenyl acetate with k_{cat}/K_M = 101700 (± 4800) $M^{-1}s^{-1}$, which is the highest esterase efficiency so far for any

CA variant. A parent mutant (V121A/V143A) has measurable K_M values for para-nitrophenyl butyrate (pNPB) and valerate (pNPV), but for V121A/V143A/T200A no K_M could be determined, showing that the additional T200A mutation has caused a decreased substrate binding. However, k_{cat}/K_M is higher with both substrates for the triple mutant, indicating that binding energy has been diverted from substrate binding to transition state stabilization.

Introduction

The bulky nature of benzoate esters makes them demanding as substrates for hydrolases. Esterases have been evaluated for their potential for benzoate ester hydrolysis, with possible applications such as mild and selective removal of protecting groups,[2] and synthesis of benzoylated compounds.[3] Another intriguing possibility is to use esterases for treatment of cocaine overdoses. By using an enzyme that hydrolyzes a benzoate ester bond in the cocaine molecule, the level of biologically active drug substance can be lowered rapidly.[4] Notably, several commonly used lipases lack any appreciable activity with model benzoate ester substrates, such as paranitrophenyl benzoate,[5] and many groups perform work on the identification and development of lipase variants with activity against bulky substrates.[5-7] In this report, we expand the range of enzymes able to hydrolyze benzoate esters by adding a human carbonic anhydrase II (HCAII) variant. The physiological role of the zinc enzyme HCAII (CA; carbonate hydro-lyase, EC 4.2.1.1) is to catalyze the reversible hydration of carbon dioxide: $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. The details of the catalytic mechanism are described elsewhere.[8] Briefly, a OH^- bound to the active site Zn^{2+} acts as a nucleophile, attacking the carbon dioxide substrate which results in a metal bound HCO_3^-. The product is replaced by a H_2O molecule, and the enzyme is regenerated when a H^+ is transferred to the bulk solution via a histidine residue.

The HCAII active site is catalytically promiscuous, for example being able to catalyze the hydrolysis of certain ester substrates.[9-12] We have previously showed that computational docking of transition state analogues (TSAs) can be used as a tool for designing HCAII mutants, allowing prediction of differences in esterase activity and specificity for variants of HCAII.[1] In this study, we extend these results to rationally design a HCAII variant for hydrolysis of a model benzoate ester. We also characterize the substrate specificity of the mutant for ester substrates of various sizes.

Experimental section

Mutagenesis

The mutant HCAII variants V121A/V143A and V121A/V143A/T200A were constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA). Primers were purchased from DNA technology A/S (Denmark). Mutant plasmids were purified and the sequences of the entire coding region were determined to verify correct sequences. The sequencing was performed by GATC (Germany). For reasons discussed elsewhere, [1]we used the plasmid pACA,[13] containing the HCAII gene with the mutation C206S,[14] as template. HCAII C206S is referred to as pseudo wild type HCAII ($HCAII_{pwt}$). The stability, the CO_2 hydration activity and the esterase activity (hydrolysis of pNPA) of $HCAII_{pwt}$ are virtually identical to that of wild type HCAII.[14, 15] Although the mutant variants also contain the C206S mutation, they will be referred to as V121A/V143A and V121A/V143A/T200A, respectively.

Preparation of enzyme variants

Enzyme variants for the kinetic measurements were produced as described previously.[1] The procedure includes IPTG induced expression of mutant enzyme in *E. coli* BL21(DE3). After cell lysis, the enzymes were purified from centrifuged lysate essentially according to the affinity chromatographic procedure used by Khalifah et al.[16] The purity of the enzyme preparations were checked with SDS-PAGE and the concentrations were determined by measuring the absorbance at 280 nm, using a molecular extinction coefficient of 55400 $M^{-1}cm^{-1}$.[17]

Measurements of nitrophenyl ester hydrolysis

Nitrophenyl ester hydrolysis reactions were monitored by measuring the absorbance at the isobestic point of the chromogenic leaving group (i.e. 348 nm for paranitrophenol). Reactions were performed

at 25 ˚C in a quartz cuvette with 1 cm lightpath. 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. Enzyme concentrations were between 0.018 - 20 μM for $HCAII_{pwt}$, V121A/V143A and V121A/V143A/T200A, and substrate concentrations were between 0.03-0.30 mM (the choice of concentrations was restricted by the low solubility of the ester substrates). The apparent second order rate constants, k_{enz} (= k_{cat}/K_M when $[S] << K_M$), were calculated from the initial slopes, that had been corrected for background hydrolysis, using the equation $v = k_{enz} \cdot [E]_0 \cdot [S]_0$.[18] To investigate if the $[S] << K_M$ assumption is valid for V121A/V143A/T200A, the activities with pNPB, pNPV and pNPC were measured for at least two different substrate concentrations (between 0.1 and 0.2 mM for pNPB and pNPV and between 0.03 and 0.1 mM for pNPC). For all substrates, control experiments were performed in which 25 μM acetazolamide was added to the enzyme-containing samples. The $\Delta\varepsilon_{348}$ 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).[12] For pNPV and pNPBenzo, $\Delta\varepsilon_{348}$ values of 5.26 $mM^{-1}cm^{-1}$ and 5.15 $mM^{-1}cm^{-1}$ were used, respectively.

Automated docking of transition state analogues to carbonic anhydrase variants

Automated docking of transition state analogues (TSA) to the active site of HCAII variants was performed as described previously.[1] The ligand molecules for the docking calculations were designed with the ester bond replaced by a phosphonate group (see Fig. 1), a transition state analogue that is frequently used experimentally to mimic the transition state for the ester hydrolysis reaction.[19] Docking calculations were performed using Autodock 3.0,[20-22] and the TSAs were built using WebLabViewer Pro (Molecular simulations). Mutants were constructed by deleting methyl groups from the wild type HCAII pdb file (pdb accession code 2cba),[23] and all water molecules were deleted except four, which have been found to be important for the docking of ligands to HCAII.[24] The zinc bound hydroxide was not included in the docking, since the oxygen

atom of the Zn-OH corresponds to one of the oxygen atoms in the TSA phosphonate group. Zn parameters used were: r = 1.1 Å, ε = 0.25 kcal/mol, q = +2.0 e. For each combination of enzyme and ligand, 100 runs of a Lamarckian genetic algorithm were performed, using a grid consisting of 60*60*60 points (with 0.375 Å between grid points) centred on the macromolecule. Population size was 100 and a maximum number of energy evaluations of 2000000 was used. Default values were used for the other parameters. Initial positions for the ligands were randomized, and all flexible bonds in the ligands were set as active. From each run, the conformation and position of the best docked molecule were retrieved, resulting in 100 docked molecules for each combination of ligand and enzyme variant.

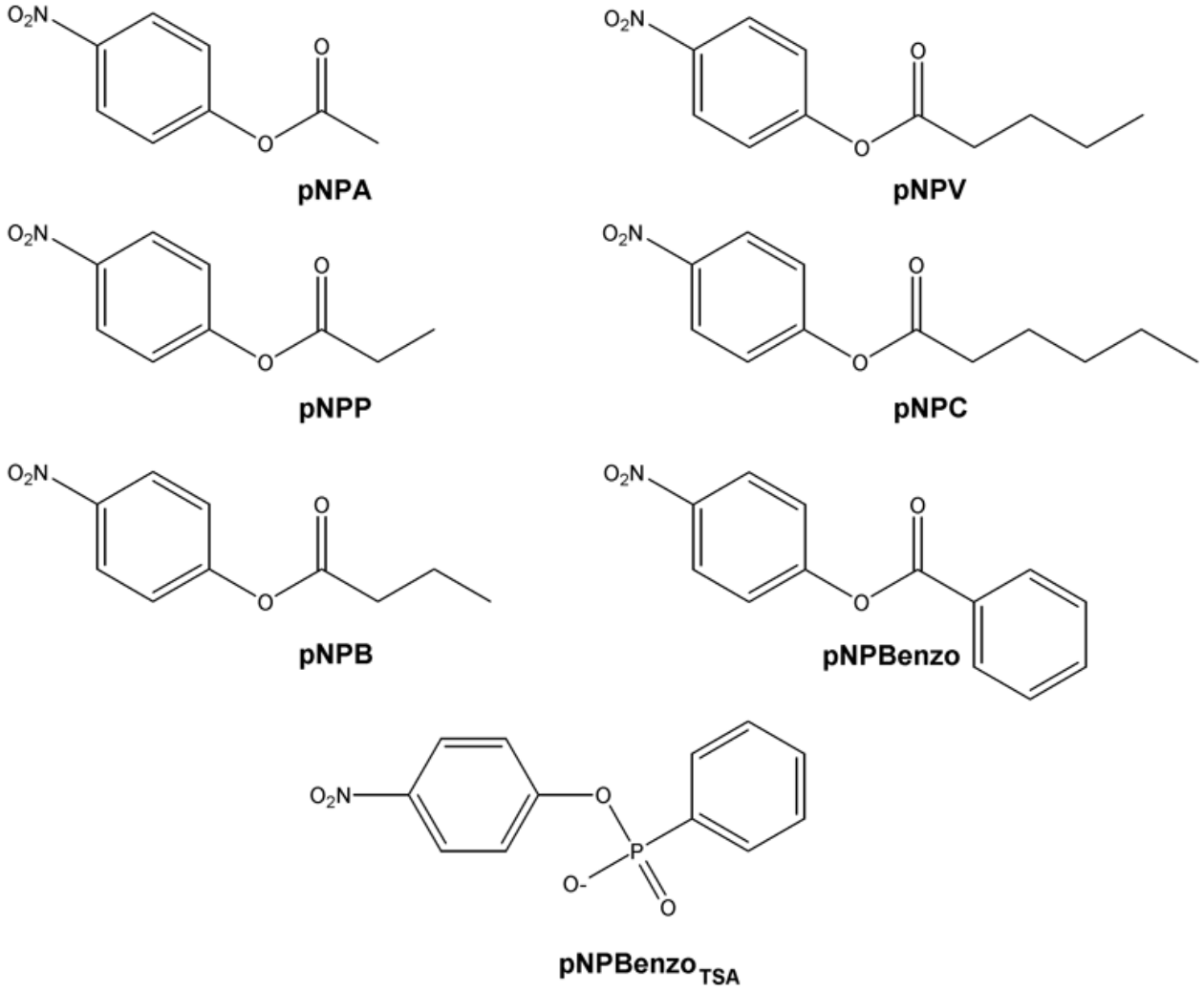

Fig. 1. Structures of the ester substrates used for kinetic measurements and a transition state analogue for the hydrolysis of para nitrophenyl benzoate (pNPBenzo$_{TSA}$). The substrates are para-nitrophenyl acetate (pNPA), para-nitrophenyl propionate (pNPP), para-nitrophenyl butyrate (pNPB), para-nitrophenyl valerate (pNPV), para-nitrophenyl caproate (pNPC) and para-nitrophenyl benzoate (pNPBenzo).

Results

Automated docking experiments

Docking of transition state analogues (TSA) of the ester hydrolysis reaction (see for example Fig. 1) to different HCAII variants was performed to guide the engineering of a mutant capable of benzoate ester hydrolysis. The results from docking of a TSA for pNPBenzo hydrolysis to $HCAII_{WT}$, V121A/V143A and V121A/V143A/T200A are shown in Table 1.

Results from docking TSAs for the aliphatic substrates pNPA, pNPP, pNPB, pNPV and pNPC to V121A/V143A/T200A are shown in Table 2.

Table 1: Fraction successfully docked molecules of 100 for a TSA of pNPBenzo hydrolysis

	WT	V121A/V143A	V121A/V143A/T200A
$pNPBenzo_{TSA}$	1	20	46

Measurements of esterase kinetics

The reaction rates for ester hydrolysis catalyzed by the HCAII mutant V121A/V143A/T200A are presented in Table 2 for various nitrophenyl esters (see Fig. 1). In Table 3, the k_{cat}/K_M values for V121A/V143A/T200A are compared to other HCAII variants.

To check for saturation kinetics, meaurements of the V121A/V143A/T200A catalyzed hydrolysis of pNPB, pNPV and pNPC were performed, in which the substrate concentrations were varied up to the limits posed by solubility. For each of the substrates, k_{enz} values were identical for the tested concentrations, indicating that the concentrations used are significantly lower than the K_M values for those substrates, i.e. the low solubilities of the substrates preclude determination of separate k_{cat} and K_M values.

Table 2: Esterase activity (k_{cat}/K_M) and fraction successfully docked TSA molecules of 100 for the HCAII variant V121A/V143A/T200A, and k_{cat}/K_M for wild type HCAII[1], for various ester substrates

	Docked TSA (%)	k_{cat}/K_M [a] ($M^{-1}s^{-1}$)	
Ester substrate	V121A/V143A/T200A	V121A/V143A/T200A	$HCAII_{WT}$
pNPA	52	101666 (± 4825)	2080 (±61)
pNPP	66	43714 (± 1857)	516 (±9)
pNPB	74	29241 (± 1632)	47 (±2)
pNPV	76	46310 (± 368)	3.2 (±0.6)
pNPC	70	55447 (± 1097)	1.7 (±1.4)
pNPBenzo	46	625 (± 38)	-[b]

[a] The esterase activities are apparent second order reaction rate constants, determined from the equation $v_0 = k_{enz}*[S]_0*[E]_0$. k_{enz} is equal to k_{cat}/K_M under the assumption that $[S] << K_M$. The values are presented as 95 % confidence intervals, based on triplicate measurements.

[b] The activity was too low to be determined. It it is estimated to be below 15 $M^{-1}s^{-1}$.

Table 3: Relative efficiency[a] of V121A/V143A/T200A compared to other HCAII variants[1]

	V121A/V143A/T200A	V121A/V143A/T200A	V121A/V143A/T200A	V121A/V143A/T200A
	HCAII	V121A	V143A	V121A/V143A
pNPA	49 (± 1.4)	215 (± 5.2)	158 (± 4.2)	65 (± 1.7)
pNPP	85 (± 1.9)	499 (± 11.3)	3 (± 0.1)	24 (± 0.6)
pNPB	621 (± 23.3)	1992 (± 63)	11 (± 0.4)	12 (± 0.3)
pNPV	14872 (± 1348)	3407 (± 37.5)	58 (± 1.1)	5 (± 0.1)
pNPC	174335 (± 113104)	13275 (± 1039)	3362 (± 274)	63 (± 2)

[a] For the various substrates, the value of k_{enz} for V121A/V143A/T200A is compared to the corresponding values for the indicated HCAII variants. Mean values and a 95% confidence interval is given, based on the range of values that can be calculated using the three individual replicates measured for each substrate.

As shown in Table 2, the mutant V121A/V143A/T200A has considerable hydrolytic activity with pNPBenzo. Catalytic activity with pNPBenzo was also tested for $HCAII_{pwt}$ and V121A/V143A, but no enzyme catalyzed reaction could be observed for these enzymes.

Control experiments were done in which the specific HCAII inhibitor acetazolamide was added to the enzyme catalyzed reaction at a concentration of 25 μM, resulting in essentially complete inhibition of the activities with all substrates (90 % inhibition or more). This demonstrates that the measured activities indeed emanate from the active site of the investigated HCAII variants.

Discussion

Rational design of a benzoate hydrolyzing HCAII variant

In a recent study, we showed that the ability of HCAII variants to discriminate between aliphatic paranitrophenyl ester substrates with different lengths of the acyl chain is highly dependent on the size and shape of the so-called hydrophobic pocket in the active site of HCAII. A double mutant variant, in which the valine residues in positions 121 and 143 were replaced by alanine residues (V121A/V143A) was found to favour esters with long acyl chains, with a maximal value of k_{cat}/K_M for pNPV.[1]

The additional space created by substituting two valine residues with alanine thus allows productive binding of larger substrates compared to non-mutated enzyme. An interesting possibility is that bulky substrates such as benzoate esters might also be productively bound in the enlarged substrate binding cavity of V121A/V143A. To evaluate this possibility, automated docking experiments were performed with a transition state analogue (TSA) for hydrolysis of paranitrophenyl benzoate (pNPBenzo), see Table 1.

By inspection of the docking to the variant V121A/V143A, it was found that the side chain of residue Thr-200 might sterically interfere with binding of the transition state. Thr-200 is positioned

in a different part of the active site cavity compared to the hydrophobic pocket, on the opposite side of the zinc binding site (see Fig. 2). Thus, the variant V121A/V143A/T200A was evaluated, giving improved docking for the $pNPBenz_{TSA}$ compared to both $HCAII_{WT}$ and V121A/V143A (Table 1). The variant V121A/V143A/T200A was produced, and kinetic measurements show that it is capable of benzoate ester hydrolysis (Table 2), while wild type HCAII and V121A/V143A are inactive. The activity measurements for the investigated variants are consistent with the docking results in Table 1, i.e. V121A/V143A/T200A has the largest pNPBenz activity and the highest number of productively docked TSAs, indicating that docking of TSAs is a useful tool for designing HCAII variants that hydrolyze pNPBenzo.

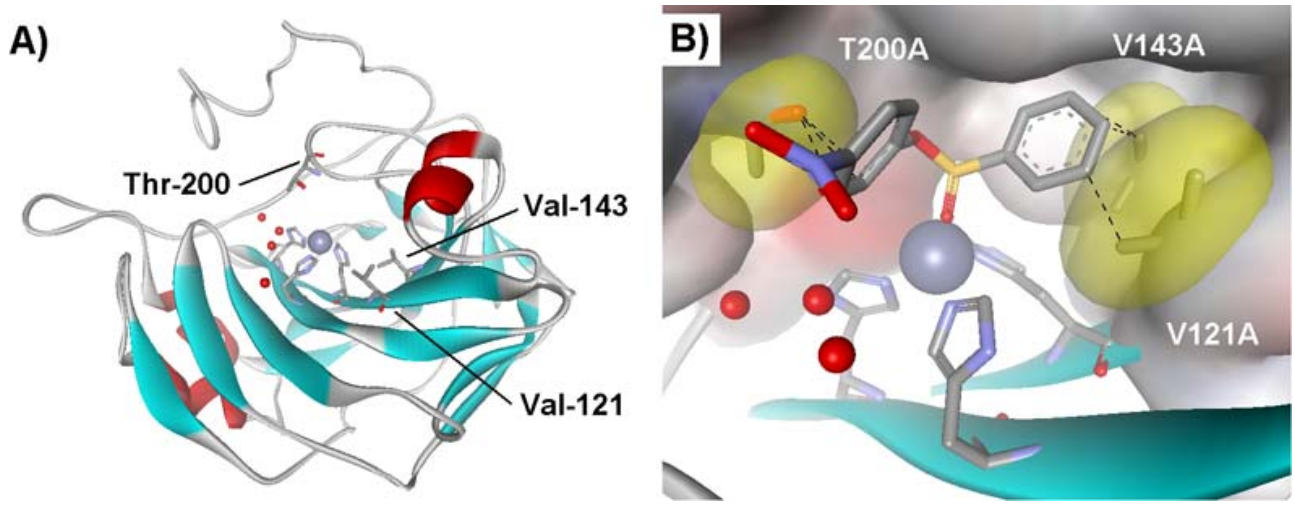

Fig. 2. The enzyme human carbonic anhydrase II (HCAII) was converted into a benzoyl ester hydrolase by three mutations, V121A, V143A and T200A. Three active site histidines coordinate a zinc ion (grey/blue sphere). Four active site water molecules are represented by red spheres. A) The residues Val-121 and Val-143 are positioned on a different wall in the active site compared to Thr-200. B) Substituting the wild type residues with alanine allows a better docking of $pNPBenzo_{TSA}$. The increased cavity volume created by the mutagenesis is indicated by yellow transparent surfaces. The potential steric clashes (in the wild type protein) between the removed atoms (yellow) and $pNPBenzo_{TSA}$ are indicated by dashed lines.

Interestingly, the activity of the rationally designed variant V121A/V143A/T200A with pNPBenzo (k_{cat}/K_M = 625 $M^{-1}s^{-1}$) is higher than liver carboxylesterase from rabbit and human (k_{cat}/K_M = 157 and 185 $M^{-1}s^{-1}$, respectively),[25] while it is much lower than the highly active chicken enzyme (k_{cat}/K_M

= $1.5*10^8$ $M^{-1}s^{-1}$).[26] The increase in activity with pNPBenzo resulting from the mutations is large compared to the parent mutants. Although the $HCAII_{pwt}$ and V121A/V143A activities are below the detection limit, an upper limit can be estimated. Based on the minimum k_{cat}/K_M value required to reach the detection limit of the assay (a conservative estimate is the k_{cat}/K_M corresponding to a reaction rate two times larger than the background rate), the variant V121A/V143A/T200A is at least 40 fold more efficient than wild type HCAII.

Having demonstrated the potential for benzoate ester hydrolysis in a HCAII variant, the next step is to design a mutant capable of hydrolyzing substrates of medical or industrial importance. We have tested V121A/V143A/T200A for hydrolysis of a benzoate ester in cocaine (data not shown). However, no activity could be detected, indicating a need for further engineering efforts.

Considering the substantial alterations of the active site caused by changing three amino acid residues in the V121A/V143A/T200A mutant, it is important to investigate the effects on the original CO_2 hydration activity. It was measured for $HCAII_{pwt}$ and the mutant V121A/V143A/T200A using a colorimetric method.[27] Sulfonamide inhibition was also evaluated, by titrating with acetazolamide to determine IC50 values. The results indicate that the triple mutant is only slightly less active than the wild type (79 (± 5) %), and it has very similar acetazolamide inhibition properties (IC50 = 3.6 (± 0.4) nM for V121A/V143A/T200A, and IC50 = 2.7 (± 0.6) nM for wild type HCAII). Thus, it appears that the structure at the catalytic Zn-OH remains fully functional in the triple mutant.

The variant V121A/V143A/T200A is a highly efficient esterase with aliphatic esters

A panel of nitrophenyl ester substrates of various shapes and sizes (Fig. 1) were used to characterize the specificity of the variant V121A/V143A/T200A, see Table 2. This mutant catalyzes the hydrolysis of aliphatic esters with greatly increased catalytic rate constants compared to the previously investigated parent mutants (see Table 3).[1] For example, the efficiencies of ester

hydrolysis for the longest substrates (pNPV and pNPC) have been increased by four orders of magnitude for V121A/V143A/T200A compared to $HCAII_{pwt}$. The highest activity of V121A/V143A/T200A is found for pNPA hydrolysis, with a catalytic rate constant of $1*10^5$ $M^{-1}s^{-1}$. This is, to our knowledge, the largest catalytic rate constant for carboxylate ester hydrolysis reported so far for any carbonic anhydrase variant.

Further, it can be seen from Table 3 that the activity of V121A/V143A/T200A is also increased compared to the highly active variants V143A and V121A/V143A that were identified in earlier studies.[1, 28]

Previously, we have found that the fraction of productively docked TSAs can be correlated with the hydrolysis rate for the corresponding substrates for some HCAII variants.[1] The results from docking of TSAs for a panel of aliphatic esters to V121A/V143A/T200A are shown in Table 2, and we observe that the variant V121A/V143A/T200A has a higher fraction of correctly docked TSAs for pNPB, pNPV and pNPC than previously examined variants (HCAII, V121A, V143A and V121A/V143A),[1] correctly predicting that the triple mutant is the most efficient enzyme for these three substrates.

Utilization of binding energy for transition state stabilization

Achieving efficient catalysis by using binding energy to stabilize the transition state of a reaction is an important and well known factor in enzyme mechanisms.[18] In an earlier study, we determined the K_M values for hydrolysis of pNPB (K_M = 2.0 ± 0.9 mM) and pNPV (K_M = 0.65 ± 0.09 mM) by the HCAII variant V121A/V143A.[1] From the k_{cat}/K_M ratios in Table 3 it can be seen that the variant V121A/V143A/T200A is more efficient than V121A/V143A for hydrolysis of these substrates by a factor of 12 and 5, respectively. This increase in k_{cat}/K_M can in principle be caused by stronger substrate binding and/or a higher value of k_{cat}. The results from attempts to determine K_M values for hydrolysis of pNPB and pNPV by V121A/V143A/T200A show that the binding is too

weak to be determined, i.e. the affinity is lowered compared to V121A/V143A for both substrates. It thus appears that the improved substrate binding capacity of V121A/V143A, compared to e.g. V143A (for which no K_M could be determined), is replaced by advantageous binding interactions between the transition state and the triple mutant leading to an increased value of k_{cat} for V121A/V143A/T200A. This seems to be a clear cut example of utilization of binding energy for transition state stabilization, rather than increased substrate binding. Further support for this conclusion comes from the observation that the k_{cat}/K_M values become progressively higher for pNPB, pNPV and pNPC. Larger aliphatic structures can have higher interaction energies with the active site cavity, and thus more binding energy is available for transition state stabilization.

The effect of the T200A mutation

As far as we know, all mutant variants of HCAII that incorporates the T200A mutation have an increased esterase efficiency compared to wild type HCAII for all substrates tested.[28-30] In this regard it is interesting to compare V121A/V143A/T200A to its parent mutant V121A/V143A.[1] The largest increase in rate constant is observed for the pNPA substrate, which is hydrolyzed 65 times more efficiently by the triple mutant compared to the double mutant (see Table 3). The efficiency is increased by at least an order of magnitude for all substrates except pNPV, for which it is increased by a factor of five. The rate constant for the T200A single mutant has previously been determined for pNPA (5000 $M^{-1}s^{-1}$ at pH 8.9),[28] and we observe that V121A/V143A/T200A has a greatly increased activity (20 times) for pNPA ($1*10^5$ $M^{-1}s^{-1}$) compared to T200A. This shows that the increased efficiency is a synergistic effect of the three mutations, and not simply a consequence of adding the contribution from the T200A mutation.

In several studies, it has been found that the T200A mutation has a large impact on the discrimination between ester substrates with different alcohol moieties. For example, the activity with oNPA is increased by a factor of 100 for the mutant T200A compared to wild type HCAII,

while the activity for pNPA is only increased by a factor of two.[28] It might thus seem reasonable to assume that alterations in position 200 mainly affect the specificity with respect to the alcohol moiety of the ester substrate, while alterations in positions 121 and 143 mainly affect discrimination between different acyl moieties. This assumption implies that discrimination between substrates with different acyl chain lengths but identical alcohol leaving groups should be similar for V121A/V143A and V121A/V143A/T200A. The present study shows that this is clearly not the case, as the triple mutant is more efficient for the hydrolysis of both the shortest substrate (pNPA) and the longest substrate (pNPC) than for substrates of intermediate length, which is the opposite pattern compared to V121A/V143A, in which pNPV is a more efficient substrate than pNPA and pNPC (see Fig. 3).[1] Apparently the T200A mutation has diverse effects on catalysis of substrates with different lengths.

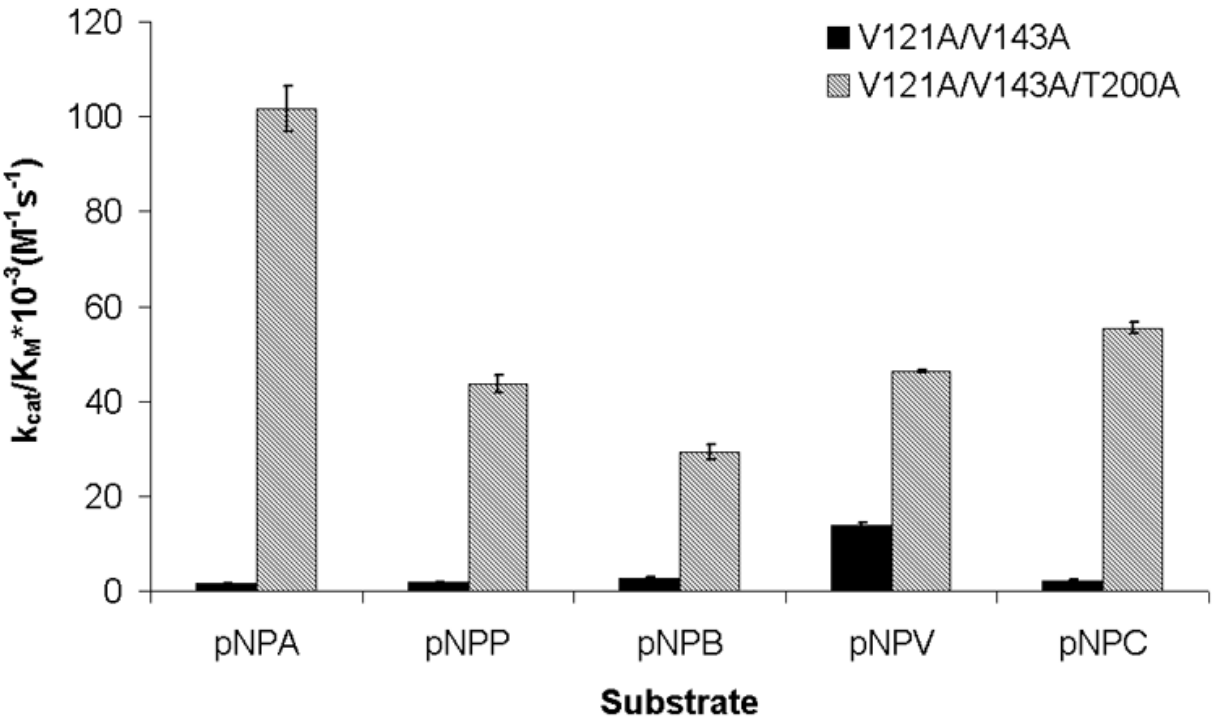

Fig. 3. Comparison between the catalytic ester hydrolysis efficiencies (k_{cat}/K_M) of the variants V121A/V143A [1] and V121A/V143A/T200A. Error bars indicate a 95 % confidence interval, based on triplicate measurements.

Conclusions

We have shown that the active site of carbonic anhydrase can be redesigned to allow hydrolysis of a bulky benzoate ester. A variant of HCAII with three mutations, V121A/V143A/T200A, was found to hydrolyze para-nitrophenyl benzoate with an efficiency that is comparable to some naturally occuring esterases. Further, it hydrolyzes para-nitrophenyl acetate with $k_{cat}/K_M = 1*10^5$ $M^{-1}s^{-1}$, which is the highest esterase efficiency reported so far for any CA variant. The successful conversion of HCAII into a benzoate ester hydrolysing enzyme is a part of our efforts to engineer variants with catalytic capabilities suitable for industrial and medical applications involving bulky substrates.

Acknowledgements

This work was supported by a grant from the Swedish Natural Science Research Council to B.-H.J.

References

[1] G. Höst, L.G. Mårtensson, B.H. Jonsson, Redesign of human carbonic anhydrase II for increased esterase activity and specificity towards esters with long acyl chains, Biochim. Biophys. Acta 1764 (2006) 1601-1606.

[2] E. Barbayianni, I. Fotakopoulou, M. Schmidt, V. Constantinou-Kokotou, U.T. Bornscheuer, G. Kokotos, Enzymatic removal of carboxyl protecting groups. 2. Cleavage of the benzyl and methyl moieties, J. Org. Chem. 70 (2005) 8730-8733.

[3] C. Zimmer, T. Platz, N. Cadez, F. Giffhorn, G.W. Kohring, A cold active (2R,3R)-(-)-di-O-benzoyl-tartrate hydrolyzing esterase from Rhodotorula mucilaginosa, Appl. Microbiol. Biotechnol. 73 (2006) 132-140.

[4] P. Ascenzi, E. Clementi, F. Polticelli, The Rhodococcus sp. cocaine esterase: a bacterial candidate for novel pharmacokinetic-based therapies for cocaine abuse, Life 55 (2003) 397-402.

[5] K. Mitsuhashi, M. Yamashita, Y.S. Hwan, F. Ihara, T. Nihira, Y. Yamada, Purification and characterization of a novel extracellular lipase catalyzing hydrolysis of oleyl benzoate from Acinetobacter nov. sp. strain KM109, Biosci. Biotechnol. Biochem. 63 (1999) 1959-1964.

[6] M. Goto, M. Kawasaki, T. Kometani, T. Nonaka, Y. Mitsui, Binding site of acyl moiety in ester hydrolysis by Candida rugosa lipase, J. Mol. Catal. B: Enzym. 11 (2001) 1029-1033.

[7] M.T. Reetz, J.D. Carballeira, J. Peyralans, H. Höbenreich, A. Maichele, A. Vogel, Expanding the substrate scope of enzymes: Combining mutations obtained by CASTing, Chem. Eur. J. 12 (2006) 6031-6038.

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

[9] G.E. Höst, J. Razkin, L. Baltzer, B.H. Jonsson, Combined enzyme and substrate design: Grafting of a cooperative two-histidine catalytic motif into a protein targeted at the scissile bond in a designed ester substrate, Chembiochem 8 (2007) 1570-1576.

[10] 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.

[11] 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.

[12] 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.

[13] 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.

[14] 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.

[15] 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.

[16] 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.

[17] 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.
[18] A. Fersht, Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding, W. H. Freeman and Company, New York, 1999.
[19] A. Tramontano, K.D. Janda, R.A. Lerner, Catalytic antibodies, Science 234 (1986) 1566-1570.
[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] K.J.P. Yoon, C.L. Morton, P.M. Potter, M.K. Danks, R.E. Lee, Synthesis and evaluation of esters and carbamates to identify critical functional groups for esterase-specific metabolism, Bioorg. Med. Chem. 11 (2003) 3237-3244.
[26] M.C. Berndt, M.R. Bowles, G.J. King, B. Zerner, Inhibition of chicken liver carboxylesterase by activated carbonyls and carbonyl hydrates, Biochim. Biophys. Acta 1298 (1996) 159-166.
[27] E.E. Rickli, S.A. Ghazanfar, B.H. Gibbons, J.T. Edsall, Carbonic anhydrases from human erythrocytes. Preparation and properties of two enzymes., J. Biol. Chem. 239 (1964) 1065-1078.
[28] 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.
[29] G. Behravan, B.H. Jonsson, S. Lindskog, Fine tuning of the catalytic properties of human carbonic anhydrase II. Effects of varying active-site residue 200, Eur. J. Biochem. 195 (1991) 393-396.
[30] S.M. Gould, D.S. Tawfik, Directed evolution of the promiscuous esterase activity of carbonic anhydrase II, Biochemistry 44 (2005) 5444-5452.