An active site tyrosine residue is essential for amidohydrolase but not for esterase activity of a class 2 histone deacetylase-like bacterial enzyme

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HDACs (histone deacetylases) are considered to be among the most important enzymes that regulate gene expression in eukaryotic cells acting through deacetylation of ϵ -acetyl-lysine residues within the N-terminal tail of core histones. In addition, both eukaryotic HDACs as well as their bacterial counterparts were reported to also act on non-histone targets. However, we are still far from a comprehensive understanding of the biological activities of this ancient class of enzymes. In the present paper, we studied in more detail the esterase activity of HDACs, focussing on the HDAH (histone deacetylase-like amidohydrolase) from *Bordetella/Alcaligenes* strain FB188. This enzyme was classified as a class 2 HDAC based on sequence comparison as well as functional data. Using chromogenic and fluorogenic ester substrates we show that HDACs such as FB188 HDAH indeed have esterase activity that is comparable with those of known esterases. Similar results were obtained for human HDAC1, 3 and 8. Standard HDAC inhibitors were able to block both activities with similar IC₅₀ values. Interestingly, HDAC inhibitors

such as suberoylanilide hydroxamic acid (SAHA) also showed inhibitory activity against porcine liver esterase and *Pseudomonas fluorescens* lipase. The esterase and the amidohydrolase activity of FB188 HDAH both appear to have the same substrate specificity concerning the acyl moiety. Interestingly, a Y312F mutation in the active site of HDAH obstructed amidohydrolase activity but significantly improved esterase activity, indicating subtle differences in the mechanism of both catalytic activities. Our results suggest that, in principle, HDACs may have other biological roles besides acting as protein deacetylases. Furthermore, data on HDAC inhibitors affecting known esterases indicate that these molecules, which are currently among the most promising drug candidates in cancer therapy, may have a broader target profile requiring further exploration.

Key words: amidohydrolase, *Bordetella/Alcaligenes*, esterase, fluorogenic assay, histone deacetylase, lipase.

INTRODUCTION

HDACs (histone deacetylases) and HATs (histone acetyltransferases) are key enzymes in the regulation of gene expression in eukaryotic cells. HATs and HDACs catalyse the addition and the removal of acetyl moieties from the ϵ -amino groups of lysine residues near the N-termini of histones respectively. In general, increased levels of histone acetylation are associated with increased transcriptional activity, whereas decreased levels of acetylation are associated with repression of gene expression [1–3]. The activity of HATs and, in particular, of HDACs, both affect angiogenesis, cell-cycle arrest, apoptosis, terminal differentiation of different cell types and the pathogenesis of malignant disease [4]. Not surprisingly, a number of HDAC inhibitors are promising antitumour agents. Several drug candidates are currently in phase I–III clinical trials [5–8] with SAHA (Zolinza) being recently approved for the treatment of cutaneous T-cell lymphoma (CTCL).

Four classes of deacetylase enzymes have been categorized on the basis of sequence homology and functional properties [9]: RPD3 (reduced potassium dependency 3)-like class 1 HDACs, HDA1 (histone deacetylase 1)-like class 2 HDACs and class 4 enzymes (HDAC11) are structurally related zinc-dependent enzymes, whereas class 3 enzymes comprise the structurally unrelated NAD-dependent sirtuins [10].

For a long time research on HDACs had focussed mainly on their role in transcriptional regulation. More recently, however,

data became available indicating that HDACs may also act on non-histone proteins and small molecules. Examples include HDAC6, which has tubulin deacetylase activity [11–13] and bacterial HDAHs (histone deacetylase-like amidohydrolases) [14,15].

Recently, the crystal structures of two class 1 enzymes, HDLP (histone deacetylase-like protein) from *Aquifex aeolicus* [16] and HDAC8 [17,18], as well as that of one class 2 enzyme, FB188 HDAH [19], have been solved. Based particularly on enzyme–inhibitor co-complex structures (see for example Figures 3A and 3B), a mechanism has been proposed which includes features of those from metallo and serine proteases [16]. By this mechanism (Figures 3C–3E), the active site zinc ion would bind to the carbonyl oxygen of the acetyl moiety, polarizing the carbonyl group and thereby increase the electrophilicity of the carbon. The zinc ion also binds to the oxygen of a water molecule such that the nucleophilicity of the water oxygen is increased. Analogous to the mechanism of serine proteases, the nucleophilicity of the water molecule is further increased by the negative charge of a buried Asp–His charge-transfer relay system, to which the water molecule is hydrogen bonded. The nucleophilic attack of the water molecule on the carbonyl carbon would lead to a tetrahedral oxyanion transition state which would be stabilized by the aforementioned zinc–oxygen contacts and by a potential hydrogen bond to the hydroxyl group of a tyrosine residue (Tyr³¹² in HDAH). Finally, the acetate would be released and the ϵ -nitrogen of the lysine residue would accept a proton from a second Asp–His

Abbreviations used: AMC, 7-amino-4-methylcoumarin; CypX, cyclopentyle-propionyle hydroxamic acid; HAT, histone acetyltransferase; HDAC, histone deacetylase; HDAH, histone deacetylase-like amidohydrolase (from strain FB188); HDLP, histone deacetylase-like protein; HMC, 7-hydroxy-4-methylcoumarin; MCA, 4-methylcoumarin-7-amide; NMM, N-methylmorpholine; SAHA, suberoylanilide hydroxamic acid.

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charge-transfer relay system not present in FB188 HDAH or any other class 2 enzymes [20]. Confirmation of the important role played by the aforementioned active site amino acid residues came from mutagenesis studies [16,19,21,22]. The proposed mechanism has been challenged in part by: (i) *ab initio* calculation studies [23], (ii) experiments with transition state analogue inhibitors designed to mimic the proposed oxyanion intermediate [10] and, (iii) experiments with substrates containing different acyl leaving groups [20]. Despite the huge body of data generated throughout many years of HDAC research, relatively little is known about natural substrates of different HDACs. Indeed, studies so far have focussed exclusively on amides as possible substrates. In the present study, we demonstrate that HDAC enzymes such as HDAH, HDAC1, HDAC3 and HDAC8 also have a very pronounced esterase activity that can be inhibited by known HDAC inhibitors. On the other hand, HDAC inhibitors are also active against known esterases. Specificity towards acyl moieties is similar for both amidohydrolase and esterase activities. However, mutation of the active site tyrosine residue (Tyr³¹² in HDAH) into a phenylalanine residue impairs only amidohydrolase activity but actually improves esterase activity. Taken together, our experimental results improve our understanding of the catalytic mechanism of HDACs. Furthermore, we provide the first evidence suggesting that at least certain members of the HDAC family may assume the biological role of an esterase.

EXPERIMENTAL

Synthesis of fluorogenic substrates

MCA (4-methylcoumarin-7-amide) and Boc-L-Lys(ϵ -acetyl)-MCA were purchased from Bachem. 4-Nitrophenyl acetate and all other reagents for organic synthesis were obtained from Sigma. The ϵ -propionyl-derivative of Boc-L-Lys(ϵ -acyl)-MCA was synthesized as described in [20]. The acetyl and propionyl esters of HMC (7-hydroxy-4-methylcoumarin) were synthesized using standard protocols. Briefly, 0.5 mmol of HMC was suspended in 2 ml of dioxane. After the addition of 90 μ l of NMM (*N*-methylmorpholine) and 180 μ l of acetyl chloride (or propionyl chloride) the suspension was incubated for 10 min at room temperature. After the addition of 2 ml of acetonitrile and 180 μ l of NMM the solution was shaken overnight at room temperature. The solvent was removed under reduced pressure and the residue was redissolved in ethyl acetate. After extraction with 3 $\times$ 20 ml of water the solvent was removed under reduced pressure. The product was analysed by reversed phase HPLC/MS using a Waters Alliance/MicromassZQ system and a 250 mm $\times$ 10 mm-C18 column (Jupiter, Phenomenex). Purities were > 90 % with no educt present. LC-MS (ESI): 4-methylcoumarin-7-acetate calculated for C₁₂H₁₀O₄ (M + H): 219.2. Found: 219.2. 4-methylcoumarin-7-propionate calculated for C₁₃H₁₂O₄ (M + H): 233.2. Found: 233.2.

Assays

HDAC1 and HDAC3/N-COR2 were obtained from BPS Bioscience. HDAC8 was expressed in *Escherichia coli* and purified as described in [20]. Porcine liver esterase (EC 3.1.1.1.) was from Sigma and *Pseudomonas fluorescens* lipase was purchased from Fluka. Mutant HDAH genes were generated by the QuikChange[®] method using the manufacturer's protocol (Stratagene). FB188 HDAH and its mutants were prepared as described in [14]. For fluorometric assays, all pipetting and fluorescence detection steps were performed with the help of a robotic workstation (CyBiTM-Screen-Machine, CyBio AG) including a

Figure 1 Small molecule substrates of FB188 HDAH

(A) Boc-L-Lys(ϵ -acetyl)-MCA, (B) Boc-L-Lys(ϵ -propionyl)-MCA, (C) 4-methylcoumarin-7-acetate, (D) 4-methylcoumarin-7-propionate, and (E) 4-nitrophenyl-acetate.

Polarstar fluorescence reader (BMG). Reactions were conducted in black 96-well microplates. For assays with chromogenic substrates, a UV-1601 spectrophotometer (Shimadzu) was used.

Fluorogenic assays

Amidohydrolase assays were performed similarly to the standard fluorogenic HDAC assay [24]. Briefly, 10 μ l of diluted enzyme solution (corresponding to 50 ng HDAH), 50 μ l of substrate solution in HD buffer {15 mM Tris/HCl (pH 8.0), 250 μ M EDTA, 250 mM NaCl and 0.1 % (w/v) PEG [poly(ethylene glycol)] 8000} including 50 mM potassium phosphate and 80 μ l of trypsin solution (1 mg/ml in HD buffer) were mixed and incubated at 30 °C. The release of AMC (7-amino-4-methylcoumarin) was monitored by measuring the fluorescence at 460 nm (λ_{ex} = 390 nm). Fluorescence intensity was calibrated using free AMC. For HDAC1 and HDAC3, the two-step variant of the assay was used. Briefly, 10 μ l of diluted enzyme solution and 50 μ l of substrate solution in HD buffer were mixed and incubated at 30 °C. After 45 min incubation, 80 μ l of trypsin solution was added. After a further incubation for 15 min at 30 °C, the released AMC was monitored by measuring the fluorescence at 460 nm. K_m and V_{max} values as well as IC₅₀ concentrations were determined as previously described [25]. Substrate concentrations between 0.5 and 50 μ M were used for standard K_m and V_{max} determinations. For IC₅₀ determinations a substrate concentration of 2 μ M was used.

Fluorescence-based esterase assays were performed by mixing 10 μ l of enzyme solution, 180 μ l of HD buffer (pH 8.0) and 10 μ l of substrate solution (from a 40 μ M stock of either substrate C or D, Figure 1) on ice. After switching the reaction temperature to 20 °C the release of HMC was monitored by measuring the fluorescence at 460 nm. The fluorescence intensity was calibrated using free HMC. Note that the esterase substrates (substrate C and D, Figure 1) showed substantial hydrolysis at higher temperatures.

Chromogenic esterase assay

In the standard chromogenic assay, 10 μ l of enzyme was mixed on ice with 980 μ l of HD buffer and 10 μ l of substrate solution. After switching the reaction temperature to 25 °C the release of 4-nitrophenol was monitored by measuring the absorbance

Table 1 Kinetic parameters for chromogenic and fluorogenic substrates

NA, no activity (background); nd, not determined.

Substrate	HDAH		Y312F HDAH		<i>P. fluorescens</i> lipase		Porcine liver esterase	
	K_m (μM)	$V_{\max}$ (pmol/s/mg)	K_m (μM)	$V_{\max}$ (pmol/s/mg)	K_m (μM)	$V_{\max}$ (pmol/s/mg)	K_m (μM)	$V_{\max}$ (pmol/s/mg)
A Boc-L-Lys(ϵ -acetyl)-MCA*	14.4 ± 3.0	136 ± 22	NA	NA	NA	NA	NA	NA
B Boc-L-Lys(ϵ -propionyl)-MCA	NA	NA	nd	nd	nd	nd	nd	nd
C 4-methyl-coumarin-7-acetate	0.90 ± 0.13	14100 ± 2100	1.68 ± 0.32	513000 ± 97700	1.66 ± 0.30	183 ± 41	2.84 ± 0.84	2150 ± 630
D 4-methyl-coumarin-7-propionate	31.2 ± 3.7	2220 ± 260	0.22 ± 0.02	52400 ± 4700	2.54 ± 0.23	313 ± 28	nd	nd
E 4-nitrophenyl-acetate	11.8 ± 1.8	33000 ± 4900	nd	nd	47.0 ± 0.8	4850 ± 1220	nd	nd

* named as in Figure 1.

at 405 nm. Absorbance was calibrated using free 4-nitrophenol ($\epsilon_{405} = 18500 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

RESULTS

The present study was initiated to examine a possible esterase activity of HDACs. Esters and amides are rather similar in structure and indeed a number of enzymes have been reported to exhibit dual functionalities accepting either substrate classes [26–29]. The work presented herein concentrates particularly on the HDAH from *Bordetella Alcaligenes* strain FB188 [14] as an HDAC homologue which can be easily produced in large quantities. FB188 HDAH exhibits 30 % identity over 291 residues with the first domain of human HDAC6 (HDAC6a), 35 % identity with the second domain (HDAC6b), and both enzymes share a number of functional similarities. They are zinc-dependent amidohydrolases that are able to efficiently deacetylate chicken histones, and they show the same substrate specificity towards small peptidic substrates [20,30]. In addition, FB188 HDAH is also inhibited by common HDAC inhibitors such as SAHA (suberoylanilide hydroxamic acid). Furthermore, the crystal structure of FB188 HDAH reveals the canonical fold of HDACs [19].

HDAH is an efficient esterase

Previously, we have shown that FB188 HDAH is an amidohydrolase that catalyses the release of acetate from ϵ -acetylated lysine residues in the context of various substrates including fluorogenic peptides ([14,20,25,31] and D. Riester, C. Hildmann, S. Grünwald, T. Beckers and A. Schwienhorst, unpublished work) as well as eukaryotic histones [14]. In addition, we demonstrated that the enzyme also converted non-peptidic amides such as *cis*-(+/-)-*N*-[4-(hydroxymethyl)cyclopent-2-enyl] acetamide [14]. For Boc-L-Lys(ϵ -acyl)-MCA substrates (Figures 1A and 1B) the acetyl derivative was the superior substrate with K_m and $V_{\max}$ values of $14.4 \pm 3.0 \mu\text{M}$ and $136 \pm 22 \text{ pmol/s/mg}$ respectively [20]. Boc-L-Lys(ϵ -propionyl)-MCA, however, did not show a significant conversion [20]. To test the possibility that FB188 HDAH could also act as an esterase we studied esterase activity in comparison to that of porcine liver esterase and *P. fluorescens* lipase using 4-methylcoumarin-7-acetate as a substrate (Figure 1C; Table 1). Cleavage of this substrate resulted in the release of fluorescent HMC, which was monitored by measuring the fluorescence at $\lambda_{\text{em}} = 460 \text{ nm}$ and $\lambda_{\text{ex}} = 390 \text{ nm}$. HDAH indeed proved to exhibit specific esterase activity that was comparable with or even superior to those of porcine liver esterase and *P. fluorescens* lipase (Table 1), which were used as controls. For 4-methylcoumarin-7-acetate K_m and $V_{\max}$ values of $0.90 \pm 0.13 \mu\text{M}$ and $14100 \pm 2100 \text{ pmol/s/mg}$ were measured respectively. For por-

Table 2 IC_{50} values of HDAC inhibitors monitored by effects on amidohydrolase and esterase activity

NA, no activity (background); nd, not determined.

Inhibitor	IC_{50} (μM)			
	HDAC assay*	Fluorogenic esterase assay†		
	HDAH	HDAH	<i>P. fluorescens</i> lipase	Porcine liver esterase
SAHA	0.95	0.43	3.82	NA
CypX	0.29	0.59	nd	NA

* [24].

† Based on 4-methylcoumarin-7-acetate as a substrate.

cine liver esterase the corresponding values were $2.84 \pm 0.84 \mu\text{M}$ and $2150 \pm 630 \text{ pmol/s/mg}$. For *P. fluorescens* lipase we obtained K_m and $V_{\max}$ values of $1.66 \pm 0.30 \mu\text{M}$ and $183 \pm 41 \text{ pmol/s/mg}$ respectively.

Esterase activity is dependent on the acyl moiety

In the aforementioned experiments with amide substrates, FB188 HDAH exhibited a clear preference for acetamides over the corresponding propionyl derivatives. We therefore also tested 4-methylcoumarin-7-propionate as a substrate (Table 1). For the latter substrate K_m and $V_{\max}$ values of $31.2 \pm 3.7 \mu\text{M}$ and $2220 \pm 260 \text{ pmol/s/mg}$ were measured respectively. Thus the enzyme again showed a preference for the acetyl derivative, reflected by both a 34-fold increase in K_m and a 6-fold decrease in $V_{\max}$ for 4-methylcoumarin-7-propionate.

Esterase activity is modulated by the nature of the alcoholic moiety

To show whether the alcohol moiety of the ester has a significant influence on the kinetic parameters of the ester substrates we compared the conversions of the aforementioned 4-methylcoumarin-7-acetate and 4-nitrophenyl acetate (Figure 1E; Table 1). For the latter, we measured K_m and $V_{\max}$ values of $11.8 \pm 1.8 \mu\text{M}$ and $33000 \pm 4900 \text{ pmol/s/mg}$ respectively, indicating a 13-fold increase in K_m and a 2-fold increase in $V_{\max}$.

Esterase activity can be blocked by HDAC inhibitors

To confirm that the esterase activity resides in the same active site of FB188 HDAH that is used to catalyse amidohydrolase reactions we tested esterase activity in the presence of known HDAC inhibitors (Table 2). For SAHA and CypX (cyclopentylpropionyle hydroxamic acid), IC_{50} values of $0.43 \mu\text{M}$ and

Figure 2 Histogram of amidohydrolase and esterase activities of different HDACs

Fluorogenic amide Boc-L-Lys(ϵ -acetyl)-MCA (black bars) or esters 4-methylcoumarin-7-acetate (grey bars) and 4-methylcoumarin-7-propionate (white bars) were used as substrates respectively. Human HDAC1, 3 and 8 substrate conversions in pmol/min were compared using the same enzyme and substrate concentrations but different substrates. Relative substrate conversions are depicted by arbitrarily setting the conversion of the HDAC substrate Boc-L-Lys(ϵ -acetyl)-MCA to 1 for each enzyme.

0.59 μ M were obtained using 4-methylcoumarin-7-acetate as a substrate. Comparing these IC_{50} values with that obtained in the fluorogenic HDAC assay ([24]; Table 2) it appeared that the inhibition of amidohydrolase and esterase activities were comparable for both hydroxamate inhibitors.

Interestingly, HDAC inhibitors also showed a significant inhibition of control esterases. For *P. fluorescens* lipase, SAHA showed an IC_{50} value of 3.82. For porcine liver esterase no inhibitory activity was observed for the hydroxamic acids (SAHA, CypX). In this case, however, MS275 proved to be a rather good inhibitor (results not shown). Furthermore, for a trifluoromethylketone derivative which we recently had identified in a screening program as a submicromolar inhibitor of HDAH and eukaryotic HDACs, we could also detect submicromolar inhibition of porcine liver esterase (results not shown).

Y312F mutation impairs amidohydrolase but not esterase activity

To further confirm that both amidohydrolase and esterase activities reside in the same active site and to study possible similarities in the catalytic mechanism between FB188 HDAH acting as an amidohydrolase or esterase we analysed the effect of mutations on enzyme activity (Table 1). Whereas the mutation H142N (inner charge-transfer relay system) proved to be inactive for both enzyme activities, Y312F only decreased amidohydrolase activity. Surprisingly, esterase activity was even improved in the mutant as compared with the wild-type enzyme. Using 4-methylcoumarin-7-acetate as a substrate, K_m and V_{max} values of $1.68 \pm 0.32 \mu$ M and $513\,000 \pm 97\,700$ pmol/s/mg respectively, were measured. This is less than a 2-fold increase in K_m but a 36-fold increase in V_{max} compared with the wild-type enzyme. Using 4-methylcoumarin-7-propionate as a substrate, K_m and V_{max} values of $0.22 \pm 0.02 \mu$ M and $52\,400 \pm 4\,700$ pmol/s/mg were measured respectively. This is a 4-fold decrease in K_m and a 23-fold increase in V_{max} as compared with the wild-type enzyme and the same substrate.

Human HDAC1, HDAC3 and HDAC8 also exhibit esterase activity

Next, we were interested to know whether human HDACs are also capable, in principle, of executing esterase activity. Human HDAC1, 3 and 8 substrate conversions (in pmol/min) were compared using the same enzyme and substrate concentrations but different substrates. In Figure 2 the relative substrate conversions

are depicted by arbitrarily setting the conversion of the HDAC substrate [Boc-L-Lys(ϵ -acetyl)-MCA] to 1. Using both 4-methylcoumarin-7-acetate and 4-methylcoumarin-7-propionate as substrates, we clearly demonstrated that HDAC1, HDAC3/N-COR2 and HDAC8 also have esterase activities. However, different specificities concerning the acyl moiety were observed. HDAC1 appeared to have a preference for the propionate derivative of the ester. As compared with Boc-L-Lys(ϵ -acetyl)-MCA, the ester substrates were more efficiently converted by a factor of 3 and 43 for the acetate and the propionate derivative respectively. For HDAC3/N-COR2 and HDAC8, similar to FB188 HDAH, a preference for the acetate derivative of the ester was observed. Here, as compared with the conversion of Boc-L-Lys(ϵ -acetyl)-MCA, the ester substrates were more efficiently converted by a factor of 48 and 20 for the acetate and the propionate derivatives respectively.

DISCUSSION

Esterase activity of FB188 HDAH

The major biological function of HDACs is believed to be the deacetylation of ϵ -acetylated lysines in histones and other proteins [33]. For their bacterial counterparts the biological role is much less clear. Experimental data available so far supports the idea that these enzymes may also function as deacetylases of proteins with ϵ -acetylated lysine residues [14] as well as polyamines [15]. In the present paper we present experimental evidence that HDAC-like enzymes are also very catalytically efficient esterases. Using chromogenic and fluorogenic substrates we demonstrated that FB188 HDAH indeed has esterase activity comparable with or superior to that of well-known lipases and esterases. In terms of specific substrate conversion, the catalytic properties of HDAC-like enzymes conform to the general reactivity of amides and esters: in contrast with esters, amides are known to be resonance-stabilized, as reflected by a more than 10^3 times slower reaction in OH^- -catalysed reactions [34–36]. In the present study we observed that the bacterial FB188 HDAH hydrolysed specific ester substrates with deacylation rates that exceeded those of amides by up to two orders of magnitude. Other researchers have reported on similar results obtained with certain serine proteases which also show amidohydrolase and esterase activity [34–38].

In both ester and amide substrates, FB188 HDAH has a significant preference for acetyl over propionyl moieties, presumably due to space limitations in the active-site cleft [25]. In line with this assumption, the larger 4-methylcoumarin-7-propionate is only bound well in the larger active site of the Y312F mutant, as reflected by a 142-fold decrease in the K_m value. On the other hand, comparing substrates based on 4-nitrophenol or HMC it appears that enzyme activity is less dependent on the alcohol moiety. However, as shown for amide substrates [14,16], it cannot be excluded that there may be more dramatic effects with other alcohol moieties.

Implications for the catalytic mechanism

Since standard HDAC/HDAH inhibitors such as SAHA and CypX inhibited not only amidohydrolase but also esterase activity, we tend to believe that both catalytic activities reside in the same active-site cleft. Although differences between IC_{50} values measured in different assays in general can be fairly large [39], for SAHA and CypX amidohydrolase and esterase assays the IC_{50} data differed only by a factor of 2. In addition, both the amidohydrolase and the esterase activities show the same preference concerning acyl moieties, thus arguing against the

Figure 3 Structure of the active site of FB188 HDAH and proposed catalytic mechanism

(A) Crystallographic structure of the inhibitor SAHA bound to the active site. (B) Schematic representation of the interactions between SAHA and the active site residues of FB188 HDAH. (C–E) Proposed mechanism for the deacetylation of amides (X = NH) and esters (X = O). HDAH residues are labelled.

possibility of two independent and rather different mechanisms. The view that both catalytic activities reside in the same active site gained further support from the fact that a H142N mutation in the ‘inner charge-transfer relay system’ was detrimental to both amidohydrolase activity and esterase function.

Consistent with the aforementioned facts we propose a mechanism for ester cleavage (Figures 3C–3E) that very much resembles the proposed mechanism for amide cleavage. In both cases a water molecule as the attacking nucleophile is activated by interaction with an Asp–His¹⁴² charge-transfer relay system and a zinc ion (Figure 3C). To increase the electrophilicity of the carbonyl carbon, the carbonyl bond of the ester or amide is polarized by interactions of the carbonyl oxygen with the zinc ion and with the hydroxyl group of Tyr³¹² (Figure 3C). Given the higher reactivity of esters compared with that of amides it seems plausible to assume that part of this carbonyl polarization is dispensable in the case of ester cleavage. This would explain the fact that a Y312F mutation did impair the energetically more demanding amidohydrolase activity but still allowed the enzyme to work as an esterase. Furthermore, in the originally proposed mechanism it was stressed that tyrosine would stabilize the tetrahedral oxyanion transition-state by hydrogen bonding to the carbonyl oxygen (Figure 3D). At least in the case of ester cleavage this function of tyrosine is obviously also non-essential.

Surprisingly, however, a Y312F mutation did stimulate esterase activity, particularly $V_{\max}$. As mentioned earlier, FB188 HDAH shows space limitations in the active-site cleft. These structural constraints may force the substrate and side chains of the enzyme into unfavourable conformations. In line with this assumption, the absence of a hydroxyl group in mutant Y312F would provide the active site with additional space, i.e. conformational freedom that could stimulate catalytic activity at least in the case of ester cleavage. Future crystallographic studies will have to specify the structural differences between wild-type HDAH and the Y312F mutant that presumably causes the observed differences in catalytic activity.

The fact that amidohydrolase activity is more sensitive to small structural perturbations in the active site as compared with esterase activity has also been observed in other systems. It is for example a well-known fact that amidohydrolase activity of serine proteases can be easily damaged by site-directed mutagenesis [40] or chemical modifications [41–45]. In contrast, esterase activity was much less affected and even increased in some cases, although never to the extent reported in the present paper for the Y312F mutant of FB188 HDAH. Thus it appears that hydrolases, in general, require a more finely tuned and stronger catalytic activity to hydrolyse energetically more demanding amides as compared with ester cleavage.

Implications for possible biological roles of HDAC-like enzymes and HDACs

In line with previous findings indicating that FB188 HDAH structurally and functionally resembles eukaryotic HDACs, we observed that not only FB188 HDAH but particularly also recombinant human HDAC1 and HDAC3 exhibited pronounced esterase activity that far exceeded their amidohydrolase activity in terms of molar substrate conversion. In regard to specificity, however, we noticed some differences. Whereas HDAC3/N-COR2 and HDAC8 resembled FB188 HDAH in favouring the acetyl derivative of the ester substrate, HDAC1 showed a clear preference for the propionyl derivative. It can be speculated that esterase activity may have preceded amidohydrolase activity, which later in evolution may have been acquired through mutation of an active-site residue (Tyr³¹² in HDAH). The mere existence of a pronounced esterase activity neither proves nor disproves that members of the HDAC family at present have biological roles as esterases. The discovery of esterase activity, however, could be taken as an opportunity to reconsider our current view of HDACs as specialized amidohydrolases and to motivate further research to identify biologically important esters as possible biological substrates of HDACs.

Implications for HDAC inhibitor development

It is a well-known fact that inhibitors of HDACs affect angiogenesis, cell cycle arrest, apoptosis, terminal differentiation of different cell types and the pathogenesis of malignant disease [4]. The development of HDAC inhibitors has become a very promising therapeutic approach in modern cancer research [8]. However, mass screening for HDAC inhibitors, particularly in miniaturized formats, is still impeded by the relatively modest activities of the recombinant enzymes available. Furthermore, the most common fluorogenic assay in primary screening [24] usually has to be performed as a two-step endpoint assay due to the susceptibility of most HDACs to trypsin [39]. In the light of our results it may be worthwhile to consider the esterase assay as a possible alternative [46]. In the esterase assay, substrate conversion rates are much more favourable for highly sensitive detection of small amounts of enzyme, particularly in miniaturized sample carriers. Furthermore, the esterase assay e.g. with substrates C or D (Figure 1), permits continuous monitoring of enzyme activity. At least for all hydroxamate and trifluoromethylketone inhibitors tested so far the results of both assays, for example the IC₅₀ values, are comparable. However, classes of compound may exist for which the transferability of results is not guaranteed. Whatever assays are used for HDAC inhibitor screening though, it appears mandatory to include major esterases in the inhibitor profiling. Our results indicate that at least certain hydroxamates and trifluoromethylketone inhibitors of HDACs are also very efficient lipase or esterase inhibitors and therefore could cause unwanted side-effects in the treatment of patients.

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