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Carbonic Anhydrase Activators: Human Isozyme II is Strongly Activated by Oligopeptides Incorporating the Carboxyterminal Sequence of the Bicarbonate Anion Exchanger AE1

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Abstract—Di-/tri- and especially tetrapeptides incorporating the sequence DADD present in the carboxyterminal region of the bicarbonate/chloride anion exchanger AE1 strongly activate human carbonic anhydrase (CA) isozyme II, whereas they act as more inefficient activators of isozymes I and IV. This discovery suggests that in the metabolon hCA II–AE1, the last protein plays a role both as a CA activator as well as a bicarbonate transporter. A synthesis of the tripeptide DAD and the tetrapeptide DADD is also presented together with the possible explanation why such highly acidic oligopeptides efficiently bind to hCA II but not to the closely related isozymes I and IV.

Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) inhibition with sulfonamides, discovered by Mann and Keilin[1] and its activation by different classes of compounds, reported by Leiner,[2] although simultaneous, had completely different consequences for research of these enzymes and their modulators of activity. Whereas CA inhibitors (CAIs) were extensively studied, leading to a detailed understanding of the catalytic and inhibition mechanisms, and also to several valuable drugs,[3] CA activators (CAAs) constituted a controversial issue immediately after they were first described.[4–7] Only recently, our group reported the X-ray crystallographic structures of adducts of the human isozyme hCA II with different activators, proving undoubtedly the existence of this class of modulators of enzyme activity as well as elucidating their mechanism of action at molecular level.[8,9]

The very recent report[10] that some CAAs (such as phenylalanine and imidazole) administered to experimental animals may produce an important pharmacological enhancement of synaptic efficacy, spatial learning and memory, proves that this class of relatively unexplored enzyme modulators may have pharmacological applications in conditions in which learning and memory are impaired, such as for example Alzheimer's disease or aging. One must also mention that it was previously reported that the levels of CA are significantly diminished in the brain of patients affected by Alzheimer's disease,[11] and these facts strongly support the involvement of different CA isozymes in cognitive functions.[10,11]

Recently Vince and Reithmeier[12] showed that the human chloride/bicarbonate anion exchanger (AE1) possesses a binding site within its 33 residue carboxyl-terminal (Ct) region for the rapid isozyme CA II. The amino acid sequence comprising this CA II binding site was determined by peptide competition and by testing the ability of truncation and point mutants of the Ct sequence to bind CA II with a sensitive microtiter plate binding assay. A synthetic peptide consisting of the entire 33 residues of the Ct (residues 879–911) region could compete with a GST fusion protein of the Ct (GST-Ct) for binding to immobilized CA II, while a peptide consisting of the last 16 residues (896–911) could not.[12] A series of truncation mutants of the GST-Ct showed that the terminal 21 residues of AE1 were also not required for binding CA II. Removal of four additional residues (887–890) from the Ct region resulted in loss of CA II binding.[12] Acidic residues in this region (D887ADD) on the other hand, were critical for

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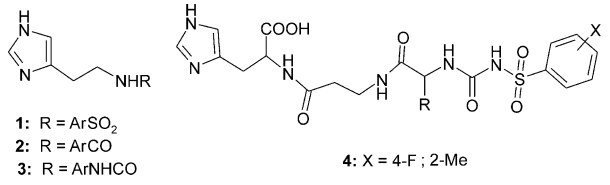
binding since mutating this sequence to DAAA, AAAA, or NANN caused loss of CA II binding. Tethering of CA II to an acidic motif within the Ct of anion exchangers was hypothesized to be a general mechanism for promoting bicarbonate transport across cell membranes.[12] This may be the case indeed, but we prove here that the tetrapeptide DADD as well as some related di/tripeptides (such as for example DAD, DDD, DA or DD) act as very potent activators of isozyme CA II and are involved in the interaction with the anion exchanger AE1 mentioned above. It is thus possible that in the complex between hCA II and AE1, the last protein acts as a natural activator of this isozyme, facilitating thus both formation and transport of bicarbonate in the erythrocytes.

Although most CAAs reported up to now[13–15] were designed by considering histamine as a lead molecule[8] (such as, for example, derivatives **1**–**3**), amino acids as well as oligopeptides were also shown to possess such a biological activity.[5,16] Recently, carnosine (β-Ala-His) derivatives of type **4** were reported to possess interesting CA activatory properties.[17] Some structurally related arylsulfonylureido-tetrapeptides of type **5** were also prepared, in order to obtain CAAs incorporating a modified tetrapeptide scaffold. Many of these tri-/tetrapeptide derivatives proved to be efficient in vitro activators of three CA isozymes, (hCA I, hCA II, and bCA IV), for which some of the new compounds showed affinities in the 1–40 nanomolar range (h = human; b = bovine isozymes).[17]



1: R = $ArSO_2$
2: R = ArCO
3: R = ArNHCO

4: X = 4-F ; 2-Me

5: X = 4-F; 2-Me

Chemistry

All amino acid and oligopeptides investigated as CAAs (Table 1) were commercially available, except for DAD and DADD.[18] Thus, a strategy to prepare these two derivatives has been developed, involving both Boc- as well as Fmoc-solution chemistry, in addition to other classical protecting groups of the carboxy moieties of these highly acidic tri/tetrapeptides (Scheme 1).

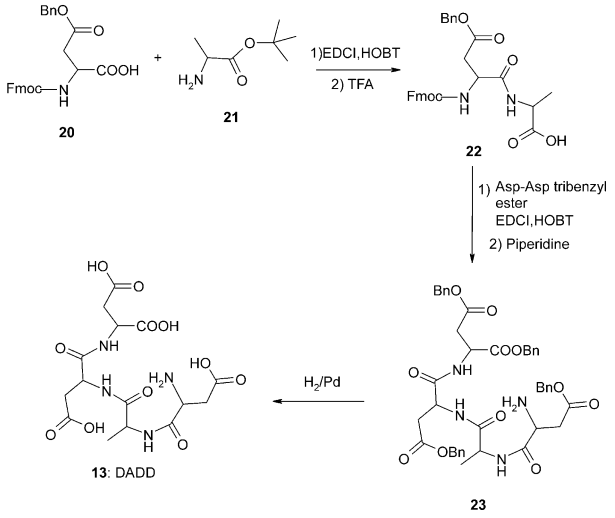
In order to prepare the tripeptide **11** (DAD), Boc-Ala was coupled with aspartic acid dibenzyl ester in the presence of carbodiimides and hydroxybenzotriazole, and the obtained intermediate was first deprotected at the aminoterminal moiety (with trifluoroacetic acid, TFA) and then coupled with *N*-Boc aspartic acid β-benzyl ester, again using the same coupling conditions. The obtained intermediate was then deprotected both at the amino (TFA) as well as carboxy (hydrogenation) moieties, affording the desired tripeptide DAD.[13–17]

The tetrapeptide **13** has been synthesized starting from *N*-Fmoc-aspartic acid β-benzyl ester **20** which was coupled with alanine *tert*-butyl ester in the presence of EDCI/hydroxybenzotriazole. After removal of the carboxy group protection (with TFA), the intermediate **22** was reacted in the same conditions with Asp-Asp tribenzyl ester (obtained from Asp-Asp and benzyl alcohol/TsOH) followed by the removal of the Fmoc moiety with piperidine. The tetrabenzyl ester **23** was converted to the desired tetrapeptide **13** by using the standard deprotection conditions for the benzyl group, that is,

Table 1. CA isozymes I, II and IV activation with standard CAAs (histamine, histidine, carnosine) and amino acids/oligopeptides investigated in the present paper of types **6**–**19**

No.	Sequence[a]	K_A[b] (μM)		
		hCA I[c]	hCA II[c]	bCA IV[d]
Histamine	—	2	125	41
Histidine	—	4	113	39
Carnosine (β-AlaHis)	—	1.3	35	18
6	A	54	150	66
7	D	48	130	60
8	DA	40	33	55
9	DD	39	21	51
10	AA	50	110	62
11	DAD	38	10	48
12	DDD	40	11	45
13	DADD	36	0.2	46
14	DDDD	35	0.1	43
15	Boc-D	> 200	> 200	> 200
16	Boc-DD	> 200	> 200	> 200
17	Boc-DDD	> 200	145	> 200
18	Boc-DADD	> 200	131	> 200
19	Boc-DDDD	> 200	124	> 200
	Ac-DA	> 200	> 200	> 200
	Ac-DD	> 200	> 200	> 200

[a]One letter amino acid code; Boc = *tert*-butoxycarbonyl.
[b]Mean from at least three determinations by the esterase method.[20] Standard error was in the range of 5–10%.
[c]Human cloned isozyme.
[d]Purified from bovine lung microsomes.



Scheme 1.

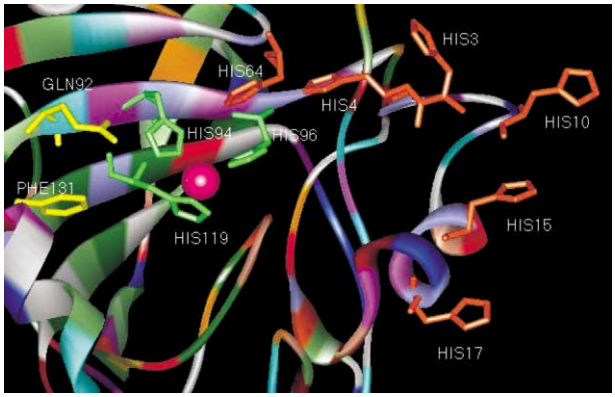


Figure 1. hCA II active site. The Zn(II) ion (central pink sphere) and its three histidine ligands (in green, His 94, His 96, His 119) are shown. The histidine cluster, comprising residues His 64, His 4, His 3, His 17, His 15 and His 10, is also evidenced, as this is considered to play a critical role in binding activators of the type **6**–**14**, reported in the paper as well as the carboxyterminal part of the anion exchanger AE1. The figure was generated from the X-ray coordinates reported by this group (PDB entry 4TST).[8]

catalytic hydrogenation (Scheme 1). The Boc-derivatives **15**–**19** were obtained from the corresponding amino acid/oligopeptides and Boc-On, whereas the acetylated derivatives by reaction of the corresponding dipeptides with acetic anhydride.[13–17,19]

CA activation

Activation data against three CA isozymes with alanine, aspartic acid and diverse di-, tri- or tetrapeptides incorporating them are shown in Table 1, together with data of standard CAAs, such as histamine, histidine or carnosine. One may see that both aspartic acid as well as alanine are weaker CAAs as compared to histamine, histidine or carnosine,[5] activators previously investigated by us and used thereafter for the design of nanomolar CAAs.[13–15,17] Di-, tri- and tetrapeptides incorporating these two amino acids, of type **8**–**14** on the other hand, showed an increased affinity for the CA active site, but important differences between the three isozymes were detected. Thus, derivatives **8**–**14** showed a rather undifferentiated affinity of 35–50 μM against hCA I and of 43–62 μM against bCA IV, which was not so different from the affinity showed by the two parent amino acids, Ala and Asp. The corresponding *tert*-butoxycarbonylated derivatives **15**–**19** did not possess at all CA activatory properties against these two isozymes.

Very different was the behavior of all these activators against hCA II. Thus, the parent amino acids act as rather weak CAAs, with activation constants of 130–150 μM. Similarly weak activatory properties showed the alanyl-alanine (AA) dipeptide **10**, but not the other di-/tri-/tetrapeptides investigated here, which all incorporated acidic amino acid moieties (aspartyl residues). Thus, an almost 5-fold increase of affinity was observed for the two dipeptides (DA and DD) as compared to the parent amino acids, whereas the two investigated tripeptides (DAD and DDD) showed a further 2–3-fold increase of affinity. This increase was indeed drastic for the tetrapeptides DADD and DDDD, which show affinity of 0.1–0.2 μM against hCA II, being thus very active CAAs. Furthermore, the Boc-derivatized compounds **15**–**19** or the *N*-acetylated dipeptides Ac-DA and Ac-DD (obtained from the corresponding dipeptides and acetic anhydride) exhibited much weaker CA activatory properties as compared to the corresponding deprotected compounds (Table 1).

Two main conclusions may be drawn from the above data. First, this is the first time that activators possessing higher affinity for hCA II than for hCA I/bCA IV are detected. Indeed, in many previous series of derivatives investigated by us, the range of affinity of a given activator for the three isozymes was hCA I > bCA IV >> hCA II.[13–15,17] For this small series of oligopeptide CAAs, the affinity is in the order: hCA II > hCA I > bCA IV. Second, one of the best activators of this series, DADD, possesses just the sequence recently evidenced to be critical for the binding of hCA II to the bicarbonate/chloride anion exchanger AE1.[12] Our data strongly suggest that in the complex formed by the two proteins, the carboxyterminal part of AE1 actually has the function of a natural CAA, facilitating catalysis and formation of bicarbonate, which is then transported by the AE1 out of the red cell. The two enzymes form in this way a metabolon, a weakly associated complex of sequential metabolic enzymes.[21] We want to stress again that the CA II activating properties of the Ct part of AE1 has not been taken into consideration up to now.

As also shown by the work of Reithmeyer's group,[12,21] basic amino acid residues at the aminoterminal part of CA II are involved in binding of the carboxyterminal part of AE1, and more precisely of the DADD sequence. In Figure 1, some of the probable residues involved in such a binding are shown, and they clearly include the histidine cluster of hCA II,[8] comprising residues 64, 4, 3, 10, 15 and 17. These histidine residues (or at least some of them) should easily interact with the highly acidic oligopeptides of the type investigated here, and more precisely with DADD or DDDD (which showed a high affinity only for this isozyme). The interaction between the positively charged imidazolium moieties of the enzyme and the carboxylate groups of the activators generated in this way, may explain the high affinity of this type of CAAs for CA II, as compared to their rather low affinity for CA I and bCA IV (which do not possess such a histidine cluster).[8] Bound at the entrance of the hCA II active site, the activators facilitate catalysis by promoting the rate-determining step of the catalytic cycle, that is, a proton transfer reaction from the zinc-bound water molecule to the environment. The proton shuttling moiety of these CAAs is not clear at this point, but it may be the aminoterminal group of the activator, since the Boc-derivatized compounds **15**–**19** showed drastically reduced activatory properties. A possible participation of the carboxylate moieties in shuttling protons should also not be excluded, since the pK_a of these functionalities when bound within the active site of the enzyme may be quite different of those in solution.

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