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Carbonic anhydrase II as host protein for the creation of a biocompatible artificial metathesase†

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An artificial metathesase results from incorporation of an Hoveyda-Grubbs catalyst bearing an arylsulfonamide anchor within human carbonic anhydrase II. The optimization of the catalytic performance is achieved upon combining both chemical and genetic means. Up to 28 TONs were obtained within four hours under aerobic physiological conditions.

Artificial metalloenzymes result from the incorporation of an abiotic cofactor within a host protein.[1] With biomedical applications in mind, it would be desirable to capitalize on a host protein which is overexpressed on the surface of cancer cells. Accumulation of the abiotic cofactor, which displays high affinity for the latter protein, may allow to site-specifically uncage a drug.[2] In this context, the ring-closing metathesis (RCM) is an attractive reaction as unactivated diolefins can be viewed as bioorthogonal. Furthermore, the intramolecular nature of the RCM may facilitate the reaction under highly dilute aqueous conditions.[3] Herein, we report on our efforts to exploit human Carbonic Anhydrase II (hCA II hereafter) for the creation of a biocompatible artificial metathesase, Scheme 1. Certain forms of cancer overexpress hCA IX, a membrane bound variant of hCA. These arylsulfonamide binding proteins are thus privileged targets for cancer therapy.[2,4a]

Introduction of an arylsulfonamide-anchor on an Hoveyda-Grubbs 2nd generation-type catalyst ensures its localization within carbonic anhydrase.[4] For this purpose, complexes **Boc-1**, **Boc-2** and **Boc-3**,[5] were deprotected *in situ* and reacted with 4-sulfamoylbenzoic acid to afford the corresponding sulfonamide-bearing metathesis cofactors **1**, **2** and **3**, Scheme 2 (see ESI† for full Experimental details).

The catalytic performance of the artificial metathesases was evaluated using the ring-closing metathesis of *N*-tosyl diallylamine in the presence of 1 mol% ruthenium. To ensure an homogeneous mixture, water/DMSO (9/1) was selected.

Comparison of catalysts **1–3** in the absence of hCA II at pH 6.0 in the presence of 0.1 M $MgCl_2$ reveals that the bulkiest catalyst **2** outperforms catalysts **1** and **3** (Table 1, entries 1–3). The same trend is observed upon incorporation of the cofactors **1–3** into WT hCA II (Table 1, entries 4–6). Catalyst **2** was thus selected for further optimization. With no $MgCl_2$ added and at pH 7.0, catalyst **2** and the corresponding metathesase **2** ⊂ WT hCA II afforded 23 and 14 turnovers after four hours at 37 °C. Performing catalysis under strict exclusion of oxygen yielded very similar results.

Reactions carried out at pH 7.0 and in the presence of 154 mM NaCl (corresponding to physiological conditions) yielded 32 and 21 TONs for **2** and **2** ⊂ WT hCA II respectively (Table 1, entries 15 and 16). As can be appreciated, the TON of the catalyst is pH dependent, both in the presence and in the absence of hCA II. The best performance is obtained at lower pH and high salt concentration (Table 1, entries 12 and 13). As for other artificial metalloenzymes, we do not believe that the pI of the host protein influences significantly the catalyst performance.[6]

Compared to the other four artificial metathesases reported to date,[3a–e] the system presented herein presents the following advantageous features (Table 2): (i) it does not require an inert atmosphere; (ii) the substrate concentration is the lowest of all systems reported to date; (iii) except for the metathesase based on FhuA (which requires SDS, a surfactant), it displays the highest turnover frequency and (iv) it catalyses RCM at pH 7.0, temperature 37 °C and physiological [NaCl] concentrations. These results thus suggest that WT hCA II is a suitable host for the creation of artificial metathesases operating under physiological conditions and at low catalyst concentrations (*i.e.* 10 μM).

To gain insight into the localization of *rac*-**2** within WT hCA II, both enantiomers were docked using the GOLD program,[7] Fig. 1. As can be appreciated, the cofactor fits nicely within the cone-shaped binding funnel of hCA II, presenting its alkylidene moiety at the surface of the protein (Fig. 1). With the aim of improving the TON of the artificial metathesase, residues I91, F131, L198, K170 were subjected to site-directed mutagenesis (Table 1, entries 20–27). Lypophylic, polar and potentially coordinating aminoacid residues were engineered into these positions. A selection of mutants tested is presented in

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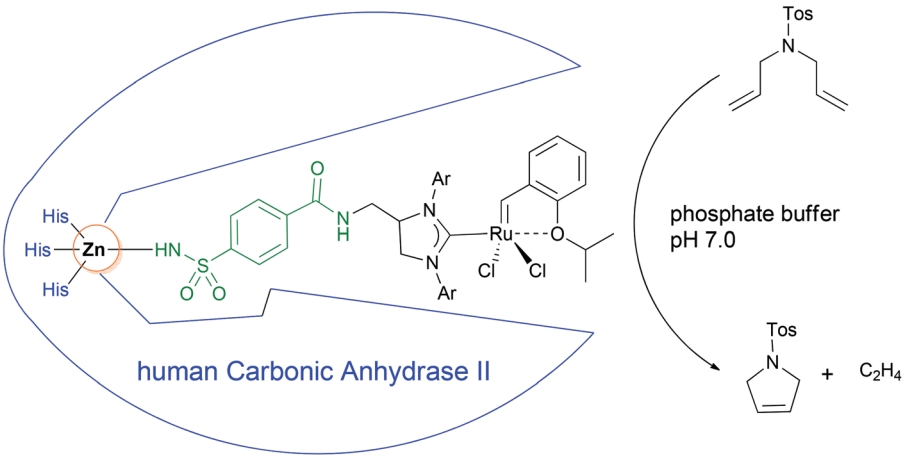
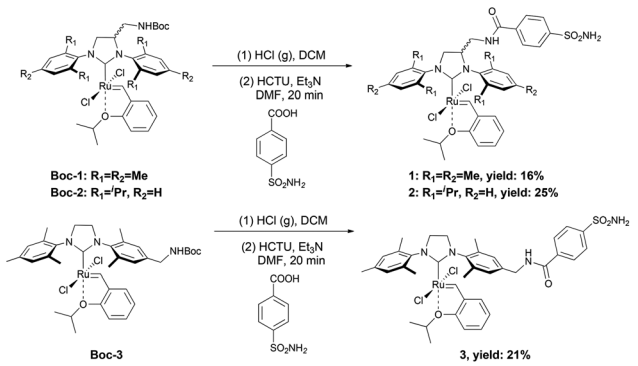
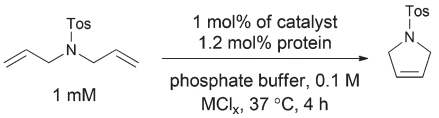


Scheme 1 Artificial metalloenzyme for ring-closing metathesis. Tethering an arylsulfonamide anchor (green) to an Hoveyda-Grubbs type catalyst (black) ensures the localization of the metal moiety within human Carbonic Anhydrase II (blue).



Scheme 2 Synthesis of olefin metathesase cofactors **1–3** bearing an arylsulfonamide anchor for incorporation within hCA II.

Table 1 Selected results for the ring-closing metathesis of *N*-tosyl diallylamine[a]



Entry	Catalyst	hCA II	pH	[MCl_x] mol/l	TON[b]
1	**1**	—	6.0	$MgCl_2$ 0.1	20 ± 0.4
2	**2**	—	6.0	$MgCl_2$ 0.1	48 ± 0.8
3	**3**	—	6.0	$MgCl_2$ 0.1	25 ± 0.7
4	**1**	WT	6.0	$MgCl_2$ 0.1	13 ± 1.3
5	**2**	WT	6.0	$MgCl_2$ 0.1	45 ± 2.0
6	**3**	WT	6.0	$MgCl_2$ 0.1	16 ± 1.0
7	**2**	WT	6.0	NaCl 0.2	40 ± 1.5
8	**2**	WT	7.0	NaCl 0.2	28 ± 1.1
9	**2**	—	7.0	—	23 ± 2.1
10	**2**	WT	7.0	—	14 ± 0.5
11[c]	**2**	WT	7.0	—	20 ± 2.3
12	**2**	—	5.0	$MgCl_2$ 0.5	85 ± 1.0
13	**2**	WT	5.0	$MgCl_2$ 0.5	78 ± 2.5
14	**2**	WT	6.0	—	23 ± 2.6
15	**2**	WT	8.0	—	21 ± 1.2
16	**2**	—	7.0	NaCl 0.154	32 ± 2.0
17	**2**	WT	7.0	NaCl 0.154	21 ± 1.8
18	**2**	WT	7.0	NaCl 0.5	32 ± 1.8
19	**2**	WT	7.0	NaCl 1.0	29 ± 1.2
20	**2**	I91A	7.0	—	18 ± 3.3
21	**2**	F131A	7.0	—	16 ± 1.3
22	**2**	L198F	7.0	—	18 ± 1.6
23	**2**	L198H	7.0	—	22 ± 0.1
24	**2**	L198H	7.0	NaCl 0.154	28 ± 0.6
25	**2**	L198A	7.0	—	15 ± 1.7
26	**2**	L198Q	7.0	—	14 ± 0.1
27	**2**	K170A	7.0	—	15 ± 2.0

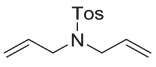
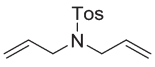
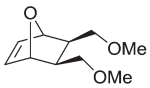
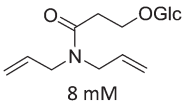
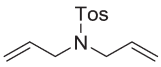
[a] Reaction conditions: [substrate] 1 mM, [catalyst] 10 μM, [hCA II] 12 μM, V_{tot} 200 μL (V_{DMSO} 20 μL), 37 °C for 4 hours. The reactions were carried out in triplicate. Very similar results were obtained under rigorous exclusion of oxygen. [b] Turnover number. [c] [substrate] 5 mM, [catalyst] 50 μM, [hCA II] 60 μM.

Table 1, entries 20–27. To our delight, **2** ⊂ hCA II L198H yielded significantly improved catalytic performance: up to 28 TONs under physiological conditions (Table 1, entry 24). Considering that both His198 and Phe198 affort similar TONs, we do not believe that the former residue coordinates to ruthenium. At this point however, it is difficult to rationalize or predict the effect a point mutations on the outcome of catalysis.

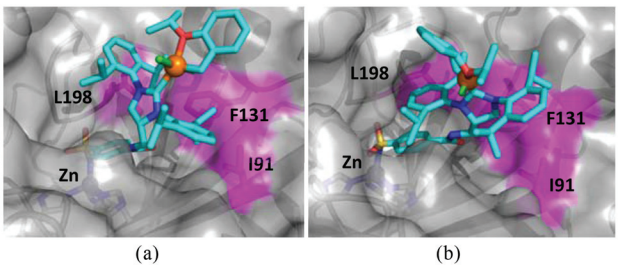
In order to ensure localisation of the cofactor **2** within hCA II under catalytic conditions, its affinity was determined using the dansylamide displacement assay.[8] Dansylamide (DNSA) displays enhanced fluorescence upon incorporation within hCA II. The non-covalent probe can be displaced by high affinity arylsulfonamide-bearing hCA II inhibitors, leading to a decrease in fluorescence.

For DNSA, we obtained a K_d = 4.83 μM and 17.35 μM for WT hCA II and L198H hCA II respectively. The displacement assay (see ESI† for details) yielded K_d = 90.40 nM and 205.10 nM for **2** ⊂ WT hCA II and **2** ⊂ hCA II L198H respectively, Fig. 2. Although we cannot exclude additional non-specific binding on the surface of hCA II, we feel that the exquisite specificity of arylsulfonamides most probably ensures selective binding to the Zn(His)$_3$ moiety. We tenta-

Table 2 Summary of the catalytic performance of artificial metathesases reported to date

	Hilvert[3b]	Ward[3a]	Schwaneberg[3d,e]	Matsuo[3c]	Ward
[substrate]	 5 mM	 15.21 mM	 100 mM	 8 mM	 1 mM
Reaction type	RCM	RCM	ROMP	RCM	RCM
Anchoring of Ru-cofactor	Covalent	Supramolecular	Covalent	Covalent	Dative
Host protein	MjHSP[a]	Avidin	FhuA ΔDCVF^{tev} [b]	α-Chymotrypsin	hCA II
	4 mol%	4.8 mol%	0.08 mol%	0.63 mol%	1 mol%
Temp.	45 °C	40 °C	25 °C	25 °C	37 °C
Time	12 h	16 h	68 h	2 h	4 h
pH	2	4	7	7	7
Reaction conditions	10 mM HCl, Water/ *t*-BuOH 4/1 under air	0.1 M acetate Water/ DMSO 5/1 0.5 M $MgCl_2$, under air	Water/THF 9/1 SDS 1% under N_2	Degassed 100 mM KCl, under N_2	0.1 M phosphate Water/ DMSO 9/1 under air
TON	25	20	955	20	28

[a] MjHSP: *M. jannaschii* small heat shock protein. [b] FhuA ΔDCVF^{tev}: engineered variants of the β-barrel ferric hydroxamate uptake protein component A.



(a) (b)

Fig. 1 Docked structure of (*R*)-**2** ⊂ WT hCA II (a) and (*S*)-**2** ⊂ WT hCA II (b). The ruthenium cofactor is displayed as stick and hCA II as solvent accessible transparent surface. Residues subjected to mutagenesis are highlighted in magenta.

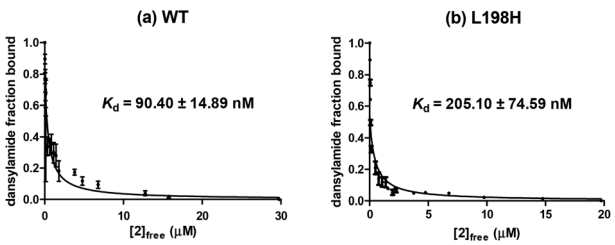


Fig. 2 Determination of the dissociation constant of **2** ⊂ hCA II using the dansylamide displacement assay: WT hCA II (a) and L198H hCA II (b) [hCA II] = 0.25 μM, [Dansylamide] = 20 μM, see ESI† for full Experimental details. All measurements were performed in duplicate.

tively attribute the modest quality of the fit to the use of a *rac*-**2** (and thus the presence of two inhibitors with potentially different affinities). Under catalytic conditions 96% and 93% of **2** is bound to WT hCA II and hCA II L198H respectively.

Conclusions

In summary, we have developed an artificial metathesase relying on hCA II as host protein. Importantly, the present system operates under aerobic physiological conditions and at low catalyst concentrations (*i.e.* 10 μM). Current efforts are directed at evaluating hCA IX, a cell-surface hCA variant which is overexpressed in various forms of cancer.

Experimental section

Typical procedure for the ring closing metathesis

The following stock solutions were prepared: catalyst **2** (200 μM) in DMSO, *N*-tosyldiallylamine (20 mM) in DMSO and hCA II isoform solution (13.3 μM) in phosphate buffer (0.1 M, pH 7.0).

To a small glass vial, the catalyst **2** stock solution (10 μL) was added to the protein stock solution (180 μL). The mixture was incubated at 37 °C for 20 min. The *N*-tosyldiallylamine stock solution (10 μL) was added and the reaction vial was placed in the incubator (37 °C) for 4 h at 200 rpm.

After completion of the reaction, 2-phenylethanol (100 μL, used as internal standard, 1 mM in H_2O) and MeOH (700 μL) were added. The mixture was transferred to an eppendorf tube and centrifuged at 14 000 rpm for 15 minutes to precipitate hCA II. The supernatant (500 μL) was transferred in an HPLC vial and water (500 μL) was added. The sample was then subjected to reversed phase HPLC for TON analysis.

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