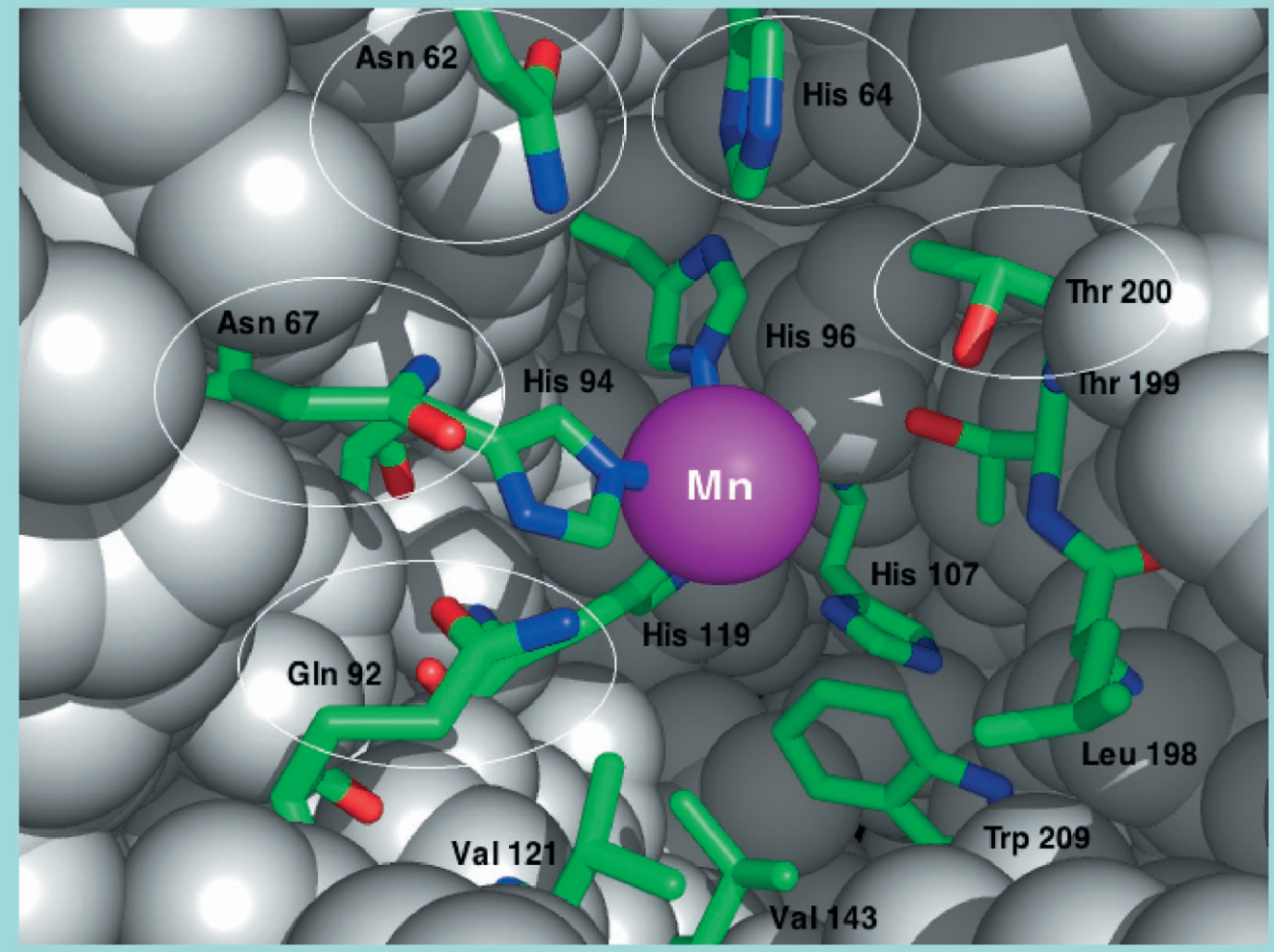
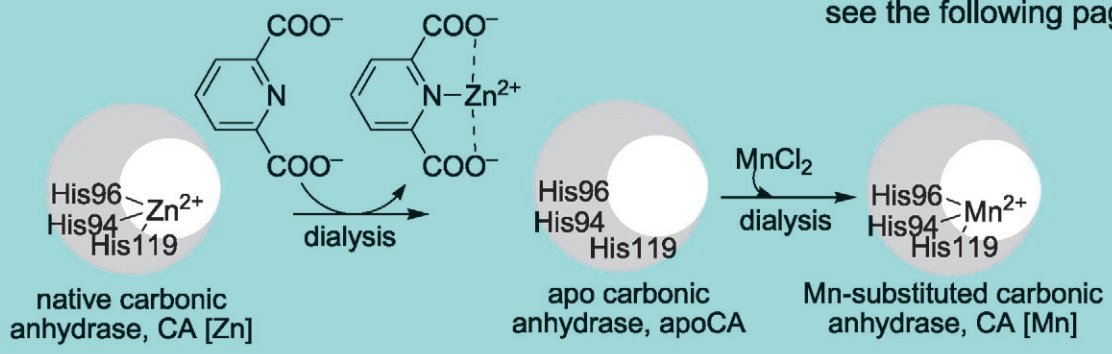
# Manganese-Substituted Carbonic Anhydrase as a New Peroxidase




For more information see the following pages



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# Manganese-Substituted Carbonic Anhydrase as a New Peroxidase

**Krzysztof Okrasa and Romas J. Kazlauskas*[a]**

**Abstract:** Carbonic anhydrase is a zinc metalloenzyme that catalyzes the hydration of carbon dioxide to bicarbonate. Replacing the active-site zinc with manganese yielded manganese-substituted carbonic anhydrase (CA[Mn]), which shows peroxidase activity with a bicarbonate-dependent mechanism. In the presence of bicarbonate and hydrogen peroxide, (CA[Mn]) catalyzed the efficient oxidation of *o*-dianisidine with $k_{cat}/K_M = 1.4 \times 10^6\,M^{-1}\,s^{-1}$, which is comparable to that for horseradish peroxidase, $k_{cat}/K_M = 57 \times 10^6\,M^{-1}\,s^{-1}$. CA[Mn] also catalyzed the moderately enantioselective epoxidation of olefins to epoxides ($E = 5$ for *p*-chlorostyrene) in the presence of an amino-alcohol buffer, such as *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES). This enantioselectivity is similar to that for natural heme-based peroxidases, but has the advantage that CA[Mn] avoids the formation of aldehyde side products. CA[Mn] degrades during the epoxidation limiting the yield of the epoxidations to $<12\%$. Replacement of active-site residues Asn62, His64, Asn67, Gln92, or Thr200 with alanine by site-directed mutagenesis decreased the enantioselectivity demonstrating that the active site controls the enantioselectivity of the epoxidation.

**Keywords:** carbonic anhydrase · enzyme catalysis · epoxidation · hydrogen peroxide · oxidation

## Introduction

Initial applications of biocatalysis involved the scale-up of naturally occurring reactions, such as glucose isomerization, protein digestion for laundry applications, or fermentation to produce natural amino acids. Later, biocatalysis extended to unnatural substrates, notably in the preparation of enantiopure pharmaceutical intermediates utilizing hydrolases or dehydrogenases.[1] An emerging trend is non-natural biocatalytic reactions—enzyme catalysis of reactions that do not normally occur in nature.[2] A commercial example of such a reaction is the pyruvate decarboxylase-catalyzed acyloin condensation of benzaldehyde and acetaldehyde to make an enantiopure hydroxyketone for ephedrine synthesis.[3] Our goal in this paper is to achieve a non-natural biocatalytic oxidation.

Most natural biocatalytic oxidations involve electron-carrying cofactors, such as nicotinamide or flavin derivatives. These cofactors allow coupling of redox reactions in cells. However, the goal of synthetic redox reactions is the direct reaction of the redox reagent with the substrate. In principle, cofactors are not needed and only complicate scale-up and reduce the stability of the biocatalyst. For example, direct oxidation of ketones to lactones by using oxygen would be ideal, but no enzymes exist for this direct oxidation. In practice, researchers continue to use cofactor-dependent enzymes. For this example, researchers use flavin monooxygenases, which use oxygen as the ultimate oxidant, but require both flavin and NADPH as cofactors. Enantioselective epoxidation of styrenes with flavin monooxygenases therefore uses whole cells to regenerate the cofactors[4] and this use of whole cells limits the concentrations. Our goal in non-natural biocatalytic oxidation is the direct use of chemical oxidants, such as hydrogen peroxide, without the use of cofactors.

Hydrogen peroxide is an ideal oxidant because it has high active oxygen content and produces water as the reduction product.[5] Since hydrogen peroxide is relatively unreactive, its use requires a catalyst. Although chemical catalysts can catalyze oxidations by hydrogen peroxide, biocatalysts should show higher stereoselectivity and remain environmentally benign.

Heme-containing peroxidases are biocatalysts that already catalyze oxidations with hydrogen peroxide.[6a] Unfortunately, these peroxidases inactivate rapidly, show moderate to low enantioselectivity, and form aldehyde side products. For example, peroxidase from *Caldariomyces fumago* (CPO)

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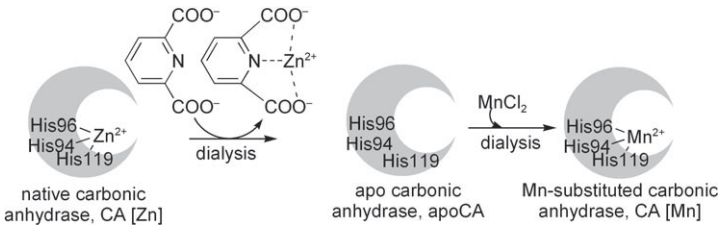
catalyzes styrene epoxidation typically with only 40–60% *ee* (*ee* = enantiomeric excess).[7,8] Aldehyde side products (up to 50%) also form during these reactions and the high enzyme cost limits its application. In some case, P450 oxidases accept hydrogen peroxide as an oxidant, but peroxide-mediated heme degradation limits the turnover number of these reactions.[6b]

In this paper, we examine a new, non-natural biocatalytic reaction catalyzed by manganese-substituted carbonic anhydrase. Carbonic anhydrase [CA, EC 4.2.1.1] is a stable zinc metalloprotein containing no cysteine residues in a mostly β-sheet structure.[9] Previous workers replaced the active-site zinc with other metals, including manganese. This manganese complex binds bicarbonate, but is a poor catalyst for dehydration to carbon dioxide. Manganese complexes often catalyze oxidations with hydrogen peroxide[10] and Burgess and coworkers reported bicarbonate-mediated oxidations with hydrogen peroxide catalyzed by manganese salts.[11] We hypothesized that the carbonic anhydrase active site could impart enantioselectivity to these manganese-catalyzed oxidations.

## Results

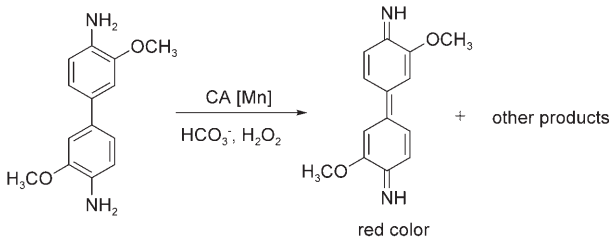
**Replacement of the active-site zinc in carbonic anhydrase with manganese**: Following literature procedures,[12] we removed the active-site zinc from carbonic anhydrase obtained from bovine erythrocytes (a mixture of isoenzymes, Scheme 1). Dialysis of carbonic anhydrase against 2,6-pyridinedicarboxylate in acetate buffer at pH 5.5 removed 90–95 mol% of the zinc as shown by inductively-coupled-plasma-atomic-emission spectrometry (ICP-AES). This amount is similar to that removed previously for bovine[12] and human[13] carbonic anhydrase. Consistent with this removal of the active-site zinc, the apo-carbonic anhydrase lost 93–97% of the original catalytic activity for the hydrolysis of *p*-nitrophenyl acetate. Similar treatment of bovine carbonic anhydrase isoenzyme II (available commercially) and human carbonic anhydrase isoenzyme II (cloned and overexpressed in *E. coli*, see below) gave the corresponding apoenzymes.




Scheme 1. Dialysis of carbonic anhydrase against a zinc chelator, 2,6-pyridinedicarboxylate, removed 90–95% of the active-site zinc. Subsequent dialysis against manganese(II) yielded manganese-substituted carbonic anhydrase (CA[Mn]). Similar procedures yielded manganese-substituted carbonic anhydrase isoenzyme II (CA II[Mn]) and manganese-substituted human carbonic anhydrase isoenzyme II (hCA II[Mn]).

Subsequent dialysis of apo-carbonic anhydrase against manganese(II) chloride at pH 6.95 yielded manganese-substituted carbonic anhydrase (CA[Mn] or CA II[Mn] for isoenzyme II and hCA II[Mn] for human carbonic anhydrase, Scheme 1). Metal analysis showed that this enzyme contained up to 80 mol% manganese and 5–10 mol% zinc. The remaining 10–15 mol% was likely to be apo-carbonic anhydrase as manganese binds to apo-carbonic anhydrase less tightly than zinc ($pK_D = < 3.4$–$4.0$ for $Mn^{II}$ vs. 12.0 for $Zn^{II}$)[13] and dissociates rapidly in the presence of zinc ($t_{1/2} =$ 27 min at pH 6.8).[12a] The samples showed 10–15% of original hydrolytic activity toward *p*-nitrophenyl acetate, likely due both to the remaining zinc and to hydrolytic activity of CA[Mn], which is 7–8% of the native carbonic anhydrase.[14]

**Efficient peroxidase activity of CA[Mn]**: CA[Mn] efficiently catalyzed the oxidation of *o*-dianisidine to the red quinonediimine with hydrogen peroxide (Scheme 2). This *o*-dianisidine oxidation is a common assay for peroxidase activity[15,20] or for peroxidase-based detection of hydrogen peroxide.[16] As control reactions, native zinc carbonic anhydrase showed < 1% of the activity of CA[Mn], while manganese(II) chloride and bicarbonate alone showed only 5% of the activity of CA[Mn]. Unlike other peroxidases,[15,20] the CA[Mn]-catalyzed oxidation of *o*-dianisidine required bicarbonate and showed only 1.5% of the activity in the absence of bicarbonate.




Scheme 2. Manganese-substituted carbonic anhydrase (CA[Mn]) catalyzed the oxidation of *o*-dianisidine by hydrogen peroxide. Reaction conditions: 25 °C, BES buffer (0.1 M, pH 7.2), CA[Mn](20 μM), sodium bicarbonate (8 mM), *o*-dianisidine (43 μM), $H_2O_2$ (400 μM). The red color was monitored at 460 nm and is apparent by eye after a few seconds.

This oxidation of *o*-dianisidine by hydrogen peroxide catalyzed by CA[Mn] is only 60–75-fold less efficient than the hydration of carbon dioxide catalyzed by native carbonic anhydrase (Table 1). We measured these apparent kinetic constants in BES buffer pH 7.2 by using variable concentrations of *o*-dianisidine (5.5–1030 μM) and constant amounts of hydrogen peroxide and sodium bicarbonate. The kinetic constants for the oxidation show an unexceptional catalytic constant ($k_{cat}$) of 17 $s^{-1}$, but a low apparent Michaelis constant ($K_M$) of 15 μM. These values give an apparent specificity constant ($k_{cat}/K_M$) of $1.1 \times 10^6\ M^{-1}\ s^{-1}$. Similarly CA II[Mn] produced a $k_{cat}$ of 140 $s^{-1}$, and a $K_M$ of 98 μM, which corre-

Table 1. Kinetic constants for reactions catalyzed by carbonic anhydrase and peroxidases.[a]

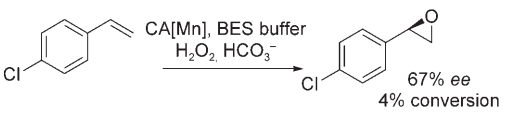
| Enzyme | Substrate | $K_M$ [$\mu$M] | $k_{cat}$ [$s^{-1}$] | $k_{cat}/K_M$ [$M^{-1}\,s^{-1}\times10^6$] |
|---|---|---|---|---|
| CA | $CO_2$ | 12000 | 1000000 | 83 |
| CA | $HCO_3^-$ | 26000 | 400000 | 15 |
| CA[Mn] | *o*-dianisidine[b] | 15 | 17 | 1.1 |
| CA II[Mn] | *o*-dianisidine[b] | 98 | 140 | 1.4 |
| HRP[a] | *o*-dianisidine | 11 | 630 | 57 |

[a] HRP = horseradish peroxidase. [b] Apparent kinetic constants for *o*-dianisidine were measured at fixed concentrations of 0.4 mM hydrogen peroxide for CA[Mn] (1.2 mM $H_2O_2$ for CA II[Mn]) and 8 mM sodium bicarbonate.

sponds to a specificity constant of $1.4\times10^6\,M^{-1}\,s^{-1}$. For comparison, carbonic anhydrase shows a high catalytic constant for the hydration of carbon dioxide, $10^6\,s^{-1}$, but a low affinity for carbon dioxide ($K_M = 12$ mM).[17] These values correspond to a specificity constant of $83\times10^6\,M^{-1}\,s^{-1}$, which is only 60–75-fold higher than the value for the CA[Mn] or CA II[Mn]-catalyzed oxidation of *o*-dianisidine. In the reverse direction (dehydration of bicarbonate), the specificity constant is lower ($15\times10^6\,M^{-1}\,s^{-1}$)[18] and only 11–14 times higher than the value for the CA[Mn] or CA II[Mn]-catalyzed oxidation of *o*-dianisidine. The active site of carbonic anhydrase contains hydrophobic patches (see below), which contribute one-hundred to one-thousand fold to the binding of hydrophobic ligands regardless of their structure.[19] It is likely that *o*-anisidine binds to this hydrophobic region.

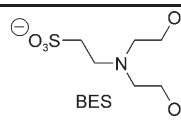
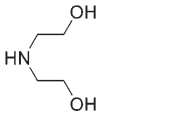
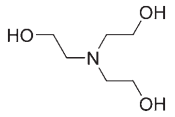
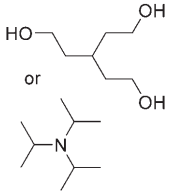
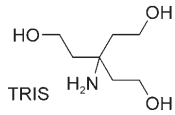
The peroxidase activity of CA[Mn] is comparable to that for true peroxidases. For horseradish peroxidase (HRP)-catalyzed oxidation of *o*-dianisidine,[20] the $k_{cat} = 630\,s^{-1}$ and $k_{cat}/K_M = 57\times10^6\,M^{-1}\,s^{-1}$, which is approximately 50-fold higher than the CA[Mn]-catalyzed reaction. Another peroxidase, vanadium chloroperoxidase (VClPO), produced a lower $k_{cat}/K_M$ of $0.31\times10^6\,M^{-1}\,s^{-1}$ (approximately 3-fold lower than for CA[Mn]), but this comparison is imperfect as the VCIPO data is for a different substrate, 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid).[21]

**Epoxidation of olefins catalyzed by CA[Mn]**: In the presence of bicarbonate, hydrogen peroxide, and BES buffer, CA[Mn] also catalyzed the enantioselective epoxidation of *p*-chlorostyrene (Scheme 3). This epoxidation was moderately enantioselective ($E = 5$ favoring the *R* enantiomer) suggesting that oxygen transfer occurs in the active site. Like the oxidation of *o*-dianisidine above, the epoxidation required bicarbonate. Replacing sodium bicarbonate with sodium phosphate or sodium acetate produced no epoxidation. Native zinc carbonic anhydrase also did not produce any epoxidation. On another hand, with a 10-fold higher concentration of manganese(II) chloride as compared to CA[Mn], we observed 1% conversion to racemic epoxide after 16 h (Table 3).




Scheme 3. Epoxidation of *p*-chlorostyrene catalyzed by CA[Mn]. Reaction conditions: 30 °C, 16 h, BES buffer (0.1 M), pH 7.2, CA[Mn] (41 μM), sodium bicarbonate (147 mM), *p*-chlorostyrene (7.4 mM), $H_2O_2$ (7.4 mM).

Table 2. Enantioselectivity of the CA[Mn]-catalyzed epoxidation of *p*-chlorostyrene in the presence of different additives.[a]

| Additive | *ee* [%] | Additive | *ee* [%] |
|---|---|---|---|
| None (water pH 7.2 or phosphate buffer) | 0 |  BES | 67 |
|  | 20 |  | 66 |
|  | 0 |  TRIS | 50 |

[a] Conditions: 30 °C, 16 h, additive (0.1 M), pH 7.2, CA[Mn] (41 μM), *p*-chlorostyrene (7.4 mM), hydrogen peroxide (7.4 mM), sodium bicarbonate (147 mM), 30 °C. Conversions ranged from 2.5–4.5%.

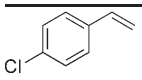
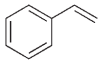
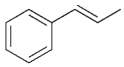
Although epoxidation also occurred without BES buffer, it was not enantioselective (Table 2). Epoxidation in bicarbonate-phosphate buffer or bicarbonate buffer produced a similar conversion to that in BES buffer, but no enantioselectivity. Triethanolamine and BES imparted the highest enantioselectivity (66–67% *ee*). Other amino alcohols, diethanolamine or tris(hydroxymethyl) aminomethane (TRIS), imparted lower enantioselectivity (20–50% *ee*), while a triol or an amine imparted no enantioselectivity.

CA[Mn] also catalyzed the enantioselective epoxidation of other olefins (Table 3). Expoxidation of styrene (56% *ee*), 5-bromobutene (45% *ee*), and *trans*-β-methyl styrene (46% *ee*) was less enantioselective than the epoxidation of *p*-chlorostyrene (67% *ee*). Epoxidation with CA II[Mn] (pure isoenzyme CA II instead of a mixture of isozymes) gave slightly higher enantioselectivity for 5-bromobutene (52% *ee*) and *trans*-β-methyl styrene (50.5% *ee*), but no change for styrene (57% *ee*) and *p*-chlorostyrene (66.5% *ee*). As reported previously by Burgess,[11] manganese(II) salts also catalyzed the epoxidation of olefins, but required approximately ten-fold higher concentrations of manganese compared to CA[Mn] and showed no enantioselectivity.

The conversions of all the CA[Mn]-catalyzed epoxidations were disappointingly low, with maximum of 12.5%, which corresponds to a turnover number of 22 (22 moles of *p*-chlorostyrene epoxide formed per mole of CA II[Mn]). These turnover numbers are similar or higher than peroxidase from *Coprinus cinereus* (Cip) (*p*-chlorstyrene TTN = 21.5; styrene TTN = 9),[22] but not as high as those for chloroperoxidase from *Caldariomyces fumago* (CPO) (styrene

Table 3. Epoxidation of terminal olefins conjugated to an aromatic or aliphatic chain by manganese-substituted carbonic anhydrases.

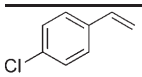
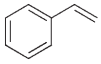
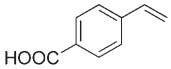
| Substrate | Catalyst[a] | $t$ [h] | *ee* [%] (config.) | Conversion [%] | TTN[b] |
|---|---|---|---|---|---|
|  | – | 16 | 0 | 0 | – |
| ″ | $MnCl_2$[c] | 16 | 0 | 1 | – |
| ″ | CA[Zn] + $MnCl_2$[c] | 16 | 0 | 2 | 3.5 |
| ″ | CA[Mn] | 2 | 66 (*R*) | 1 | 2.2 |
| ″ | CA[Mn] | 4 | 66.5 (*R*) | 1.8 | 4 |
| ″ | CA[Mn] | 16 | 67 (*R*) | 4 | 7 |
| ″ | CA II[Mn] | 16 | 66.5 (*R*) | 12.5 | 22 |
| ″ | hCA II[Mn][d] | 16 | 55 (*R*) | 6.5 | 9.5 |
| ″ | Cip[22,1] | 16 | 21 (*S*) | 43 | 21.5 |
|  | CA[Mn] | 16 | 56 (*R*) | 6 | 10.5 |
| ″ | CA II[Mn] | 16 | 57.5 (*R*) | 12 | 21 |
| ″ | Cip[22] | 16 | 35 (*S*) | 18 | 9 |
| ″ | CPO[1,23] | 5 | 49 (*R*) | 40 | 1500 |
|  | CA[Mn] | 16 | 46 (*R*,*R*) | 1.8 | 3 |
| ″ | CA II[Mn] | 16 | 50.5 (*R*,*R*) | 4.3 | 7.5 |
| ″ | Cip[22] or CPO[23] | | no epoxide formed | | |
|  | CA[Mn] | 16 | 45 (nd)[e] | 2.5 | 4.5 |
| ″ | CA II[Mn] | 16 | 52 (nd)[e] | 4 | 7 |

[a] Cip = peroxidase from *Coprinus cinereus*; CPO = chloroperoxidase from *Caldariomyces fumago*. Reaction conditions as in Scheme 3. [b] Total turnover: number of μmol of epoxide formed per μmol of enzyme, [c] 412 μM $MnCl_2$. [d] 50 μM hCA II[Mn]. [e] nd = not determined.

TTN = 1500).[23] Importantly, we did not observe any aldehyde byproducts during epoxidation, but heme peroxidases Cip and CPO formed up to 50% aldehyde byproducts. The time course of the CA[Mn]-catalyzed epoxidation of olefins showed good activity over the first four hours, followed by a rapid decrease. For example, the turnover number of CA[Mn] was equal to four over the first four hours, but increased to only seven over the next twelve hours. This result and the MS analysis below suggest that CA[Mn] degrades during epoxidation.

**Slower initial rate of epoxidation suggests that protein hinders access to manganese**: The initial rate of the CA[Mn]-catalyzed epoxidation of *p*-chlorostyrene was slower than that for nonenzymatic manganese-dependent epoxidation catalysts (Table 4). The CA[Mn]-catalyzed formation of *p*-chlorostyrene epoxide is constant initially ($t$ = 4 h) and corresponds to a turnover frequency (TOF) of 0.013 per min. This value is 440-fold lower than the corresponding manganese/bicarbonate catalyst without the carbonic anhydrase ligand (5.7 per min). The slower reaction of the CA[Mn] catalyst is consistent with the notion that the protein hinders access of the olefin to the manganese reaction center thereby imparting enantioselectivity to the epoxidation. Jacobsen's enantioselective epoxidation catalyst is also slower than free manganese/bicarbonate (0.06 per min)[24] but about 4.6 times faster than CA[Mn].

Table 4. Turnover frequency during epoxidation of styrenes catalyzed by different catalysts with hydrogen peroxide as a source of oxygen.

| Substrate | Catalyst | TOF [$min^{-1}$][a] |
|---|---|---|
|  | CA[Mn]/bicarbonate | 0.013 |
|  | chiral complex $Mn^{III}$-salen | 0.06[b] |
|  | $Mn^{II}$/bicarbonate | 5.7[c] |

[a] Turnover frequency: number of μmol of epoxide formed per μmol of enzyme per minute. [b] From reference [24]. [c] Calculated from data in reference [11]; reaction conditions: $MnSO_4$ (0.2 mM), 4-vinylbenzoic acid (20 mM), bicarbonate (100 mM), of $H_2O_2$ (10 equiv).

**Degradation of CA[Mn] limits the turnover number**: ESI-MS analysis of bovine carbonic anhydrase II manganese showed a molecular weight of 29024 D (Figure 1) which agrees with a previous report of 29025 D[25] and with the calculated molecular weight of 29024 D. This calculated molecular weight omits the zinc ion and initial methionine, and adds an acetyl group to the *N*-terminus. The zinc ion likely dissociated before MS analysis because the preparation of the protein sample involved dissolving the protein in solutions contain-

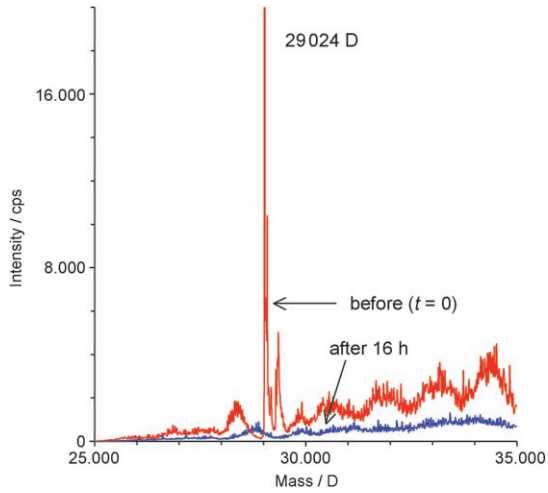



Figure 1. ESI-MS spectra of CA[Mn] before ($t$ = 0) and after 16 h of epoxidation of *p*-chlorostyrene with hydrogen peroxide. The peak at 29024 D corresponds to carbonic anhydrase, while the absence of this peak after the expoxidation indicates degradation of the protein. Reaction conditions are in the note to Scheme 3 (cps = counts per second).

ing 5–75% acetonitrile and 0.1% formic acid. Removal of the initial methionine and acetylation of the *N*-terminus are common post-translational modifications in *E. coli*.

As the epoxidation proceeds, the ESI-MS spectrum of CA[Mn] broadens and after 16 h shows no clear protein peak (Figure 1). This disappearance suggests oxidation of the protein during epoxidation. CA[Mn] was stable to added peracid or hydrogen peroxide. Incubation of CA[Mn] with *m*-chloroperoxybenzoic acid or hydrogen peroxide (0.01 M, 8 h) showed no detectable degradation or increase in mass corresponding to added oxygen atoms; this concentration of oxidants was twice as high as during epoxidation. This stability to added oxidant suggests that intermediates generated during catalysis, possibly radicals, cause the degradation of bovine carbonic anhydrase.

**Residues in the active site influence enantioselectivity**: To show that amino acid residues in the active site impart enantioselectivity, we used site-directed mutagenesis of human carbonic anhydrase isoenzyme II to replace selected amino acids in the active site by alanine. Fierke's group previously cloned and overexpressed human carbonic anhydrase II (hCA WT) in *E. coli*[26] and several X-ray crystal structures are available,[27,28] including a structure of the manganese-substituted form (Figure 2a).[29] We chose six positions in the substrate-binding site for mutagenesis: Asn62, His64, Asn67, Gln92, Thr199, and Thr200. We expected that replacing these amino acids with alanine would create more room in the active site, thereby allowing alternative *p*-chlorostyrene orientations and thus lower enantioselectivity. We also prepared Thr200His, a mutant that forms a more stable zinc-bicarbonate complex.[30]

Site-directed mutagenesis yielded genes encoding all seven desired mutants of human carbon anhydrase II. Six of these mutants showed good protein expression by SDS PAGE (data not shown), while the seventh mutant, Thr199Ala, yielded 4–5 times less protein than the others and was not further investigated. The resulting proteins produced the expected decreases in molecular weight by ESI-MS (Table 5). The calculated molecular weight for hCA II WT was 29099 D, while the experimental ESI-MS showed 29098 or 29095 D. Drift of the apparatus from calibration likely causes such 1–4 mass unit variation in the experimental molecular weights.

All six mutants catalyzed the hydrolysis of *p*-nitrophenyl acetate. His64Ala showed the same hydrolytic activity as wild type, while the other mutants showed lower activity (Table 5). His64Ala, Gln92Ala, and Thr200Ala produced 70% of wild-type activity, while

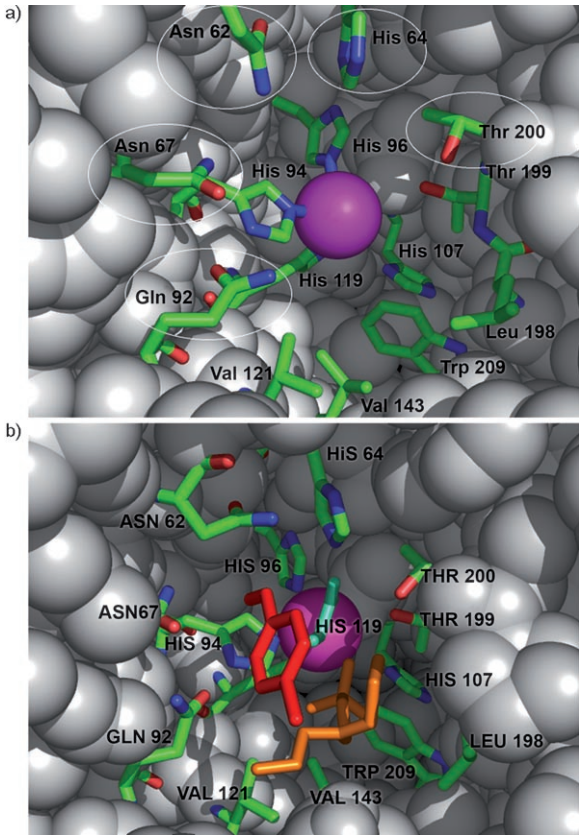



Figure 2. Structures of the active site of manganese-substituted human carbonic anhydrase II. a) X-ray crystal structure. The surrounding protein atoms are illustrated by a grey space-filling representation and the manganese ion is a violet ball. Amino acids nearest the active site are shown as sticks in CPK colors. White circles mark the five amino acids that were replaced with alanine by using site-directed mutagenesis (see text). b) Computer model created by adding peroxycarbonate (pastel blue sticks), BES (orange sticks) and *p*-chlorostyrene (red sticks) to an X-ray crystal structure of manganese-substituted human carbonic anhydrase.[29] The peroxybicarbonate lies closest to the favored face of *p*-chlorostyrene for epoxidation.

Table 5. Calculated and experimental molecular weights, hydrolytic activity, and enantioselectivity of *p*-chlorostyrene epoxidation of hCA II mutants.

| Mutant | Molecular weight of enzyme [Daltons] | | Hydrolytic activity [μmol min$^{-1}$ mg$^{-1}$] | *ee* [%][c] |
|---|---|---|---|---|
| | Calculated [difference: mutant vs. WT][a] | Determined by ESI-MS [difference: mutant vs. WT][b] | | |
| hCA II WT | 29099 | 29095 | 0.21 | 55 |
| Asn62Ala | 29056 (43) | 29052 (43) | 0.01 | 6 |
| His64Ala | 29033 (66) | 29029 (66) | 0.21 | 13 |
| Asn67Ala | 29056 (43) | 29053 (42) | 0.16 | 33 |
| Gln92Ala | 29042 (57) | 29038 (57) | 0.14 | 31 |
| Thr200Ala | 29069 (30) | 29066 (29) | 0.15 | 0 |
| Thr200His | 29135 (36) | 29132 (37) | 0.04 | 32 |

[a] Calculated molecular weights from the amino acid sequences omit the initial methionine and the zinc ion, but add an acetyl group. [b] Molecular weights of all samples were measured on the same day. Samples run on another day showed small differences in experimental values (e.g., wild-type hCA II 29097.52), are likely due to a drift in the instrument calibration. [c] Conditions as in Scheme 3. Epoxidation with hCA II[Mn] wild type used 50 μM of protein solution and gave 6.5% conversion. Epoxidation with the hCA II[Mn] mutants used a 23.5 μM solution and gave ~1% conversion.

Asn62Ala and Thr200His produced only 4 and 20%, respectively.

After substitution of zinc with manganese in hCA II, the resulting hCA II[Mn] produced similar enantioselectivity and conversion for the oxidation of *p*-chlorostyrene (55% *ee*, 6.5% conversion) as to the bovine CA[Mn]. All six mutants of hCA II produced lower enantioselectivity than wild-type hCA II (Table 5). Asn62, His64, and Thr200 produced the largest decrease in *ee* (0–13% *ee*; a 4–9-fold decrease) and thus, appear to be the key residues that impart enantioselectivity. It also possible that these mutations alter the enantioselectivity indirectly by altering the binding of the BES buffer molecule. There was no correlation between the hydrolytic activity of zinc hCA II mutants and enantioselectivity of epoxidation catalyzed by the corresponding hCA II[Mn] mutants.

**Molecular modeling of a possible reactive complex**: Computer modeling yielded a model of a possible reactive complex for the human carbonic anhydrase II containing manganese, peroxybicarbonate, BES, and *p*-chlorostyrene (Figure 2b). Modeling started from the X-ray crystal structure of human carbonic anhydrase II[27] and first replaced the active-site zinc with manganese. Next, we added peroxybicarbonate, BES, and *p*-chlorostyrene stepwise. After each addition the new atoms were geometry optimized and then the entire complex was geometry optimized. The peroxybicarbonate bound in the same location as bicarbonate in a previous X-ray crystal structure.[29b] The BES bound to the manganese via its sulfonate group and filled a hydrophobic site formed by Val121, Val143, Leu198, and Thr209. This BES position agrees with previous X-ray structures, which showed a sulfate bound to manganese at the sulfonate location[30] and a Tris-buffer molecule bound in the hydrophobic site.[31] The BES molecule also limits the space available to bind *p*-chlorostyrene. Modeling *p*-chlorostyrene in the remaining space yields an orientation with one olefin face near (~3 Å) the active oxygen of peroxybicarbonate. This orientation is consistent with the observed preference for the (*R*)-epoxide. One side of the aromatic ring of *p*-chlorostyrene faces BES, consistent with the requirement for BES or other bulky aminoalcohol for an enantioselective epoxidation. The other side of the aromatic ring faces residues Asn 62, His 64, Asn 67, and Gln 92, consistent with the lower enantioselectivity observed after site-directed mutagenesis of these residues to the smaller alanine residue. This model is a good working hypothesis for the reactive complex and is consistent with the current experimental data.

## Discussion

A number of groups have added metal ions to metal-free proteins to create enantioselective catalysts. For example, Whitesides linked an achiral rhodium hydrogenation catalyst to the surface of albumin and demonstrated moderate enantioselectivity.[32] More recently, Ward and coworkers linked hydrogenation catalysts to streptavidin and found high enantioselectivity (up to 96% *ee*) in the reduction of olefins and ketones.[33] Van de Velde et al.[34] added a vanadate ion to the active site of phytase to create a catalyst for enantioselective oxididation of thioanisole to the sulfoxide in 66% *ee*. Hilvert introduced selenoserine into the protease subtilisin to create an enantioselective catalyst for the reduction of hydroperoxides.[35]

On the other hand, *replacement* of a metal ion in a metalloenzyme has not yet yielded an enantioselective catalyst. Yamamura and Kaiser replaced the zinc in carboxypeptidase with copper(II) to catalyze the slow air oxidation of ascorbate.[36] Bakker et al.[37] replaced the zinc in thermolysin with anions, such as molybdate, selenate, or tungstate to give an enzyme that catalyzed the nonenantioselective oxidation of thioanisoles with hydrogen peroxide.

In this paper, we showed that substitution of manganese for zinc in carbonic anhydrase converted this hydrolase to an enantioselective peroxidase. Native carbonic anhydrase catalyzes the enantioselective hydrolysis of methyl mandelate (40–51% *ee*) and of several *N*-acetyl-DL-amino acid methyl esters (>95% *ee*).[38] Substitution of manganese for zinc extends this enantioselectivity to a different reaction class. The enantioselectivity of the CA[Mn]-catalyzed epoxidation of styrenes was moderate, but comparable to or better than that for natural peroxidases. For epoxidation of *p*-chlorostyrene and styrene, CA[Mn] produced 67 and 58% *ee*, respectively. Chloroperoxidase from *Caldariomyces fumago* (CPO) produced 66 and 49% *ee*,[23,39] while peroxidase from *Coprinus cinereus* (Cip) produced 21 and 35% *ee*.[22] Wild-type horseradish peroxidase oxidized styrene with ~11% *ee*, but the His42Gln mutant produced 58% *ee*.[40]

The affinity of carbonic anhydrase for manganese is low ($pK_D = < 3.4$–4.0, corresponding to a binding enthalpy of ~3 kcal mol$^{-1}$), so that solutions of CA[Mn] inevitably contain some free manganese. Fortunately, the oxidative activity of CA[Mn] is higher that that of free manganese, so that the observed products are primarily from the CA[Mn]-catalyzed reaction. Adding up to ten equivalents of free manganese to solutions of CA[Mn] did not change the reaction rate or the product distribution.

Two advantages of CA[Mn] are a broader substrate range and the lack of aldehyde side products. CA[Mn] catalyzed epoxidation of *trans*-β-methyl styrene (46% *ee*), but this substrate was not oxidized by CPO.[23] We did not detect any aldehyde side products in the CA[Mn]-catalyzed epoxidations. In contrast, the CPO and Cip-catalyzed epoxidation of styrene formed 24[23] and 52%[22] benzaldehyde side-product. These aldehyde side products form simultaneously with epoxide during styrene epoxidation catalyzed by heme peroxidases.[22] Although the mechanism of aldehyde formation is unknown, this simultaneous formation of aldehyde suggests that it forms from a reaction intermediate of epoxidation. As the mechanism of epoxidation for the CA[Mn] percarbonate reaction differs from that for the heme peroxidases,

this different mechanism may account for the lack of aldehyde side products.

Although conversions for the CA[Mn]-catalyzed epoxidation of styrenes were only ~12%, conversions for the CPO and Cip-catalyzed expoxidations were also low: 40% for *p*-chlorostyrene and 18–40% for styrene.[22,33] ESI-MS analysis of the CA[Mn]-catalyzed epoxidation suggested that the enzyme degrades during epoxidation. Soluble manganese complexes are powerful bleaching agents when using hydrogen peroxide and may involve radical intermediates.[41] Such radical intermediates may also form in the CA[Mn]-catalyzed epoxidation and damage the protein. It may be possible to eliminate this degradation by using protein engineering, as protein engineering yielded a more than 100-fold stabilization of a heme peroxidase toward oxidative degradation.[42]

Because of this catalyst degradation, organometallic catalysts are currently the best synthetic reagents for enantioselective epoxidation of olefins. Chiral $Mn^{III}$-salen complexes yield up to 99% *ee* for *cis*-disubstituted, tri- and tetra-substituted alkenes,[43] but the best results require less desirable oxidants, such as iodosyl benzene or hypochlorite. Other catalysts accept a more limited substrate range: the Sharpless-Katsuki titanium-tartrate ester[44] for allylic alcohols and the Juliá-Colonna epoxidation for α,β-unsaturated ketones.[45]

The likely mechanism of the CA[Mn]-catalyzed epoxidation of olefins involves direct transfer of oxygen from peroxybicarbonate to the olefin while both are bound in the active site of carbonic anhydrase. Previous work on the epoxidation of olefins catalyzed by free manganese and bicarbonate suggested peroxycarbonate as the key intermediate in epoxidation.[11] CA[Mn] forms a stable manganese-bicarbonate complex in the active site.[46] Hydrogen peroxide may add to the carbonyl of this bicarbonate complex and displace water, thereby forming a peroxycarbonate, which may then epoxidize a bound olefin. Computer modeling shows that both the olefin and peroxycarbonate can fit in the active site of carbonic anhydrase in an orientation consistent with the observed enantioselectivity.

Both BES and the amino acid residues in the active site are likely to be controlling the enantioselectivity of epoxidation. Omitting BES (or a similar amino alcohol) from the reaction mixture eliminated enantioselectivity. Previous work, including an X-ray crystal structure, showed that Tris buffer (an amino alcohol) binds to the active site of carbonic anhydrase.[25] Our modeling showed that such binding of BES restricts the ways that *p*-chlorostyrene can bind to the active site. Site-directed mutagenesis of six amino acid residues in the active site to a smaller amino acid residue (alanine) decreased the enantioselectivity of epoxidation. The largest decrease (from 55% *ee* in wt to 0–13% *ee* in the mutants) occurred for Asn62Ala, His64Ala, and Thr200Ala, indicating that these residues may orient *p*-chlorostyrene during catalysis.

## Experimental Section

**General**: All chemicals and enzymes were purchased from Sigma-Aldrich. Carbonic anhydrase (mixture of isoenzymes) from bovine erythrocytes (Sigma-Aldrich C-3934) was a lyophilized powder containing 93% of protein, and carbonic anhydrase isoenzyme II from bovine erythrocytes (Sigma-Aldrich C-2522) was a lyophilized powder containing 95% of protein. ESI-MS used a QSTAR quadruplole-TOF MS (Applied Biosystems) with a nanospray infusion to introduce the protein solution. Proteins were desalted by using a PorosR2 (Applied Biosystems) column before analysis first by washing with water: acetonitrile 95:5 v/v containing 0.1% formic acid and then eluting the protein with water: acetonitrile 25:75 v/v containing 0.1% formic acid. BioAnalyst Software Tool (Applied Biosystems) was used to calculate the expected molecular weight from the amino acid sequence, the average multi-isotopic mass of amino acids, and also to deconvolute the multiplycharged ion envelope to yield the experimental molecular weights (Bayesian Protein Reconstruction Tool).

**Replacement of zinc in carbonic anhydrase with manganese**: We used a modification of a literature method.[12] A dialysis tube (Spectra/Por 2, molecular weight cut off = 12–14 000 g mol$^{-1}$) containing a solution of carbonic anhydrase from bovine erythrocytes (75 mg, 2.4 μmol) in buffer (0.1 M *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), pH 7.2, 5.0 mL) was dialyzed overnight against 2,6-pyridinedicarboxylic acid (2,6-PDCA, 557 mg, 3.34 mmol in acetate buffer (500 mL, 0.1 M, pH 5.5). The tube was removed and dialyzed for 16 h against buffer (0.05 M BES, pH 6.95, 500 mL) to remove the excess of 2,6-PDCA and yield apo-carbonic anhydrase. The manganese ion was added by dialysis for 16 h against manganese(II) chloride (250 mg, 2 mmol in 500 mL of 0.05 M BES buffer, pH 6.95). To remove the traces of unbound manganese the dialysis tubing was dialyzed for 2 h against buffer (0.05 M BES, pH 6.95); this washing step should not exceed 2 h to avoid loss of manganese. (After 3 h we observed 12% less manganese and after 4 h, 25–30% less.) Total volume of manganese-substituted CA solution increased about 10% (to 5.5 mL) during the entire process.

**Determination of kinetic constants, CA[Mn]-catalyzed oxidation of *o*-dianisidine with hydrogen peroxide**: CA[Mn] (5 μl of 0.49 mM), bicarbonate solution (10 μl of 0.1 M), and the desired volume of *o*-dianisidine in acetonitrile (1–18 μl of a 0.54 mM to 8.6 mM solution) was added to BES buffer (99–82 μl, 0.1 M, pH 7.2). To start the reaction, hydrogen peroxide (10 μl of 5 mM for CA[Mn] or 15 mM for CA II[Mn]) was added. The $K_M$ and $k_{cat}$ were calculated from spectophotometrical measurements at 460 nm of the resulting red-oxidation product ($\varepsilon = 11.3$ mM$^{-1}$ cm$^{-1}$ at pH 7.2[47]) for the following *o*-dianisidine concentration: 5.4–299 μM for CA[Mn] and 33.2–1030 μM for CA[Mn]. The final concentration of hydrogen peroxide was constant during measurements and equal to 500 μM for CA[Mn] and 1500 μM for CA II[Mn].

**Determination of esterase activity of carbonic anhydrase**: Assay adapted from a previous method[48] to 96-well microplate by mixing enzyme solution (10 μl of 0.0045–0.05 mM) with assay solution (90 μl prepared from BES buffer (11 mL, 100 mM, pH 7.2), 4-nitrophenyl acetate (20 μl of 200 mM), and acetonitrile (880 μl)). During this assay 4-nitrophenyl acetate was hydrolyzed to 4-nitrophenol ($\varepsilon = 17.3$ mM$^{-1}$ cm$^{-1}$)[49] the formation of which was measured spectrophotometrically at 404 nm. Typical activity for zinc-containing bovine CA was 0.15 μmol min$^{-1}$ mg$^{-1}$ and for manganese-containing bovine CA, 0.02 μmol min$^{-1}$ mg$^{-1}$.

**CA[Mn]-catalyzed epoxidation of olefins**: A closed vial containing BES buffer (500 μl of 0.1 M, pH 7.2), sodium bicarbonate (100 μl of 1 M), manganese-substituted carbonic anhydrase (60 μl, 0.028 μmol), a methanol solution of appropriate olefin (*p*-chlorostyrene, styrene, 5-bromopentene, or methyl-β-styrene (10 μl of 0.5 M)), and hydrogen peroxide (10 μl of 0.5 M) was stirred for 16 h at 30 °C. After this time, the product and unreacted substrate were extracted with EtOAc and the extracts were analyzed by GC on the chiral column Chirasil-Dex CB 25 m × 0.25 mm (oven temperature: 150 °C for *p*-chlorostyrene and 100 °C for styrene, 5-bromopentene, or methyl-β-styrene). Retention times: 3.37 (*R*, major enantiomer) and 3.48 min for *p*-chlorostyrene oxide; 8.02 (*R*, major enantiomer) and 8.66 min for styrene oxide; 8.46 (major enantiomer) and 8.74 min for

5-bromo-1-pentene oxide; 9.46 and 9.64 min (*R,R* major enantiomer) for methyl-β-*trans*-styrene oxide. Comparing the GC traces for the reaction products with commercially available epoxide samples with known configuration established the absolute configuration of the products.

**Expression of human carbonic anhydrase II**: Plasmid encoding human carbonic anhydrase isoenzyme II with a T7 RNA polymerase promoter and an ampicillin resistance gene (pACA) was a gift from Carol Fierke.[26] Their construction of this plasmid changed the amino acid at position 2 from serine to alanine. This change has no apparent effect on the enzyme expression or catalytic properties. The ESI-MS data was consistent with an alanine at position 2. The *Escherichia coli* strain BL21-(DE3) containing pACA was grown in induction media (20 g $L^{-1}$ tryptone, 10 g $L^{-1}$ yeast extract, 80 mM NaCl, 0.4% glucose, 0.36 × M9 salts solution, 200 μM $ZnSO_4$, 0.1 g $L^{-1}$ ampicillin) at 37 °C to $A_{600}=1$. Addition of isopropyl-β-D-thiogalactopyranoside (250 μM, final concentration) and $ZnSO_4$ (690 μM, final concentration) induced protein expression. After 5–6 h incubation at 37 °C the cells were pelleted (4500 rpm for 15 min at 4 °C) and lysed by the addition of hen egg white lysozyme in a buffer containing 200 μM $ZnSO_4$, 1 mM dithiothreitol, 10 mM EDTA, and the protease inhibitors phenylmethanesulfonyl fluoride (10 μg $mL^{-1}$) and *N*-α-*p*-tosyl-L-arginine methyl ester (1 μg $mL^{-1}$). Nucleic acids were precipitated by the addition of 10% streptomycin-sulfate, followed by centrifugation (4500 rpm for 45 min at 4 °C). The supernatant, containing crude hCA II, was dialyzed (Spectra/Por 2, MWCO: 12–14000) overnight against zinc buffer (10 mM Tris-sulfate, pH 8.0, 0.1 mM $ZnSO_4$, 1 mM dithiothreitol) and then mixed with DEAE-Sephacel gel (7.5 mL of gel for 250 mL of growth media). After stirring for 15 min at 4 °C, the solution was filtered and the remaining gel washed with zinc buffer. All initial flows contained protein and should be collected. This procedure yielded 100–200 mg of hCA II per liter of culture and was typically 90–95% pure by SDS-PAGE. The final protein concentration was calculated by using $\varepsilon_{280}=5.4\times10^4\ M^{-1}\ cm^{-1}$.[50]

**Site-directed mutagenesis of human carbonic anhydrase II**: Site-directed mutagenesis was carried out by using the Stratagene QuikChange® site-directed mutagenesis kit according to the manufacturer's instructions. Polymerase chain reaction (PCR) conditions: reaction buffer (5 μl, 10×), plasmid (1 μl, 50 ng), forward primer (1 μl, 125 ng, see Table 6), reverse primer (1 μl, 125 ng, see Table 6), dNTP mix (10 μl, 20 mM), and $ddH_2O$ (31 μl) were mixed to give a final volume of 49 μl; then PfuTurbo DNA polymerase (1 μl, 2.5 U $\mu l^{-1}$) was added. PCR machine conditions: 1 segment cycle: 95 °C for 30 s; 2 segment cycle (repeated 20 times): 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 5 min; 3 segment cycle: 4 °C for 10 min. Dpn I restriction enzyme (1 μl, 10 U $\mu l^{-1}$) was added directly to each PCR reaction and thoroughly mixed by pipetting. Then each PCR reaction was centrifuged for 1 min at 13,200 rpm and incubated at 37 °C for 1 h to digest the nonmutated plasmid. Mutated DNA was transferred to *E. coli* BL21 (DE3) and protein was expressed as above for the wild-type protein.

**Molecular modeling**: Molecular modeling was performed with InsightII/Discover (Accelrys, San Diego, CA) by using the extensible and systematic force field (ESFF)[51] to allow modeling of the manganese ion. The dielectric constant was distance dependent and set to 1 to mimic the solvation effects of water. The starting structure was the X-ray crystal structure of zinc human carbonic anhydrase II at 2.0 Å resolution, PDB file ID: 4CAC.[21] We used the X-ray crystal structure of hCA II containing zinc instead of the one containing manganese due to its higher resolution. Hydrogen atoms were added (pH set to 7.2) and the atom types for His 94, 96, 119 were set to HisP (protonated histidines) to allow metal coordination. The structure was geometry optimized stepwise. First, the hydrogen atoms on water were geometry optimized, next the hydrogen atoms on the protein, then all water molecules, and finally the whole protein with water molecules. Each geometry optimization step involved a minimization with steepest descent algorithm until the RMS derivative was $<1$ kcal $mol^{-1}$ $Å^{-1}$ and then with conjugate gradient (Polak-Ribiere) algorithm until the RMS derivative was $<0.001$ kcal $mol^{-1}$ $Å^{-1}$.

The reactive complex for epoxidation was also prepared stepwise. Water molecules (#3, 55, 56, 75, and 122) were removed from the active site, the zinc atom was replaced with manganese, peroxycarbonate and BES were added to the manganese atom, and *p*-chlorostyrene was added so that the double bond was near the peroxycarbonate. Each added fragment was minimized (the rest of the protein was fixed) with steepest descent algorithm (2000 steps) and then with conjugate gradient (Polak-Ribiere) algorithm until the RMS derivative was lower than 0.001 kcal $mol^{-1}$ $Å^{-1}$. After these steps, the entire unrestricted structure was minimized as described above.

Table 6. List of primers used.

| Primer | Sequence | Length |
|---|---|---|
| forward Asn62 ALA | CCCTGAGGATCCTCAACGCGGGTCATGCTTTCAACGTG | 38 |
| reverse Asn62 ALA | CACGTTGAAAGCATGACCCGCGTTGAGGATCCTCAGGG | 38 |
| forward His64 ALA | GAGGATCCTCAACAATGGTGCGGCTTTCAACGTGGAGTTTG | 41 |
| reverse His64 ALA | CAAACTCCACGTTGAAAGCCGCACCATTGTTGAGGATCCTC | 41 |
| forward Asn67 ALA | CAACAATGGTCATGCTTTCGCGGTGGAGTTTGATGACTCTC | 41 |
| reverse Asn67 ALA | GAGAGTCATCAAACTCCACCGCGAAAGCATGACCATTGTTG | 41 |
| forward Gln92 ALA | GGCACTTACAGATTGATTGCGTTTCACTTTCACTGGGG | 38 |
| reverse Gln92 ALA | CCCCAGTGAAAGTGAAACGCAATCAATCTGTAAGTGCC | 38 |
| forward THR200 ALA | CCCAGGCTCACTGACCGCGCCTCCTCTTCTGGAATG | 36 |
| reverse THR200 ALA | CATTCCAGAAGAGGAGGCGCGGTCAGTGAGCCTGGG | 36 |
| forward THR200His | CTACCCAGGCTCACTGACCCATCCTCCTCTTCTGGAATGTG | 41 |
| reverse THR200His | CACATTCCAGAAGAGGAGGATGGGTCAGTGAGCCTGGGTAG | 41 |

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