

Histidine 64 is not required for high CO₂ hydration activity of human carbonic anhydrase II

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To test the hypothesis that histidine 64 in carbonic anhydrase II has a crucial role as a 'proton shuttle group' during catalysis of CO₂-HCO₃⁻ interconversion, this residue was replaced by lysine, glutamine, glutamic acid and alanine by site-directed mutagenesis. All these variants turned out to have high CO₂ hydration activities. The *k*_{cat} values at pH 8.8 and 25°C were only reduced by 1.5–3.5-fold compared to the unmodified enzyme. These results show that intramolecular proton transfer via His 64 is not a dominating pathway in the catalytic reaction. The variants also catalyze the hydrolysis of 4-nitrophenyl acetate. The p*K*_a values for the activity-controlling group are between 6.8 and 7.0 for all studied forms of the enzyme except the Glu 64 variant which shows a complex pH dependence with the major p*K*_a shifted to 8.4

Carbonic anhydrase; Site-directed mutagenesis; Proton transfer; Catalytic mechanism

1. INTRODUCTION

Carbonic anhydrase isoenzymes of type II are extraordinarily efficient catalysts of the reversible reaction CO₂ + H₂O ⇌ HCO₃⁻ + H⁺ [1]. During catalysis, protons must be transported rapidly between the active site and the reaction medium. This proton transfer reaction is complex and seems to comprise an intramolecular transfer from a zinc-bound water molecule to a 'proton shuttle group' in the enzyme molecule and a subsequent transfer from this group to surrounding buffer molecules [1,2]. We have previously proposed that His 64 functions as the proton shuttle group [3]. To test this hypothesis we decided to investigate variants of human carbonic anhydrase II (HCAII) where His 64 has been replaced by lysine, glutamine, alanine or glutamic acid. Therefore, we have isolated cDNA encoding HCAII and determined its nucleotide sequence. We have also constructed

an expression system for production of active HCAII in *Escherichia coli*. Here, we report that all the studied enzyme variants produced by site-directed mutagenesis have high CO₂ hydration activities. These results show that His 64 is not an obligatory proton shuttle group as previously hypothesized. However, the catalytic activity and the pH-rate profile depend to some extent on the nature of the side chain in position 64.

2. MATERIALS AND METHODS

A human fetal liver cDNA library was obtained from Clontech Laboratories and cDNA encoding HCAII was isolated using standard cloning techniques, and sequencing according to the Sanger dideoxy chain termination method using 2'-deoxyadenosine 5'-[α-³⁵S]thiotriphosphate as tracer [4]. For in vitro site-directed mutagenesis we have used the Amersham system which is based on the method of Taylor et al. [5]. The mutations were verified by nucleotide sequencing. Unmutated and mutated HCAII cDNA were expressed in an *lon*⁻ strain of *E. coli* from a plasmid containing the *tac* promoter and the *lac* repressor gene. Details of the expression system will be published elsewhere. The cells were grown at 37°C in Luria broth medium supplemented with 10 μM ZnSO₄, and protein syn-

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thesis was induced by the addition of 0.5 mM isopropyl- β -D-thiogalactopyranoside. Modified and unmodified carbonic anhydrases were purified from *E. coli* lysate by affinity chromatography according to Khalifah et al. [6]. Enzyme concentrations for all variants were estimated spectrophotometrically at 280 nm using $A_{280}^{1\%} = 18.7 \text{ cm}^{-1}$ [7] and $M_r = 29300$ [8]. Amino acid sequences were determined in an Applied Biosystems model 477A sequencing system. Analytical isoelectrofocusing was performed at 4°C in precast polyacrylamide gel plates (LKB), pH range 5.5–8.5, using an LKB Multiphore unit. The pH was measured with a surface glass electrode (Ingold M2145).

Initial rates of CO_2 hydration were measured in a Hi-Tech stopped-flow apparatus at 25°C by the changing pH-indicator method [3,9]. The buffer-indicator system was 1,2-dimethylimidazole/metacresol purple monitored at 578 nm, and the ionic strength was kept at 0.1 M with Na_2SO_4 . The hydrolysis of 4-nitrophenyl acetate was monitored spectrophotometrically at 348 nm and 25°C using a substrate concentration of 0.4 mM which is well below K_m for HCAII from red blood cells [10]. We have also assumed a high K_m for the different cloned enzyme variants. The apparent second-order rate constants, $k_{\text{enz}} (= k_{\text{cat}}/K_m)$, for the catalyzed reactions were calculated from initial rates after subtraction of the nonenzymic reaction using $\Delta\epsilon_{348} = 5.15 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [10].

3. RESULTS AND DISCUSSION

The NH_2 -terminal amino acid sequence of cloned HCAII was found to be $\text{H}_2\text{N-Ser-His-His-Trp-}$ showing that the initiator methionine residue has been enzymatically removed by *E. coli*. Thus, cloned HCAII differs slightly from the red cell enzyme, since the α -amino group of the NH_2 -terminal serine residue of the latter is blocked by an acetyl group [11]. This difference is reflected in a difference of 0.2 unit in isoelectric points as shown in table 1 which also lists the isoelectric points of the variants.

The CO_2 hydration activities of red cell HCAII, the unmodified, cloned enzyme and the variants were measured at pH 8.8, the results being summarized in table 1. The kinetic parameters for the unmodified, cloned enzyme do not differ significantly from those of the red cell enzyme. All the variant enzymes have quite high CO_2 hydration activities showing a decrease of k_{cat} of only 1.5–3.5-fold compared to unmodified enzyme. The K_m values of the variants also decreased so that k_{cat}/K_m varies relatively little.

These results were quite unexpected since they do not agree with the hypothesis that His 64 is required for rapid proton transfer between metal-

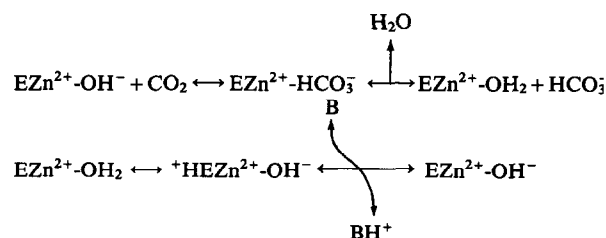
Table 1

Isoelectric points (pI) of variants of human carbonic anhydrase II and Michaelis-Menten parameters for CO_2 hydration at pH 8.8 and 25°C

Enzyme source	Residue 64	pI	$k_{\text{cat}} \times 10^{-5}$ (s^{-1})	K_m (mM)	$k_{\text{cat}}/K_m \times 10^{-8}$ ($\text{s}^{-1} \cdot \text{M}^{-1}$)
Red cells	His	7.1	8.8 ± 0.6	7.0 ± 1.1	1.3
<i>E. coli</i>	His	7.3	7.6 ± 0.8	8.0 ± 1.7	1.0
<i>E. coli</i>	Lys	7.6	4.9 ± 0.4	6.3 ± 1.0	0.8
<i>E. coli</i>	Gln	7.1	4.6 ± 0.2	4.3 ± 0.5	1.1
<i>E. coli</i>	Ala	7.1	4.3 ± 0.2	4.9 ± 0.8	0.9
<i>E. coli</i>	Glu	7.0	2.1 ± 0.1	3.6 ± 0.4	0.6

Values of k_{cat} and $K_m \pm \text{SD}$ were estimated by a nonlinear least-squares procedure

bound water and the reaction medium. A wide body of kinetic data on HCAII can be rationalized by a mechanism model that, in essence, consists of two, temporally separate, half-reactions [2], a $\text{CO}_2/\text{HCO}_3^-$ interconversion half-reaction occurring at the metal site (eqn 1) and a proton transfer half-reaction containing a rate-limiting intramolecular proton transfer step and a buffer-dependent step (eqn 2).



In eqn 2, H^+ to the left of E symbolizes a protonated proton shuttle group in the enzyme. With His 64 in this role one predicts that variants having a nonionizing amino acid side chain in this position should have drastically reduced k_{cat} values. This is clearly not the case since the variants with Gln 64 and Ala 64 are potent catalysts of CO_2 hydration. Until more data have been obtained we shall refrain from speculating about alternative mechanism hypotheses.

The pH profiles of the 4-nitrophenyl acetate hydrolase activities of the unmodified, cloned enzyme and the variants were measured. All variants have substantial activities as shown in table 2. The

Table 2

Maximal k_{enz} values and pK_a values derived from pH-rate profiles of the 4-nitrophenyl acetate hydrolase activities of the different variants of human carbonic anhydrase II

Enzyme source	Residue 64	k_{enz}^{max} ($s^{-1} \cdot M^{-1}$)	pK_a
Red cells	His	2800	6.9
<i>E. coli</i>	His	2800	6.8
<i>E. coli</i>	Lys	2900	6.8
<i>E. coli</i>	Gln	2100	7.0
<i>E. coli</i>	Ala	1300	7.0
<i>E. coli</i>	Glu	1000	8.4

(and 5.6)

See legend to fig.1 for additional experimental details. Data for the red cell enzyme are from [3]

pH profiles for red cell HCAII, the unmodified, cloned enzyme and the variants with Lys 64, Gln 64 and Ala 64 follow simple titration curves with the pK_a values given in table 2. The Glu 64 variant has a more complex behavior. Its pH-rate profile, shown in fig.1 together with the profile of the Lys

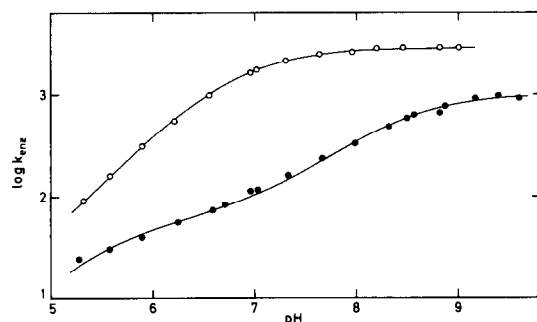


Fig.1. pH dependences of the apparent second-order rate constant, k_{enz} , for the hydrolysis of 4-nitrophenyl acetate catalyzed by variants of human carbonic anhydrase II with Lys (○) and Glu (●) in sequence position 64. The following buffers were used at 47 mM: 4-morpholineethanesulfonic acid-NaOH (pH 5.2–7.0), Hepes-NaOH (pH 6.7–9.1), Tris- H_2SO_4 (pH 8.5–9.6). The ionic strength was maintained at 0.1 M with Na_2SO_4 . The curve for the Lys 64 variant has been calculated as a simple titration curve with a maximal $\log k_{enz}$ value of 3.46 and $pK_a = 6.82$, whereas that for the Glu 64 variant has been calculated using two pK_a values (8.35 and 5.6) with a maximal $\log k_{enz}$ value of 3.00 and $\log k_{enz} = 1.80$ for the monoprotonated species.

64 variant, can formally be described using two pK_a values; an inactive, doubly protonated species titrates with $pK_a = 5.6$ to give rise to the monoprotonated species with 6.3% of the activity of an unprotonated species that is formed with $pK_a = 8.4$. It has been shown previously, in studies of Co(II)-substituted isoenzyme II [12], that the esterase pH-rate profile closely parallels the titration of metal-bound water. Thus, our interpretation of the profile of the Glu 64 variant implies that the microscopic pK_a of metal-bound water depends strongly on the ionization state of Glu 64 (cf. [12]). It is interesting to note that while the negative charge from Glu 64 shifts the pK_a of metal-bound water about 1.4 units upwards from the value observed when position 64 is occupied by an uncharged amino acid side chain, the positive charge from Lys 64 appears to give a much smaller effect. Hopefully, a crystal structure investigation of these variants will provide further information on this apparent asymmetry.

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