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Two point mutations convert a catalytically inactive carbonic anhydrase-related protein (CARP) to an active enzyme

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Abstract **A murine carbonic anhydrase-related protein (CARP) has been expressed in *Escherichia coli* and purified to near homogeneity. The polypeptide chain consists of 290 amino acid residues and has a calculated molecular mass of 32 950 Da. By introducing two mutations, $Arg^{117} \rightarrow His$ and $Glu^{115} \rightarrow Gln$, we created a metal-binding center homologous to that in the carbonic anhydrases from the animal kingdom. In contrast to unmodified CARP, this double mutant was isolated as a 1 : 1 zinc–protein complex. While unmodified CARP is catalytically inactive, the mutant catalyzes CO_2 hydration with a significantly higher efficiency than the mammalian low-activity carbonic anhydrase isozyme III. The activity is strongly inhibited by the powerful and selective carbonic anhydrase inhibitor, acetazolamide.**

Key words: Carbonic anhydrase-related protein; Mutagenesis; CO_2 hydration; Zinc binding; Acetazolamide

1. Introduction

A number of proteins have been found to contain amino acid sequences with significant homologies with the sequences of various forms of the zinc-containing enzyme carbonic anhydrase (CA, EC 4.2.1.1) from animal sources [1]. Most of these proteins lack one or more of the three histidines which act as zinc ligands in the functional enzyme. Since the metal ion is required for catalytic activity [2], and all three histidines are essential for tight zinc binding [3], it might be presumed that these proteins have little or no CO_2 hydration activity. However, a human tumor-associated protein, MK, containing a CA-like domain, has been reported to have some activity, but all the zinc-binding histidines are conserved in this case [4].

One of the first of these CA-like proteins to be discovered was described by Kato [5], who obtained cDNA encoding a carbonic anhydrase-related protein (CARP) from a mouse brain cDNA library. He also showed that CARP is expressed in Purkinje cells. As yet, its function is unknown. The sequence of the human homologue was reported by Skaggs et al. [6], who found a 98% sequence identity at the amino acid level with mouse CARP. Bergenhem et al. [7], who named the protein CA VIII despite the fact that no CA activity has been demonstrated, have localized the human gene (*CA8*) to chromosome 8 at q11 → q12.

CARP is an acidic protein, mainly depending on a glutamate-rich amino-terminal extension comprising about 25 residues. This sequence segment has no counterpart in any isozyme form of CA, and its function is unknown. The remainder of the sequence shows 30–40% identity with sequences of different mammalian CA isozymes [5]. Moreover, 26 of the 28 residues, that are invariant in all sequenced animal CAs, are also found in CARP. The two exceptions are located in the active site of CA. Thus, the zinc ligand His^{94} corresponds to Arg^{117} in mouse CARP. In addition, Gln^{92}, which forms a hydrogen bond with His^{94} in CA, corresponds to Glu^{115} in CARP.

We have used site-specific mutagenesis to replace Arg^{117} and Glu^{115} in CARP with His and Gln, respectively. This double mutant binds zinc and catalyzes the hydration of CO_2, suggesting that the structural similarities between CA and CARP include subtle features at the three-dimensional level.

2. Materials and methods

The cDNA encoding murine CARP was kindly provided by Dr. N.D. Carter, London, and had been cloned into the *Not*I site of the plasmid pBluescript KS(+) (Stratagene). To obtain efficent expression in *Escherichia coli*, we used the plasmid pACA1, which is derived from the plasmid pACA [8]. Like pACA, pACA1 contains the cDNA encoding human CA II under control of the T7 RNA polymerase promoter, an F1 origin of replication allowing the production of single-stranded DNA, and a gene conferring ampicillin resistance. In pACA1, a *Hin*dIII site has been removed, and the plasmid has a single *Nco*I site located at the initiator ATG codon and a single *Hin*dIII site further downstream. The nucleotide sequence of the original CARP cDNA insert had no *Hin*dIII site but three *Nco*I sites located at the initiator ATG and at positions 412 and 482 in the coding region. The *Nco*I sites at positions 412 and 482 were removed by introducing 'silent' mutations and a *Hin*dIII site was introduced at position 984 downstream of the stop codon. The plasmid harboring the altered CARP cDNA and pACA1 were cleaved by *Nco*I and *Hin*dIII. The purified pACA1 fragment was ligated to the CARP DNA fragment using T4-DNA ligase. The mixture was used to transfect *E. coli* BL21(DE3) [9]. Positive colonies were identified by SDS-PAGE and Coomassie staining after growth in liquid cultures containing ampicillin and induction of protein synthesis by IPTG. For a clone producing a 30 kDa protein in high yield the complete coding region was checked by plasmid sequencing before the clone was used for protein production. The plasmid was called pCARP. All nucleotide sequencing was performed using the chain termination method [10].

In vitro site-directed mutagenesis was performed essentially according to the method of Kunkel [11]. Uracil-containing single-stranded DNA of the plasmids pBluescript KS(+) and pCARP was produced in *E. coli* strain CJ236 (*dut*$^-$, *ung*$^-$) after infection with the helper phage M13K07. Mutants were obtained by in vitro second-strand synthesis from oligonucleotides containing mismatches. The resulting duplex DNA was transformed into *E. coli* (*dut*$^+$, *ung*$^+$), and mutations were then identified by direct sequencing of the plasmid DNA.

CARP was produced in *E. coli* BL21(DE3) harboring the plasmid pCARP. The cells were grown at 23°C in 2× Luria broth containing 1 mM $ZnSO_4$ and 50 μg/ml ampicillin. Protein production was initiated by adding IPTG to a final concentration of 0.5 mM at a cell

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Abbreviations: CA, carbonic anhydrase; CARP, carbonic anhydrase-related protein; IPTG, isopropyl thio-β-D-galactoside

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density corresponding to OD^{660}=0.5 and then allowing the cells to grow for 12–14 h. The cells were broken using a Bead-Beater (Biospec Products, Bartlesville, OK). After removal of cell debris by centrifugation, the lysate was applied to an anion exchanger, Q-Sepharose (Pharmacia), equilibrated with 25 mM Mes-NaOH (pH 6.0) containing 50 mM NaCl. The column was washed with the same buffer until the absorbance at 280 nm of the eluate was close to zero. Further washing was performed with five column volumes of 25 mM Mes-NaOH (pH 6.0) containing 250 mM NaCl. CARP was then eluted by five column volumes of the same buffer containing 300 mM NaCl. The fractions containing CARP were pooled, concentrated and passed through a gel-filtration column, Sephacryl 200 (Pharmacia), using a buffer of the same composition. SDS-PAGE analysis showed the presence of some impurities after this step, but we estimated that CARP represented about 95% of the total protein.

The extinction coefficient at 280 nm (ε_{280}) of CARP was determined by the method of Gill and von Hippel [12] using the amino acid composition derived from the nucleotide sequence. Zinc analyses were performed at 213.9 nm with a Varian AA-375 atomic absorption spectrometer with an air-acetylene flame. All glassware had been treated with 2 M HNO_3 to remove metal ion impurities. A 477A Pulsed Liquid Phase Sequencer with an online PTH 120A Analyzer (Applied Biosystems, Foster City, CA) was used for determination of the N-terminal amino acid sequence. The protein was resolved by SDS-PAGE and transblotted onto a polyvinylidene difluoride membrane (0.2 μm) from where it was excised and eluted into solution. Sodium thioglycolate (0.1 mM) was included in all solutions to prevent oxidation of the protein.

Initial rates of CO_2 hydration were measured at 25°C in an Applied Photophysics DX.17MV stopped-flow apparatus (Leatherhead, UK) by the changing pH-indicator method [13]. The buffer-indicator system was Taps-NaOH/metacresol purple monitored at 578 nm. The ionic strength was kept at 0.1 M with Na_2SO_4. Rate data were fitted to the Michaelis-Menten equation using the program GraFit (Erithacus Software Ltd., UK).

3. Results

The nucleotide sequence of the coding region of mouse CARP cDNA was found to be identical to that published by Kato [5] except at the positions engineered to remove restriction sites (see Section 2) and at two additional positions. Thus, we found C instead of T at position 782. This involves a Val→Ala interchange at amino acid sequence position 261. Furthermore, we found an additional T after T^{869}. This would result in a 12-residue shorter polypeptide chain than the sequence derived by Kato, since a TAG stop codon would occur at nucleotide positions 874–876. Both of these sequence differences were found for unmodified CARP as well as for the Glu^{115}→Gln/Arg^{117}→His mutant, so it seems unlikely that our results depend on sequencing errors. In addition, at these points our sequence corresponds exactly with that obtained by Skaggs et al. [6] for human CARP cDNA.

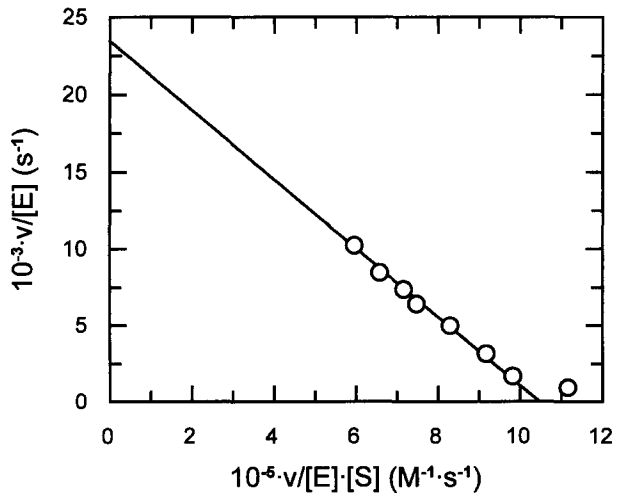

Fig. 1. Eadie-Hofstee plot of initial rates of CO_2 hydration catalyzed by murine Glu^{115}→Gln/Arg^{117}→His CARP, (E), in 50 mM Taps-NaOH buffer at 25°C and pH 9.0. The ionic strength was kept at 0.1 M with Na_2SO_4.

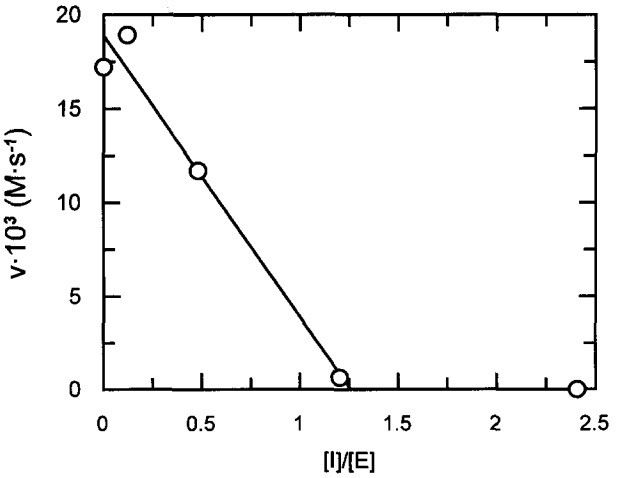

Fig. 2. Inhibition by acetazolamide, (I), of CO_2 hydration catalyzed by murine Glu^{115}→Gln/Arg^{117}→His CARP in 50 mM Taps-NaOH buffer at 25°C and pH 9.0. The ionic strength was kept at 0.1 M with Na_2SO_4. The CO_2 concentration was 16.8 mM and the concentration of the CARP mutant, (E), was 2.1 μM.

The N-terminal amino acid sequence of purified CARP was found to be Ala-Asp-Leu-Ser-Phe- in accordance with the nucleotide sequence assuming that the initiator Met residue has been cleaved off. The resulting polypeptide should consist of 290 residues with a calculated molecular mass of 32 950 Da. The extinction coefficent of unmodified CARP at 280 nm (ε_{280}) was estimated as 48.0 mM^{-1} cm^{-1}. The results of zinc analyses gave 0.12 mole of zinc per mole of unmodified CARP after dialysis against metal-depleted buffer, while the mutant contained 1.10 equivalents of zinc, indicating that a rather strong zinc-binding site has been created.

Unmodified CARP showed no detectable CO_2 hydration activity. However, the mutant is active. As shown in the Eadie-Hofstee plot in Fig. 1, this 'reconstructed' CA follows Michaelis-Menten kinetics. Estimated values and standard errors are $k_{cat}=(2.3\pm0.1)\cdot10^4$ s^{-1}, $K_m=22\pm1$ mM, and $k_{cat}/K_m=(1.0\pm0.02)\cdot10^6$ M^{-1} s^{-1} (25°C, pH 9.0). This activity could be inhibited by acetazolamide (2-acetylamido-1,3,4-thiadiazole-5-sulfonamide). The results illustrated in Fig. 2 demonstrate that acetazolamide is a strong inhibitor of the CO_2 hydration activity of the mutant and that there is a 1 : 1 stoichiometry between inhibitor and mutant protein. While several isozyme forms of CA are efficient catalysts of the hydrolysis of 4-nitrophenyl acetate, we could not detect any such esterase activity associated with the CARP mutant.

4. Discussion

The amino acid sequence of murine CARP derived from our nucleotide sequence differs from that of human CARP [6] in four places only, namely positions 10, 25, 109, and 246. Thus, the degree of sequence identity is 98.6% on the amino acid level. Two of these positions, 10 and 25, are lo-

cated in the N-terminal extension, whose function is unknown. Preliminary results seem to exclude this glutamate-rich sequence as a calcium-binding region [14]. However, when applying the program PEST-FIND [15], we found that part of the N-terminal extension plus the first 30, or so, residues of the CA-homologous region is a potential PEST sequence, indicating that this part of the protein might contain a degradation signal. The putative PEST sequence is as follows. Note also the underlined 7-residue repeat.

16-KEEDEEEEEEEGVEWGYEEGVEWGLVFPDANGEYQSPINLNSR-58

Our observation that two point mutations restore zinc binding and catalytic activity to CARP is strong evidence that the three-dimensional structure of the active-site cavity of CA is well conserved in CARP. Additional evidence is provided by the strong inhibition of the activity by acetazolamide, a powerful and specific CA inhibitor [16]. After the initiation of our study, Kiefer et al. [17] have shown that the replacement of Gln^{92} with Glu in human CA II has only a marginal effect on zinc affinity. Thus, it seems plausible that the single mutation, $Arg^{117} \rightarrow$ His, is sufficient to convert CARP to an active CA. We are presently testing this possibility.

The CO_2 hydration activity of the $Glu^{115} \rightarrow Gln/Arg^{117} \rightarrow$ His mutant is low compared to that of the most potent of the human CA isozymes, CA II, which has $k_{cat} = 1 \cdot 10^6$ s^{-1} and $k_{cat}/K_m = 1 \cdot 10^8$ $M^{-1}s^{-1}$ near pH 9 at 25°C [18]. However, the specific activity of the CARP mutant is significantly higher than that of one mammalian CA isozyme, namely the muscle-specific form, CA III. Bovine CA III, for example, was found to have $k_{cat} = 6.4 \cdot 10^3$ s^{-1} and $k_{cat}/K_m = 4.3 \cdot 10^5$ M^{-1} s^{-1} at 25°C and pH 8.9 [19]. Thus, the CARP mutant has 3.6 times the activity of bovine CA III on the basis of k_{cat} and 2.3 times the activity on the basis of k_{cat}/K_m at pH near 9.

In addition to some invariant amino acid residues, such as the zinc ligands and residues hydrogen bonded to them, the active sites of the CAs contain a number of residues showing a certain degree of interspecies and interisozyme variation [20]. At least some of these variations might be important for the fine tuning of the catalytic properties [21]. Most of the putative active-site residues in the CARP mutant fall within the range of these, previously observed, variations. For example, His^{88} in CARP corresponds to His^{64} in CA II, where this residue has a crucial proton transfer role during catalysis [22].

The only striking exception is Ile225 in CARP corresponding to residue 200 in CA. Most CA isozymes have threonine in this position, but all sequenced CAs I have His^{200}, which seems to be a major determinant of the specific properties of this isozyme [21,23]. A $Thr^{200} \rightarrow$ Ile mutant of human CA II has been produced, and it has a reduced CO_2 hydration activity with k_{cat} about 20% and k_{cat}/K_m about 50% of the values for unmodified enzyme at 25°C and pH 8.9 [18]. This result suggests that it might be possible to increase the specific CO_2 hydration activity of CARP further by introducing additional mutations, such as $Ile^{225} \rightarrow$ Thr.

Other candidates for further mutations of CARP are Ile^{143} and Ile^{165} with positions homologous to those of Val^{121} and Val^{143}, respectively, in human CA II. These residues form part of the wall of a hydrophobic pocket, where the substrate, CO_2, has been assumed to bind [24,25]. With isoleucine in these two positions, it is probable that this pocket is smaller in the CARP mutant than in human CA II. In the human CA II framework, a $Val^{143} \rightarrow$ Ile mutation was found to have no effect on k_{cat} for CO_2 hydration, while a 9-fold increase of K_m was observed, presumably as a consequence of the decreased size of the pocket [24]. The $Val^{121} \rightarrow$ Ile mutant of human CA II was reported to have 15% of the CO_2 hydration activity of the unmodified enzyme [8].

Another residue which might have a modifying influence on the catalytic activity of the CARP mutant is Asp^{86} with a sequence position homologous to that of residue 62 in CA. All sequenced CAs of the animal type have a neutral amino acid in this position, in most cases asparagine [1]. While the introduction of a negative charge near the zinc ion or in the substrate-binding pocket has a strong, detrimental effect on the catalytic activity [18,26], this residue is located in the outer part of the active-site cavity, and the effect of an Asn → Asp interchange is difficult to predict.

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