Reconstitution of carbonic anhydrase activity of the cell-surface-binding protein of vaccinia virus

Anna OHRADANOVA*, Daniela VULLO†, Juraj KOPACEK*, Claudia TEMPERINI†, Tatiana BETAKOVA*, Silvia PASTOREKOVA*, Jaromir PASTOREK*¹ and Claudiu T. SUPURAN†^{1,2}

*Centre of Molecular Medicine, Institute of Virology, Slovak Academy of Sciences, Dubravská cesta 9, 845 05 Bratislava, Slovak Republic, and †Università degli Studi di Firenze, Polo Scientifico, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, 50019 Sesto Fiorentino, Italy

The N-terminal region of a 32 kDa cell-surface-binding protein, encoded by the D8L gene of vaccinia virus, shows sequence homology to CAs (carbonic anhydrases; EC 4.2.1.1). The active CAs catalyse the reversible hydration of CO₂ to bicarbonate participating in many physiological processes. The CA-like domain of vaccinia protein [vaccCA (vaccinia virus CA-like protein)] contains one of the three conserved histidine residues required for co-ordination to the catalytic zinc ion and for enzyme activity. In the present study, we report the engineering of catalytically active vaccCA mutants by introduction of the missing histidine residues into the wild-type protein. The wild-type vaccCA was inactive as a catalyst and does not bind sulfonamide CA inhibitors. Its position on a phylogram with other hCAs (human CAs) shows a relationship with the acatalytic isoforms CA X and XI, suggesting that the corresponding viral gene was acquired from the human genome by horizontal gene

transfer. The single mutants (vaccCA N92H/Y69H) showed low enzyme activity and low affinity for acetazolamide, a classical sulfonamide CA inhibitor. The activity of the double mutant, vaccCA N92H/Y69H, was much higher, of the same order of magnitude as that of some human isoforms, namely CA VA and CA XII. Moreover, its affinity for acetazolamide was high, comparable with that of the most efficient human isoenzyme, CA II (in the low nanomolar range). Multiplication of vaccinia virus in HeLa cells transfected with the vaccCA N92H/Y69H double mutant was approx. 2-fold more efficient than in wild-type vaccCA transfectants, suggesting that the reconstitution of the enzyme activity improved the virus life cycle.

Key words: acetazolamide, carbonic anhydrase, cell-surface-binding protein, *in vitro* mutagenesis, poxvirus, vaccinia virus.

INTRODUCTION

Poxviruses constitute a family of viruses characterized by a large, linear dsDNA (double-stranded DNA) genome, a cytoplasmic site of replication and complex virion morphology [1,2]. The most notorious member of the family is the variola virus, the causative agent of smallpox, whereas the laboratory prototype virus used for the study of poxviruses is vaccinia, the virus used as a live, naturally attenuated vaccine for the eradication of smallpox [1–3]. The poxviruses have a long history of causing disease, and their biological effects in humans and other mammals have been extensively studied [4]. In the 1980s and 1990s, genetic engineering techniques were applied to vaccinia in order to create replicating recombinant vectors that could express inserted genes encoding other proteins, which were able to elicit a specific adaptive immune response to foreign antigens in animal models, creating the basis of gene delivery approaches for the treatment of diseases (mainly viral infections and tumours) [4,5]. Although intensively studied, much is still to be learned about vaccinia virus, considering the fact that its approx. 200-kbp-long genome encodes more than 200 different proteins, some of which have not been much investigated up to now [1,6].

In 1990, Maa et al. [7] identified a 32 kDa protein of vaccinia, which was shown to be a part of the virus envelope binding machinery to cells. The gene encoding this 32 kDa viral protein was then mapped and sequenced, and was found to encode a 35426

Da protein with a large N-terminal domain, showing sequence homology to α -CAs (α -carbonic anhydrases; EC 4.2.1.1), a family of zinc enzymes widespread over the phylogenetic tree [8–10], and a C-terminal domain with sequence similarity to the attachment glycoprotein VP7 of rotavirus and some other transmembrane proteins [7]. The 32 kDa protein was basic, with a pI of ~8.7, and was probably dimeric due to a disulfide bridge involving the unique cysteine residue at amino acid position 262 [7]. It was also shown to be a vaccinia virus antigen, with predicted antigenic sites located near amino acids 108–110 (CA domain) and 298–299 (transmembrane domain). This protein was found to bind to cell-surface chondroitin sulfate of cultured cells and participate in efficient virus replication both in cultured cells and in infected animals [7–9,11]. As there are no other known viral CAs or CA-like proteins [10,12–14], we decided to investigate this 32 kDa protein discovered by Maa et al. [7]. In the present study, we report the cloning of vaccCAs (vaccinia virus CA-like proteins) in which we have mutated two amino acids belonging to the putative enzyme active site in such a way as to bind zinc ions, the metal cofactors essential for catalytic activity in all α -CAs [10,12,13]. We were able to engineer a variant of vaccCA possessing enzyme activity for the physiological reaction catalysed by CAs, i.e. CO₂ hydration to bicarbonate, of the same order of magnitude as exhibited by some hCAs (human CAs). Furthermore, this active vaccCA was also inhibited by sulfonamide CA inhibitors (some of which are clinically used drugs [15,16]) in the low nanomolar

Abbreviations used: AAZ, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide; CA, carbonic anhydrase; CARP, CA-related protein; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione transferase; hCA, human CA; pfu, plaque-forming unit; RPTP, receptor protein tyrosine phosphatase; RT, reverse transcriptase; wt, wild-type; vaccCA, vaccinia virus CA-like protein (vaccinia virus cell-surface-binding protein).

¹ Equal senior contributors.

² To whom correspondence should be addressed (email claudiu.supuran@unifi.it).

Table 1 List of primers used for cloning and RT-PCR analysis of vaccCA and its mutant variants

Source DNA (GenBank® accession no.)	Name of primer	Sequence 5' → 3'
Vaccinia virus cell-surface-binding protein gene (J05190)	VaccCAs	gaattcATGCCGCAACAACATCTCCTATT
	VaccCAa	ctcgaGGAGGCTCAACAAGGTATCGAAT
	N92Hs	ACTCTGGAGAGATT C ATCTTGTTCATTGG
	N92Ha	CCAATGAACAAGAT G AATCTCTCCAGAGT
	Y69Hs	CATCACTACATATA C ATTGGGGAAAGGAAG
	Y69Ha	CTTCTTTCCCAAT G TATATGTAGTGATG
pBluescript KS-(X52329)	R	GGAAACAGCTATGACCATGATTACGCC
	F	GTGAGCGCGCGTAATACGACTCACTA
pGEX-4T-1 (U13853)	pGEXS	TACTTGAAATCCAGCAAGTATATA
	pGEXA	AGAGGTTTTCACCGTCATCA
β -Actin (NM_001101)	BactinS	CCAACCGCGAGAAGATGACC
	BactinA	AGGATCTTCATGAGGTAGTCAGTC

range, indicating that the engineered protein was indeed a viral CA.

EXPERIMENTAL

Cell culture, viral and bacterial strains

HeLa cells derived from human cervical carcinoma were cultivated in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) FCS (foetal calf serum; BioWhittaker, Verviers, Belgium), 100 i.u. (international units)/ml penicillin, 100 μ g/ml streptomycin sulfate and 0.25 μ g/ml amphotericin B (BioWhittaker) in a humidified atmosphere with 5% CO₂ at 37 °C.

Vaccinia virus, strain CR19 (wild-type), was a gift from Dr Gustav Russ (Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovakia). *Escherichia coli* strains JM109 and BL21-CodonPlus (DE3)-RIPL (Stratagene, La Jolla, CA, U.S.A.) were used for cloning and protein expression respectively.

Plasmids, primers and cloning

The gene for vaccCA [vaccinia virus cell-surface-binding protein – the NCBI (National Center for Biotechnology Information) accession number J05190] was amplified from a boiled culture medium containing viral particles by PCR using oligonucleotides 'VaccCAs' and 'VaccCAa' (sequences of all primers used are listed in Table 1; nucleotides introduced for cloning are in lower-case; mutations are in boldface) using Phusion polymerase (Finnzymes, Espoo, Finland). The PCR product was first subcloned into the EcoRV site of pBluescript KS– (Stratagene), creating the plasmid pBKS-vaccCAwt (where wt is wild-type). The target fragment was retrieved by digestion with restriction enzymes EcoRI and XhoI and cloned into pGEX-4T-1 (Amersham Biosciences, Chalfont St Giles, U.K.), creating a plasmid designated pGEX-4T-1-vaccCAwt.

Mutants of vaccCA designated N92H and Y69H, according to the amino acids mutated, were generated by two-step PCR-directed mutagenesis using the pBKS-vaccCAwt plasmid as a template. One reaction of the first round was performed using the sense mutagenic primers N92Hs or Y69Hs respectively and the antisense pBluescript-specific external primer R. In the second reaction, the antisense mutagenic primers N92Ha or Y69Ha were used with sense pBluescript-specific external primer F. For the second round, PCRs were carried out using sense and antisense pBluescript-specific external primers for both mutants and the PCR products from the first round were used as templates. Final

PCR products of single mutants were gel-purified, processed with EcoRI and XhoI and inserted into the pGEX-4T-1 vector linearized with EcoRI and XhoI, creating plasmids pGEX-4T-1-N92H and pGEX-4T-1-Y69H.

A double mutant N92H/Y69H of vaccCA was similarly generated later using the plasmid construct pGEX-4T-1-N92H as a template. First PCRs were carried out using the sense and antisense Y69H-mutagenic primers and the corresponding pGEX-4T-1-specific external primers pGEXA and pGEXS. The PCR product obtained was inserted into the pGEX-4T-1 vector using EcoRI and XhoI, yielding a plasmid designated pGEX-4T-1-vaccCA_{dm}.

All PCR reactions were performed using the following amplification programme: an initial denaturation at 98 °C for 30 s, then denaturation at 98 °C for 10 s, annealing at 62–68 °C for 30 s and extension at 72 °C for 20–35 s for a total of 30 cycles, and finally 5 min at 72 °C. All pGEX-vaccinia constructs were confirmed by sequencing using an ABI Prism BigDye terminator V3.1 sequencing kit for fluorescent detection and an ABI Prism 3100 genetic analyser (Applied Biosystems, Foster City, CA, U.S.A.).

For eukaryotic expression, cDNAs of the wild-type and the double mutant of vaccCA were cloned from the plasmids pGEX-4T-1-vaccCAwt and pGEX-4T-1-vaccCA_{dm} respectively into the pHCMV1 expression vector (Genlantis, San Diego, CA, U.S.A.) using EcoRI and NotI enzymes.

Recombinant protein expression and purification

Verified plasmid constructs pGEX-4T-1-vaccCAwt, -N92H, -Y69H and -vaccCA_{dm} were transformed into BL21-CodonPlus (DE3)-RIPL cells. An expression of GST (glutathione transferase)-fusion proteins was induced with 0.4 mM IPTG (isopropyl β -D-thiogalactoside) at 30 °C for 4 h. Recombinant proteins were purified using glutathione-Sepharose 4B medium and cleaved from their GST tag overnight at 4 °C with thrombin (Sigma-Aldrich) while still attached to Sepharose beads, as reported previously by our laboratory for the purification of several mammalian CA isoenzymes [17–19]. After elution from the beads (which retained the GST part of the fusion protein), the concentration of recombinant proteins was determined by measuring its absorption A₂₆₀ (absorbance) as well as by SDS/PAGE followed by Coomassie Brilliant Blue R-250 staining. For the double mutant possessing good CA activity (see below), the obtained bacterial lysate was applied to a prepacked glutathione-Sepharose 4B column (Amersham), the column was extensively washed with ice-cold PBS and the GST-vaccCA_{dm} (where dm is double mutant) fusion protein was eluted with a buffer consisting of 5 mM GSH in 50 mM Tris/HCl (pH 8.0). Finally, the GST part of the fusion protein was cleaved with thrombin and the vaccCA_{dm} was further purified by sulfonamide affinity chromatography [20], the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO₂ as the substrate [21]. The CO₂ hydrase activity was measured as described below.

CA activity/inhibition assay

An Applied Photophysics stopped-flow instrument was used for assaying the CA/vaccCA-catalysed CO₂ hydration activity [21]. Phenol Red (at a concentration of 0.2 mM) was used as an indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as the buffer and 0.1 M Na₂SO₄ (for maintaining constant the ionic strength). The CA-catalysed CO₂ hydration reaction was followed for a period of 10–100 s. The CO₂ concentrations ranged from 1.7 to 17 mM for the

determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction were used for determining the initial velocity. The uncatalysed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 nM were made thereafter with distilled-deionized water. Inhibitor and enzyme solutions were pre-incubated together for 15 min at room temperature (25°C) prior to the assay, in order to allow for the formation of the E–I complex (enzyme–inhibitor complex). The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, from Lineweaver–Burk plots, as reported earlier, and represent the means for at least three different determinations [21].

Generation of HeLa cells expressing vaccCA proteins

HeLa cells plated on to 35 mm Petri dishes were transfected with 2 µg of the pHCMV1-vaccCAwt, pHCMV1-vaccCAadm plasmids and an empty pHCMV1 vector using GenePorter transfection reagent (Genlantis). At 2 days after transfection, the cells were split (on to seven 60 mm Petri dishes) and subjected to a 2-week selection in a medium containing Geneticin (G418; Invitrogen, Carlsbad, CA, U.S.A.) at a concentration of 600 µg/ml. The resistant colonies were pooled and tested for the expression of ectopic genes by RT (reverse transcriptase)–PCR using vaccCAs and Y69Ha primers and primers for β-actin that served as an internal standard (see the list in Table 1).

Plaque assay

The effect of the wild-type and the double mutant of vaccCA on vaccinia virus replication was compared after the infection of stably transfected HeLa cells where ectopically expressed vaccCA variants could be packed into virus particles. Confluent HeLa cell monolayers grown in 96-well tissue culture dishes were infected with 5-fold serial dilutions of vaccinia virus starting at an MOI (multiplicity of infection) of 1 pfu (plaque-forming unit) per cell. After 1 h of adsorption, the monolayers were washed and covered with DMEM containing 0.5% methylcellulose. After 2 days, the cells were stained with Crystal Violet and the pfu/ml for the vaccinia virus grown in different transfectants was determined.

Computer analysis of CA sequences

Amino acid sequence alignments were carried out using the program ClustalW, version 1.83 [22]. Protein phylogeny tree reconstructions were performed using the MEGA3.1 software [23]. Trees were obtained using the distance/nearest neighbour joining method and bootstrap statistical analyses were performed with 1000 replicates. Three-dimensional protein models were obtained with the program PHYRE, a successor of the program 3D-PSSM [24].

RESULTS AND DISCUSSION

By catalysing the interconversion between CO₂ and bicarbonate, with generation of a proton, the CAs operate on three very simple molecules/ions involved in a variety of critical life processes [10,12–15]. Among them, the most important ones are pH regulation, respiration, secretion of electrolytes, biosynthesis of some important biomolecules such as urea, glucose, lipids and pyrimidine bases, excretion of acid and salts, carcinogenesis and signalling etc. [10,12–15]. Different isoenzymes among the 16 currently known isoforms in mammals participate in such processes. These isoforms display very diverse cellular localiza-

tion, catalytic activity and susceptibility for inhibition/activation by various endogenous or exogenous regulators of activity. In humans, only 15 of the 16 mammalian isoforms are present, due to the fact that CA XV is encoded by a pseudogene in all primates investigated so far [25]. Among these remaining 15 isoforms, 12 possess catalytic activity for the CO₂ hydration reaction, whereas CA VIII, X and XI [also known as CARPs (CA-related proteins)] are devoid of this activity due to a lack of one or more histidine residues co-ordinating the catalytically critical Zn(II) ion within the enzyme active site [26]. Indeed, CA I–III, VII and XIII are soluble cytosolic isoenzymes, CA IV and XV are extracellular membrane-anchored enzymes by means of GPI (glycosylphosphatidylinositol) tails, CA VA and VB are mitochondrial enzymes, CA VI is the only secreted CA isoform, initially identified in sheep saliva and parotid glands, whereas CA IX, XII and XIV are transmembrane proteins with the active site situated outside the cell [10,15,25,26].

Whereas the physiological function of most catalytically active CAs is rather well understood, the three CARPs (i.e. isoenzymes CA VIII, X and XI) still represent a biochemical and physiological enigma [26]. The CARPs lack one or more histidine residues required to bind the zinc ion, which is essential for the CO₂ hydration activity of all CAs. The Zn(II) ion within the enzyme active site is tetrahedrally co-ordinated to the imidazole moieties of three histidine residues (His⁹⁴, His⁹⁶ and His¹¹⁹, CA II numbering; [10]), whereas the fourth ligand is a water molecule/hydroxide ion which acts as a strong nucleophile on the substrate (CO₂) bound within a hydrophobic pocket in its neighbourhood [10,12–18]. The CARPs have been shown to be consistently expressed in the brain, and CARP VIII was also found to be expressed in some peripheral tissues [26]. In a similar manner to the catalytically active transmembrane isoforms CAs IX and XII, CARP VIII has also been reported to be overexpressed in certain types of carcinoma [26].

An inactive CA-like domain has also been identified in the N-terminal regions of two RPTPs (receptor protein tyrosine phosphatases), RPTPβ and RPTPγ [27,28]. RPTPβ is expressed on the surface of glial cells and utilizes the CA domain to bind the cell recognition molecule contactin on neuronal cells, thus supporting its role in neuronal adhesion and neurite outgrowth [29]. RPTPγ is expressed in a variety of tissues. It appears developmentally regulated and is implicated in haemopoietic differentiation [30,31]. Both these phosphatases contain only one of the three histidine residues that bind zinc and are crucial for CA activity. On this basis it has been proposed that their CA domains function rather as a protein-binding pocket.

In a similar manner to CARPs and RPTPβ/γ, the N-terminal CA-like domain of the cell-surface-binding protein of vaccinia virus identified by Maa et al. [7] shows a high sequence homology with mammalian CAs, but lacks two of the zinc-co-ordinating histidine residues present in the active site of these enzymes. More precisely, a tyrosine residue is present at position 69, and an asparagine residue is found at position 92, instead of histidine at the corresponding positions 96 and 119 in CA II. Figure 1 shows the sequence alignment of the CA domains of all human isoenzymes (including CARPs), RPTPβ/γ and vaccCA. The sequence homology between the CA domains of these proteins ranges from 22 to 67%. A phylogenetic analysis of these proteins (Figure 2) revealed that vaccCA is evolutionarily similar to CARPs XI and X, whereas it is more distantly related to human CARP VIII, CA XIII, CA VII, CA VA and CA VB. On the other hand, all other CAs are clustered on different branches of the phylogenetic tree, suggesting that they are less evolutionarily related to vaccCA.

hCAI	HSFHVNFEDNDNRSVLKGGPFYSYRLQFHHFWG--STNEHGSEHTVDGVKYSAEHLVAHWNSA-KYS	130
hCAIII	HSFNVDFFDTENKSVLRGGPLSYRLRQVHLHWG--SADHGHSEHIVDGVSYAAELHVVHWNST-KYP	130
hCAII	HAFNVEFDDSDQKAVLKGGPLTYRLIQFHHFWG--SLDGQSEHTVDKKKYAAELHLVHWN-T-KYG	128
hCAIII	KTCRVVFFDDTYDRSMLRGGPLPYRLRQFHLHWG--SSDDHGSEHTVDGVKYSAAELHLVHWN-P-KYN	128
hCAVII	HSVQVDFNDSDDRTVVTGGPLPYRLKQFHHFWG--KKHVDGSEHTVDGKSFPSSELHLVHWNK-KYS	131
hCAVA	YLFQVEFDDATEASGISGGPLHYRLKQFHHFWG--AVNEGSEHTVDGHAYPAELHLVHWNST-KYQ	127
hCAVB	YSFLVEFEDSTDKSVIKGGPLNYRLKQFHHFWG--AIDAWGSEHTVDSKCFPAELHLVHWNST-RFE	132
hCAVIII	HTIQVILK---SKSVLSGGPLEFELYEVRFHWG--RENQRGSEHTVNFKAFFMELHLIHWNST-LFG	125
hCAX	GRHVSRLDKLKEHLVNISSGPMShRLLEIRLHFG--SEDSQGSEHLLNGQAFSGEVQLIHYNHE-LYT	142
hCAXI	GRHVSFLPAPRPVVNVSGGPLSHRLSELRLHFG--ARDGAGSEHQINHQGFAEVQLIHFNQE-LYG	142
hCAXII	HSVKNLNPDSMHIQ-GLQS--RYSATQLHLHWG-NPNDPHGSEHTVSGQHFAAELHIVHYNST-LYP	130
hCAXIV	HTVQLSLPSTLYLG-GLPR--KYVAAQLHLHWG-QKGSPPGGSEHQINSEATFAELHIVHYDSD-SYD	127
hCAIX	HSVQLTLPPGLEMALGPGR--EYRALQHLHLHWG-AAGRP-GSEHTVEGHRFPAEIHVHLST--AFA	124
hCAVI	HTVQISLPSTMRMTVADGT--VYIAQQMHFHWGGASSEISGSEHTVDGIRHVIEIHIHVHNS--KYG	130
hCAIV	HSVMMLEN---KASISGGGLPYQAKQLHLHWS--DLPYKGSEHSLDGEHFAMEMHIVHEKEKGTSR	133
RPTP β	KTVEINLTN---DYRVSGGVSFVKASKITFHWGKCNMSSDGSEHSLGQKFPLEMOICYFDAD-RFS	120
RPTP γ	KTVAILLKD---DYFVSGAGLRFKAEKVEFHWGHSNGS-AGSEHSINGRRFPVEMOIFYNPD-DFD	119
vaccCAwt	GKLVRIINFKGG---YISGGFLEYVLSSLIHFWG--KEDDYGSNHLIDVYKYSGEINHLVHWNKK-KYS	102

Figure 1 Alignment of amino acid residues 64–128 (CA II numbering, relevant for the catalytic mechanism) of hCA isoenzymes I–XIV, human RPTP β/γ and vaccCA using the ClustalW program

The zinc ligands of catalytically active isoforms are highlighted in black, whereas the corresponding residues in the non-catalytic CAs are highlighted in grey. The two missing potential Zn(II) ligands of vaccCA (mutated to histidine in the experiments described in the paper) are indicated with arrows. For the alignment, the NCBI reference sequences were used with the following accession numbers: hCAI, NP_001729; hCAII, NP_000058; hCAIII, NP_005172; hCAIV, NP_000708; hCAVA, NP_001730; hCAVB, NP_009151; hCAVI, NP_001206; hCAVII, NP_005173; hCAVIII, NP_004047; hCAIX, NP_001207; hCAX, NP_064563; hCAXI, NP_001208; hCAXII, NP_001209; hCAXIII, NP_940986; hCAXIV, NP_036245; RPTP β , NP_002842; RPTP γ , NP_002832.

Figure 2 Unrooted phylogenetic tree of hCA isoenzymes I–XIV, human RPTP β/γ and vaccCA based on the alignment of CA domains corresponding to amino acid residues 1–260 of hCA II

The tree was constructed using the distance (neighbour joining) method by using MEGA3.1 software. A bootstrap of 1000 replicates was performed and the number on each node indicates the percentage with which each branch topology was supported. The bar shown represents the genetic distance.

In order to investigate the possibility of engineering a CA-like active site in this viral protein, we decided to mutate the vaccCA amino acids Tyr⁶⁹ and Asn⁹² (corresponding to positions 96 and 119 in CA I and II) to histidine residues. Thus we have generated three mutant vaccCAs, i.e. N92H vaccCA, Y69H vaccCA and

the double mutant, N92H/Y69H vaccCA (see the Experimental section for details). GST-fusion proteins were obtained for the wild-type and the three mutant vaccCAs that were purified by a procedure previously optimized for the production of high amounts of various CA isoenzymes for medicinal chemistry purposes. Two-column chromatographic passages were utilized, the first involving a glutathione–Sephacore column, which retained the GST–vaccCA fusion protein. After thrombin cleavage of the chimaeric protein, a second column based on aromatic sulfonamide–Sephacore allowed the purification of the proteins with CA-like activity (this second column could not be used for the wild-type vaccCA, which was not retained on sulfonamide affinity columns). The average yield after two purification procedures ranged from 1 to 3 mg per litre of culture. The CO₂ hydrase activity of the obtained proteins has been measured by a stopped flow assay. Their inhibition by the prototypic sulfonamide CA inhibitor, acetazolamide [AAZ (5-acetamido-1,3,4-thiadiazole-2-sulfonamide)], was also assessed [10].

Data included in Table 2 show that the wild-type vaccCA protein is devoid of CO₂ hydrase activity, as expected for a protein unable to bind zinc, being thus similar to CARP VIII, X and XI [26]. On the other hand, the two single mutants N92H and Y69H, and much more the double mutant, vaccCA N92H/Y69H, show enzymatic activity typical of a CA-like protein. Nevertheless, the single mutants showed a low activity, with k_{cat} and k_{cat}/K_m values similar to those of CA III (the least effective catalyst among the mammalian CAs for the physiological reaction [10]). This may be explained by the presence of only two histidine residues within the active site leading to a rather low affinity for Zn(II), and as a consequence, probably the active site of these enzymes is only partially occupied by zinc. This hypothesis is also confirmed by the finding that the sulfonamide CA inhibitor acetazolamide binds to these two proteins with a rather low affinity (inhibition constants in the range of 76–89 nM). However, the fact that the sulfonamide does bind represents a clear sign that the active site of the mutated viral proteins can also be formed when only two histidine residues are present, but the catalytic activity is low. In the case of the double mutant, vaccCA N92H/Y69H, the CO₂ hydrase activity

Table 2 Kinetic parameters for the CO₂ hydration reaction catalysed by the cytosolic α -hCA isoenzymes I–III, the mitochondrial isoenzymes VA and VB, the secreted isoform hCA VI (full-length) and the transmembrane isoenzymes hCA IX (catalytic domain), and hCA XII (catalytic domain), at 20 °C and pH 7.5, vaccCA (wt) and the three mutants prepared during the present study, and their inhibition data with acetazolamide AAZ, a clinically used drug

Isoenzyme	Activity level	k_{cat} * (s ⁻¹)	k_{cat}/K_m * (M ⁻¹ · s ⁻¹)	K_i (AAZ) * (nM)
hCA I	Moderate	2.0×10^5	5.0×10^7	250 ± 13
hCA II	Very high	1.4×10^6	1.5×10^8	12 ± 0.6
hCA III	Very low	1.0×10^4	3.0×10^5	300000 ± 5000
hCA VA	Low	2.9×10^5	2.9×10^7	63 ± 4
hCA VB	High	9.5×10^5	9.8×10^7	54 ± 5
hCA VI	Moderate	3.4×10^5	4.9×10^7	11 ± 1
hCA IX	High	3.8×10^5	5.5×10^7	25 ± 3
hCA XII	Moderate	4.2×10^5	3.5×10^7	5.7 ± 0.4
vaccCAwt	Devoid	–	–	–
vaccCA N92H	Low	7.8×10^4	$(3.7 \pm 0.2) \times 10^6$	76 ± 6
vaccCA Y69H	Low	8.3×10^4	$(2.3 \pm 0.1) \times 10^6$	89 ± 7
vaccCA N92H/Y69H	Moderate	1.2×10^5	$(2.5 \pm 0.2) \times 10^7$	12 ± 1

* Means $\pm$ S.E.M. (from three different determinations); kinetic data for hCA I–hCA XII are from the literature [10,12–17], whereas vaccCA data are reported in the present study for the first time.

is much higher as compared with the single mutants, with k_{cat} and k_{cat}/K_m values similar to those of some catalytically efficient human isoforms, such as CA VA and CA XII. The other enzymes considered in Table 2 are much better catalysts for this reaction, especially CA II, which is in fact one of the best catalysts known in Nature (Table 2) [10]. Furthermore, acetazolamide binds with very high affinity to the double mutant, showing an inhibition constant of the same order of magnitude as that against CA II. This is clear proof that we were able to engineer the active site of a viral CA isoenzyme, which similarly to other α -CAs shows activity for the CO₂ hydration reaction and binds the typical CA inhibitors, sulfonamides such as acetazolamide, with nanomolar affinity.

How can we explain these findings? Owing to the high sequence homology between vaccCA and other α -CAs, most of the amino acid residues needed for the construction of the active site architecture useful for CO₂ hydration are already present in the wild-type vaccinia protein. By inserting the amino acid residues (two histidine residues) in positions equivalent to His⁹⁶ and His¹¹⁹ in the CA II sequence, these features are further accentuated, leading to a viral CA with a moderate CA activity for the physiological reaction (in the same range as the activity of the human isoenzymes CA VA and CA XII), which also binds the sulfonamide CA inhibitors efficiently. In fact, other residues in the neighbourhood of the Zn(II) ion, more precisely Thr¹⁹⁸ (CA II numbering), which forms a hydrogen bond to the water coordinated to Zn(II) as the fourth ligand in all other α -CAs [10,12–17], and Thr¹⁹⁹, which is involved in the binding of inhibitors, together with Thr¹⁹⁹ [10,12–17], are also present in vaccCA, and this probably helps in stabilizing the tetrahedral geometry of Zn(II) during the catalytic cycle. In close proximity of another important residue for the catalytic cycle, His⁶⁴ (in CA II), vaccCA (and CA III) contain lysine residues (see Figure 1). This residue is involved in the proton shuttling between the active site and the environment, with generation of the zinc hydroxide, a catalytically effective species of the enzyme [10,12–17]. Obviously, the ϵ -amino group present in the lysine residue is a less efficient proton acceptor as compared with the imidazole moiety of a histidine residue, and this may explain the lower catalytic activity

Figure 3 A plaque assay of vaccinia virus, strain CR19, on HeLa cells expressing vaccCA protein variants

Representative wells of HeLa cells stably transfected with cDNA of vaccCAwt and vaccCAadm and infected with 5-fold serial dilutions of vaccinia virus, strain CR19. After 1 h of adsorption, the monolayers were washed and covered with DMEM containing 0.5% methylcellulose. Two days later, the cells were stained with Crystal Violet and pfu/ml for the vaccinia virus grown in the transfectants was determined.

of vaccCA (double mutant), which is of the same order of magnitude as that of CA III. However, our double mutant is in fact approx. 83 times more efficient as a catalyst for CO₂ hydration as compared with CA III (Table 2). This is probably due to the fact that CA III has Phe¹⁹⁷, a very bulky amino acid that highly restricts its active site, whereas the equivalent amino acid residues in vaccCA all possess smaller side chains, and thus the steric impairment is not present in its active site, allowing for a better catalytic efficiency.

To learn whether introduction of the CA catalytic activity into the vaccinia virus cell-surface-binding protein could have any biological effect on infectious properties of the virus, we attempted to produce virions containing ectopically expressed vaccCA protein variants. Three stable transfectants of HeLa cells had been created that expressed either full-length vaccCA or its catalytically active counterpart designated here as vaccCAadm. As a control, HeLa cells had been transfected with an empty vector carrying a neomycin-resistance gene. Prior to infection with the CR19 strain of vaccinia virus, expression of vaccCAwt and vaccCAadm was confirmed by RT-PCR (results not shown). By plaque assay, the titre of recombinant vaccinia virus containing vaccCAadm in its envelope was determined as 4.3×10^7 pfu/ml, whereas the titre of the virus grown on HeLa cells expressing the wild-type vaccCA was close to the titre of the control (2.4×10^7 pfu/ml compared with 2.7×10^7 pfu/ml). The size of plaques was similar in all samples (see Figure 3).

On the basis of recent knowledge, it is difficult to anticipate a possible function of the CA domain of vaccCA. This viral cell-surface-binding protein has been shown to contribute to virus entry, but the CA domain was considered dispensable for its binding capacity [7]. On the other hand, others have demonstrated that the N-terminal region (amino acids 1–264) of the 32 kDa vaccCA is important in vaccinia virus adsorption on cells [11]. As a binding partner, a cell-surface glycosaminoglycan chondroitin sulfate was presented [11], but the precise region of interaction between the two molecules has so far not been mapped.

To ascertain how the gained CO₂ hydrase function affected the overall structure of the protein, which may prove important for adsorption properties of the virus, we also derived three-dimensional models of the CA domain of vaccCA proteins in comparison with that of RPTP β and CA II, which is

well characterized from the structural point of view. In a set of Supplementary Figures 1A–1E (<http://www.BiochemJ.org/bj/407/bj4070061add.htm>), we present the X-ray crystal structure of CA II and the corresponding three-dimensional model, which was obtained with the program PHYRE. This model matches well with the crystal structure (compare Supplementary Figures 1A and 1B). The model of the RPTP β CA domain was also included for comparison (Supplementary Figure 1C), since the pocket structure of the catalytically inactive CA domain of this protein was shown to serve as a receptor site that binds contactin and modulates adhesion and differentiation of neurons [27]. The vaccCA three-dimensional models were obtained from the amino acid sequences of the CA domains of the wild-type protein (Supplementary Figure 1D) and the catalytically active double mutant (Supplementary Figure 1E). It may be observed that the globular shapes of CA II, RPTP β and vaccCA are quite similar. The active site of CA II forms a wide and deep pocket, but also the acatalytic RPTP β and vaccCA display a pocket-like structure. Interestingly, introduction of two mutations leading to the acquisition of zinc ion-binding capacity did not show any remarkable effect on the tertiary structure of the double mutant, suggesting that the formation of the deep pocket-like structure does not depend on the binding of the zinc ion, but seems to be affected by the amino acid composition of the entire CA domain. This, however, supports our experimental results where no dramatic changes in properties of vaccCAwt and vaccCA Δ virions were observed.

Genome analyses of poxviruses revealed the existence of numerous genes homologous with eukaryotic genes present in their host organisms. Therefore it has been suggested that the evolution of poxviruses involved multiple events of gene acquisition [32]. Due to this horizontal gene transfer, poxviruses express many proteins that are functionally and structurally similar to host proteins, such as DNA polymerase, thymidine kinase, ribonucleotide reductase, DNA topoisomerase etc. It is well conceivable that vaccCA has also been acquired by means of a horizontal gene transfer. Its close similarity to CARP X and XI preferably points to an ancestral gene that led to CARPs (in mammals) and in parallel to vaccCA (Figure 2). However, taking into account the results of our mutagenesis study, it also cannot be excluded that the virus originally acquired an evolutionary even older gene, i.e. a common ancestor of the catalytic and acatalytic CA isoforms, which lost its catalytic activity over evolution in the virus, since it was not critical for the vaccinia virus life cycle.

In conclusion, we engineered the active viral CA active site by mutating two amino acid residues in the vaccCA protein. The double mutant vaccCA N92H/Y69H showed a catalytic activity of the same order of magnitude as that of some physiologically relevant hCA isoforms. The double mutant (and to a lesser degree also the two single mutants reported in the present study) also bound sulfonamide CA inhibitors, such as acetazolamide, with a good efficacy. Moreover, when expressed in HeLa cells, the double mutant was able to slightly increase the titre of the virus. However, the biological significance of this finding cannot merely be judged based on *in vitro* data from cell culture, as the CA activity is particularly relevant at the physiological level in the living organisms.

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