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Biochemical Characterization of a S-glutathionylated Carbonic Anhydrase Isolated from Gills of the Antarctic Icefish *Chionodraco hamatus*

Antonia Rizzello,[1] M.Antonietta Ciardiello,[2] Raffaele Acierno,[1] Vito Carratore,[2] Tiziano Verri,[1] Guido di Prisco,[2] Carlo Storelli,[1] and Michele Maffia[1,3]

Gill cytoplasmic carbonic anhydrase of the haemoglobinless Antarctic icefish *Chionodraco hamatus* (Ice-CA) was directly sequenced and consists in 259 residues with an acetylated N-terminus. The molecular mass, deduced from the sequence, was 28.45 kDa, while mass spectrometry analysis of the native protein gave higher values. Treatment with dithiothreitol abolished this difference, indicating possible post-translational modifications. Isoelectric focusing analysis of Ice-CA suggested S-thiolation, which was identified as S-glutathionylation by immunostaining. Deglutathionylated Ice-CA maintained the anhydrase activity but showed higher susceptibility to hydrogen peroxide, suggesting that glutathione binding to Cys residues may have a role in the defence against oxidative damage. Ice-CA is characterized by lower thermal stability, higher activity and lower activation energy than its homologue gill CA of the temperate European eel, confirming the adaptation of the catalytic capacity to low temperatures. Alignment of Ice-CA with homologous enzymes from other fish shows high identity; the enzyme is grouped with a previously described fish CA monophyletic clade although Ice-CA shows several characteristics that can increase protein-solvent interaction and structural flexibility.

KEY WORDS: Gill carbonic anhydrase; Antarctic fish; haemoglobinless *Chionodraco hamatus*; amino acid sequence; S-glutathionylation.

1. INTRODUCTION

Carbonic anhydrase (CA, E.C. 4.2.1.1) represents a family of zinc metalloenzymes that catalyse the reversible hydration of CO_2. They are widespread in nature and are divided into five distinct classes (α, β, γ, δ and ε) that appear to have evolved independently (Hewett-Emmett and Tashian, 1996; So *et al.*, 2004; Sawaya *et al.*, 2006). In vertebrates, the α class isoforms are found in many different tissues and play important roles in many homeostatic processes, including CO_2 transport, ion exchange and acid-base balance (Henry, 1996; Chegwidden and Carter, 2000). Many mammalian α-CA isozymes have been characterized, each with different kinetic properties, molecular structure and sub-cellular distribution (Hewett-Emmett, 2000), namely five cytoplasmic (CAI, II, III, VII and XIII), five membrane-associated (CAIV, IX, XII, XIV and XV), two mitochondrial (CAVA and VB) and one

[1] Laboratory of General Physiology, Department of Biological and Environmental Science and Technology, University of Salento, via Monteroni, 73100, Lecce, Italy.
[2] Institute of Protein Biochemistry, C.N.R., Via Marconi 12, 80125, Naples, Italy.
[3] To whom correspondence should be addressed.
e-mail: m.maffia@physiology.unile.it.

Abbreviations: CA, carbonic anhydrase; Ice-CA, icefish carbonic anhydrase; DTT, dithiothreitol; DTNB, 5,5′-dithio-bis 2-nitrobenzoic acid; IEF, isoelectric focusing; RBC, red blood cell, erythrocyte; ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase

secreted (CAVI). There are also three carbonic-anhydrase-related proteins (CARPs) belonging to the same gene family (Tashian *et al*., 2000) whose physiological function is still unknown. All the isozymes have a molecular mass of 29–30 kDa and contain one zinc atom per molecule. Appleton and Sarkar (1974) indicated that CA activity is related to the ionization of a group close to the zinc. Zinc may be replaced by cobalt and the activity of cobalt-substituted enzyme is dependent upon the state of oxidation of the metal (Shinar and Navon, 1974).

Very few CA isozymes have been structurally and functionally characterized in fish, in which most of the available information pertains to erythrocyte CA (Lund *et al*., 2002; Esbaugh and Tufts, 2006a, b; Esbaugh *et al*., 2004, 2005). All the erythrocyte CAs of modern teleosts are high-turnover isozymes, catalytically comparable to the mammalian CAII. Besides, recent studies on rainbow trout have shown structural and kinetic evidence of the presence of other two CA isozymes: a cytoplasmic isozyme with a wide tissue distribution and very abundant in gills and a membrane-bound isoform (CAIV) mostly expressed in kidney (Esbaugh *et al*., 2004, 2005; Georgalis *et al*., 2006a, b). In addition, there is genetic evidence of a CAVII-like isoform in zebrafish (SWISS-PROT accession number Q6PB17). Phylogenetic analyses suggest that fish CA isozymes with high-turnover rate belong to a group distinct from mammalian CAs, with the only exception of the CAVII and CAIV isoforms (Esbaugh and Tufts, 2006a; Esbaugh *et al*., 2004, 2005)).

The Antarctic icefish *Chionodraco hamatus* (sub-order Nototheniodei, family Channichthyidae) is characterized by the absence of erythrocytes, haemoglobin and circulating CA (Ruud, 1965; MacDonald and Wells, 1991; Feller *et al*., 1994). In a previous study (Maffia *et al*., 2001), the carbonic anhydrase activity was measured in blood, intestine, kidney and gill of two Antarctic teleosts, the haemoglobinless *Chionodraco hamatus* and the red-blooded *Trematomus bernacchii*, and of the temperate teleost *Anguilla anguilla*. In all species, the highest CA activity was in the gills, with the greatest activity in *C. hamatus*. CA activity in the blood was highest in *A. anguilla*, while the activity was low in the intestine and kidney of all three species. The high catalytic rate found in the gills of the haemoglobinless Antarctic teleost has been putatively related to the equilibration of blood CO_2/bicarbonate content in the absence of erythrocytes and circulating CA. Moreover, part of this characteristic can be related to the impairing effects of the low temperature on the reaction rates of psychrophilic enzymes, which are often characterized by low activation energy of the enzymatic reaction (Hoyoux *et al*., 2004; D'Amico *et al*., 2001; Arnold *et al*., 2001) and by high structural flexibility, mainly near the active site (Lonhienne *et al*., 2000; Fields and Somero, 1998; Kim *et al*., 1999). The objective of the present work is to better define the structural and functional characteristics of the branchial CA from the haemoglobinless icefish *C. hamatus* (Ice-CA) and to investigate the molecular basis of its high catalytic efficiency.

2. MATERIALS AND METHODS

2.1. Chemicals and Materials

All reagents, of the highest purity commercially available, were purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (St Louis, MO, USA), Fluka (St Louis, MO, USA), Applied Biosystems (Foster City, CA) and Amersham-Pharmacia Biotech (Uppsala, Sweden). The endoproteinases Lys-C and Asp-N were from Boehringer (Mannheim, Germany), HPLC-grade acetonitrile from Lab-Scan Analytical (Dublin, Ireland). Monoclonal anti-GSH antibody was obtained from Virogen (Watertown, MA, USA).

Specimens of the Antarctic haemoglobinless teleost *Chionodraco hamatus* (240–360 g) were caught by gill nets in Terra Nova Bay, Antarctica, during the 19th and 20th Italian Antarctic Expeditions (January-February 2004, 2005). Fish were kept without feeding in open circulating seawater (3.5% salinity) aquaria at 0°C and allowed at least 6 days to recover from the stress of capture.

2.2. Protein Purification

Fish were killed by a sharp blow to the head and pithed; polyethylene cannulae were then inserted into the ventral aorta to start the systemic perfusion with marine teleost saline (Pellegrino *et al*., 2003) by means of a peristaltic pump (100 ml min^{-1} kg^{-1} body mass) until clear saline left the circulatory system. Tissues were then frozen in liquid nitrogen and stored at −80°C until experiments.

The cytosolic fraction of gill filaments was obtained as previously described (Maffia *et al.*, 2001). Ice-CA was purified by FPLC affinity chromatography on *p*-aminomethylbenzene-sulphonamide immobilized on cyanogen-bromide-activated agarose gel (Whitney, 1974; Maffia *et al.*, 2001). The gel column (1.6×20 cm), fitted to an AKTA-Pharmacia FPLC system, was equilibrated with 0.25 M Tris, 0.1 M Na_2SO_4, adjusted to pH 8.7 with HCl and then rinsed with 0.25 M Tris, 0.3 M $NaClO_4$, adjusted to pH 8.7 with HCl. The enzyme was eluted at 8 ml hr^{-1} by 0.1 M CH_3COOH, 0.5 M $NaClO_4$, pH 5.6 at 4°C. Protein elution was monitored by measuring the absorbance at 280 nm and all fractions containing CA activity, measured by the electrometric method (Maffia *et al.*, 2001), were pooled and concentrated by ultra filtration with a YM10 membrane (Amicon Corp, Lexington, U.S.A), under nitrogen pressure (7×10^5 Pa). All purification steps were carried out between 0 and 4°C. Protein concentration was measured by the Bio-Rad DC protein assay kit, using lyophilized bovine serum albumin as standard.

2.3. SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli on one-dimensional 15% polyacrylamide gel with the Bio-Rad Mini-Protean II cell (Laemmli, 1970). Gels were stained with Coomassie blue R-250. The molecular mass of isolated Ice-CA was calculated from a calibration curve obtained by plotting the relative mobility on the gel of standard proteins (Bio-Rad SDS-Page standards-Broad Range) against the log of their respective molecular mass in five different SDS-gel-electrophoreses.

2.4. Amino Acid Sequencing

Denaturation and alkylation of the Ice-CA sulfhydryl groups with 4-vinylpyridine was carried out as already described (Camardella *et al.*, 1995). Two enzyme aliquots of 200 μg were subjected to different enzymatic cleavages. The first aliquot was dissolved in 75 μl of 40% acetonitrile and incubated at 37°C for 6 hr after addition of 125 μl of 0.125 M sodium phosphate buffer, pH 8.0 and 4 μg of Asp-N in 100 μl of 0.01 M Tris/HCl, pH 7.5; the second aliquot was resuspended in 20 μl of 50% acetonitrile and incubated for 2 hr at 37°C after addition of 200 μl of 0.25 M Tris/HCl, pH 8.5, 0.001 M EDTA, 9 μg of Lys-C in 90 μl of 0.05 M tricine pH 8.0, 0.01 M EDTA. The peptides were separated without drying by reverse-phase HPLC on a μ-Bondapak C_{18} column (0.39×30 cm, 5 μm, Waters), using a Beckman System Gold apparatus (Beckman Instruments, Irvine, CA). Elution was accomplished by a multistep linear gradient of eluant B in eluant A at a flow rate of 1 ml min^{-1}, monitoring the eluate at 220 and 280 nm. The separated fragments were collected and sequenced with an automated repetitive Edman degradation performed on a Procise™ Protein Sequencer model 492 (Applied Biosystems Division of Perkin–Elmer Corporation, Foster City, CA).

2.5. Mass Spectrometry Analysis

The molecular mass of the native Ice-CA and S-pyridylethylated peptides (less than 10 kDa) was measured by MALDI-TOF mass spectrometry on a PerSeptive Biosystems Voyager-DE Biospectrometry Workstation (PerSeptive Biosystems Inc., Framingham, MA, USA). Analyses were performed on pre-mixed solutions prepared by diluting samples (final concentration, 5 pmol μl^{-1}) in four volumes of matrix, namely 10 mg ml^{-1} sinapinic acid in 30% acetonitrile containing 0.3% trifluoroacetic acid (native enzyme), and 10 mg ml^{-1} α-cyano-4-hydroxycinnamic acid in 60% acetonitrile containing 0.3% trifluoroacetic acid (peptides). Electrospray MS and MS/MS analyses were performed on a Finnigan LCQ ion trap mass spectrometer (Finnigan Corp., San Jose, CA, USA), equipped with a reverse-phase HPLC column for the N-terminal peptide purification directly connected to the ion source, and the spectra were acquired and elaborated with the software provided by the manufacturer.

2.6. Reverse-phase HPLC

Native, dithiothreitol (DTT)-treated and denatured and S-pyridylethylated Ice-CA were subjected to reverse-phase HPLC on a Vydac C18 column (4.6 mm×250 mm, 5 μm), fitted to a Beckman System Gold apparatus (Beckman Instruments, Irvine, CA). Elution was carried out by linear gradient of eluant B (0.08% trifluoracetic acid in acetonitrile) in

eluant A (0.1% trifluoroacetic acid) at a flow rate of 1 ml min^{-1}, monitoring the eluate at 220 and 280 nm.

2.7. Measurement of Protein Sulfhydryl Groups

The sulfhydryl groups of the native and denatured enzyme were determined according to the method of Ellman (1959) as modified by Riener *et al.* (2002). Briefly, the native and denatured proteins (with 6 M urea) were reduced by incubation with 0.1 M DTT for 1 hr; DTT in excess was removed by dialysis for 6 hr against 0.1 M NaH_2PO_4, 0.0002 M EDTA adjusted to pH 8.0, with and without 6 M urea, respectively. For the measurement of the thiol concentration by the Ellman method, the protein samples were adjusted to a final sulfhydryl concentration of ≤ 40 μM by dilution with PBS (137 m*M* NaCl, 10.1 m*M* Na_2HPO_4, 1.76 m*M* KH_2PO_4 adjusted to pH 7.4). Then, 200 μl of a buffer containing 0.1 M boric acid, 0.2 m*M* EDTA, pH 8.2, were added, to adjust the pH at values ≥8.0. After addition of 20 μl of 10 m*M* Ellman reagent [5,5′-dithio-bis 2-nitrobenzoic acid (DTNB) in 100 m*M* NaH_2PO_4, 2 m*M* EDTA adjusted to pH 7.0] the absorbance was measured at 412 nm and the sulfhydryl content was calculated using a molar extinction coefficient of 14150 M^{-1} cm^{-1} (Riener *et al.*, 2002).

2.8. Isoelectric Focusing (IEF)

IEF of Ice-CA was performed in a vertical polyacrylamide minigel (pH 4–9, 5% acrylamide) running for 1.5 hr at 200 V constant voltage, then increased to 400 V constant voltage for an additional 1.5 hr. Gels were fixed with 10% trichloroacetic acid for 10 min, transferred into 1% trichloroacetic acid for a minimum of 2 hr to remove ampholytes and stained with Coomassie Blue R-250. To determine the pI of Ice-CA, IEF markers were used (Sigma IEF Markers: myoglobin, pI 6.8 and 7.2; glucose oxidase, pI 4.2). To assess the presence of post-translational modifications of Ice-CA, the isoelectric focusing was repeated after treatment with DTT, in which ten μg of Ice-CA were incubated with increasing concentration of DTT (0, 10, 50, 100 and 200 m*M*) for 2 hr at 4°C and the reaction was stopped by treating the sample with 45 m*M* iodoacetamide.

2.9. Detection of S-glutathionylation by Immunoblotting

Glutathione bound to Ice-CA was immunologically detected by Western blot analysis. Ten μg of three different preparations of Ice-CA were separated by SDS-PAGE and transferred to nitrocellulose. After SDS-PAGE, proteins were blotted onto a nitrocellulose membrane at 200 mA for 60 min and blocked for 1 hr in PBS/0.1% Tween-20 (PBST) with 5% non-fat milk. The membrane was incubated overnight with the monoclonal mouse anti-GSH antibody (1:600 dilution) in 5% milk/PBST and then washed three times with PBST for 10 min each. The horseradish peroxidase-conjugated anti-mouse antibody was used as the secondary antibody (1:3000 dilution). Visualization of immunoreactive bands was accomplished by chemiluminescence and exposure of the blots to luminescence detection film (ECL Western Blotting detection reagents and Hyperfilm-ECL, Amersham).

2.10. Carbonic Anhydrase Activity

Carbonic anhydrase activity was measured either by electrometric or by radioactive method, both described in details elsewhere (Maffia *et al.*, 2001). The electrometric (ΔpH) method was used to measure the effect of H_2O_2 on the activity of the native and reduced Ice-CA. The enzymatic activity was measured at 0°C before and after treatment of the enzyme with 0.045 M H_2O_2 for 15 min, by adding CO_2-saturated H_2O to the reaction medium and measuring the H^+ developed per unit of time by the conversion of carbon dioxide to bicarbonate. The specific activity of Ice-CA was reported in μmol H^+ min^{-1} mg $protein^{-1}$. To investigate the thermodependence, the radioactive method allowed to measure the enzymatic activity in the 0–40°C range using 500 ng of purified branchial CA for each determination and a saturating concentration of 100 m*M* $NaH^{14}CO_3$. The maximal velocity of $H^{14}CO_3^-$ dehydration produced by one nmol of CA was expressed as the number of cts min^{-1} produced by the $^{14}CO_2$ developed by the enzymatic reaction. The Ice-CA specific activities were reported in an Arrhenius plot (*ln CA activity* as a function of $1/T$) and the activation energy (E_a) was calculated from the slope ($-E_a/R$) of the regression line, where R is the gas constant. The same measurements were performed on the carbonic anhydrase isolated from the gills of the temperate teleost *Anguilla anguilla* (Eel-CA).

2.11. Sequence Analysis and Computational Procedures

The amino acid sequence of Ice-CA was compared to those of CAs of other organisms. Accession numbers are SWISS-PROT for all CA isozymes used. Teleosts: Mozambique tilapia (*Oreochromis mossambicus*) CA (Q6JRS3); rainbow trout (*Oncorhynchus mykiss*) erythrocyte (RBC) CAII (TCAb; Q7T2K6); rainbow trout gill CAII (TCAc; Q6R4A2); rainbow trout kidney CAIV (Q6R4A1); zebrafish (*Danio rerio*) embryo CAII (Q6PFU7), retina CA (CAHZ; Q92051) and eye CAVII (Q6PBI7); Japanese dace (*Tribolodon hakonensis*) cytoplasmic gill CAII (Q8UWA5); carp (*Cyprinus carpio*) RBC CA (Q3Y545); winter flounder (*Pseudopleuronectes americanus*) RBC CA (Q5MCN0). Ancient fish: longnosed gar (*Lepisosteus osseus*) RBC CA (Q8JG56); sea lamprey (*Petromyzon marinus*) RBC CA (Q3Y546); dogfish (*Squalus acanthias*) kidney CAIV (Q4FCT9). Mammals: man (*Homo sapiens*) HCAI (P00915), HCAII (P00918), HCAIII (P07451), HCAIV (P22748), HCAVA (P35218), HCAVB (Q9Y2D0), HCAVI (P23280), HCAVII (P43166), HCAVIII (P35219), HCAIX (Q16790), HCAX (Q9NS85), HCAXI (O75493), HCAXII (O43570), HCAXIII (Q8N1Q1) and HCAXIV (Q9ULX7); mouse *(Mus musculus)* CAI (P13634), CAII (P00920), CAIII (P16015), CAVA (P23589), CAVB (Q9QZA0), CAVII (Q9ERQ8) and CAXIII (Q9D6N1); rat *(Rattus norvegicus)* CAII (P27139), CAIII (P14141), CAVA (P43165) and CAVB (Q66HG6); sheep *(Ovis aries)* CAI (P48282) and CAII (P00922); bovine *(Bos Taurus)* CAII (P00921); rabbit *(Oryctolagus cuniculus)* CAI (P07452) and CAII (P00919); pig *(Sus scrofa)* CAI-II (Q5S1S4). The theoretical isoelectric point was calculated with the online program "ProtParam" (http://www.expasy.org/tools/protparam.html).
Amino acid sequences were aligned with ClustalW (version 1.8; http://www.ebi.ac.uk/clustalw), a bioinformatics application developed for the alignment of closely related sequences. Phylogenetic analysis of cytoplasmic CA isoforms was carried out by neighbour joining (NJ) with the MEGA program (version 3.1) (Kumar *et al.*, 2004), using complete deletion with *P*-values corrected by a Poisson distribution with 1000 bootstrapping replicates. Analyses were performed using mouse, human and zebrafish CAVII as a monophyletic outgroup, as previously described by Hewett-Emmett and Tashian (1996). In addition, a comparative analysis of the active sites was performed between Ice-CA and other fish and human cytosolic CA isoforms, as described by Tashian *et al.* (2000).

2.12. Statistical Analyses

All measures were performed repeatedly in at least three separate experiments. The data are reported as the mean (±SEM) of at least three different enzyme preparations. Means were compared by Student's *t*-test and significant differences are indicated by asterisks (*$P < 0.05$; **$P < 0.01$).

3. RESULTS

3.1. Amino Acid Sequence of Cytosolic Carbonic Anhydrase of the Icefish C. hamatus Gills (Ice-CA)

Ice-CA isolation procedure gave a purified fraction showing a single band on SDS-PAGE, with an estimated apparent molecular mass of 28.76 ± 0.7 kDa (Fig. 1). The primary structure was obtained by direct protein sequencing (Edman degradation). Attempts to obtain the N-terminal amino acid sequence of the native and S-pyridylethylated Ice-CA were unsuccessful, suggesting a blocked N-terminus. Sequencing of internal Ice-CA fragments (Fig. 2) was obtained after enzymatic digestions of the S-pyridylethylated enzyme with Asp-N (D-fragments) and Lys-C (K-fragments). Peptide mixtures were separated by HPLC. The molecular mass of each peptide was measured by MALDI-TOF mass spectrometry; then each peptide was sequenced. The complete amino acid sequence was obtained by peptide alignment with homologous enzymes and by overlapping fragments. Only the most significant peptides necessary to elucidate the complete amino acid sequence are indicated in Fig. 2. The enzymatic digestions with Lys-C and Asp-N essentially produced the expected peptides; sometimes Asp-N cleaved at Glu (D5, D6). Fragments K2, K11, D7 and D11 were not extensively sequenced due to the low yield; the sequence of these regions was established by sequencing other peptides. The blocked N-terminus fragment was analysed by ES mass spectrometry, showing the occurrence of a mass signal at m/z 757.63, corresponding to the doubly charged ion of the peptide, from which a molecular

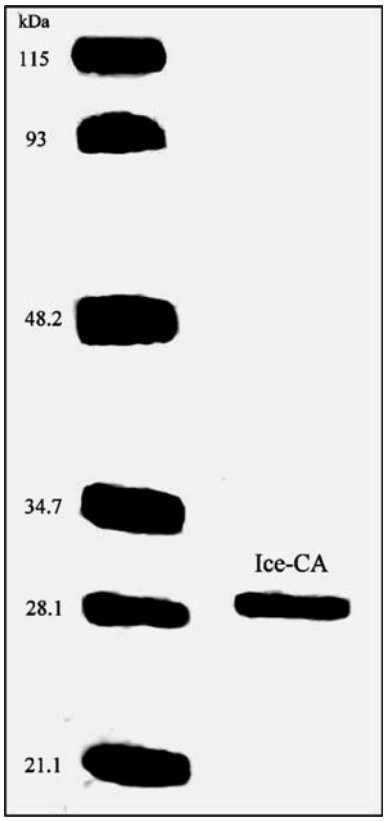

Fig. 1. One-dimensional SDS-PAGE of carbonic anhydrase from *C. hamatus* gill (Ice-CA). The Ice-CA migrated as single protein band of 28.76 ± 0.7 kDa, as determined from five different experiments. The positions of molecular-mass protein markers (kDa) are shown on the left of the figure.

mass of 1513.26 ± 0.12 Da (compatible with that of a 14 residues peptide) was calculated. The doubly charged ion was then isolated into the ion trap and fragmented to establish the entire sequence of this peptide. The MS/MS spectra showed the presence of fragment ions from both y and b series and the complete sequence of the blocked peptide was Ac-Ala-His-Ala-Trp-Gly-Tyr-Gly-Pro-Thr-Asp-Gly-Pro-Asp-Lys. The two C-terminal residues (Asp13-Lys14), also identified in peptide D1 (see Fig. 2), were helpful for the exact alignment of the fragments. This short sequence had 79% identity with the corresponding region reported for zebrafish CAHZ, showing the same residue (acetyl-Ala) at the N-terminus. The complete amino-acid sequence of Ice-CA contained 259 residues and the calculated molecular mass, including the acetyl group linked to the N-terminal residue and the zinc atom, was 28.45 kDa.

3.2. Reverse-phase HPLC of Ice-CA

Native Ice-CA was eluted as a large and asymmetric peak by HPLC (Fig. 3), indicating heterogeneity. DTT treatment of the native protein was effective in producing a sharp peak, suggesting that the heterogeneity was probably due to the linkage of ligands to Cys residues exposed on the molecular surface. Denaturation (in 6 M guanidinium hydrochloride) and S-pyridylethylation of cysteine residues abolished the heterogeneity.

3.3. Ice-CA Molecular Mass

The molecular mass of native Ice-CA obtained by MALDI-TOF mass spectrometry ranged from 28.74 ± 0.022 to 29.22 ± 0.022 kDa. These values were higher than that calculated based on the sequence, including the acetyl group linked to the N-terminus and the Zn atom at the active site (28.45 kDa). The molecular mass obtained after incubation in 0.1 M DTT for 1 hr and removal of reagents by HPLC was 28.37 ± 0.022 kDa, strengthening the hypothesis of the presence of ligands at the exposed Cys residues. Data from the Ellman method on DTT-reduced enzyme both in native and denatured form suggest that four of six Cys residues are exposed on the Ice-CA surface, therefore being available to possible post-translational modifications.

3.4. Ice-CA Isoelectric Point and S-glutathionylation

IEF of native Ice-CA showed three bands in correspondence to pI 5.5, 5.1 and 4.8 (Fig. 4A, lane 2), consistent with the possibility of partial titration. The presence of low molecular-mass thiols such as glutathione bound to the protein sulfhydryl groups was hypothesized, which would explain also the higher molecular mass of native Ice-CA. Treatment with DTT (Fig. 4A, lanes 3–6) determined the disappearance of the more acidic bands confirming the presence of reversible oxidation events of cysteine residues (i.e., S-glutathionylation). The specific binding of glutathione molecules to Ice-CA was demonstrated by Western blot analysis using a monoclonal anti-GSH antibody (Fig. 4B). A specific immunolabelling occurred as a result of S-glutathionylation in three different enzyme preparations under non-reducing condition (without DTT; Fig. 4B lanes 1–3); this specific labelling was

```
   1                         25                          50
Ac-AHAWGYGPTDGPDKWVSNFPIADGPRQSPIDILPGGASYDSGLKPLSLKY
   <----K1------><------------K2-------------******><
                <---D1---->        <---D2--><----D3--->

                            75                          100
   DPSNCLEILNNGHSFQVTFADDSDSSTLKEGPISGVYRLKQFHFHWGASN
   -------------K3-------------><----K4---><----K5---
   <-D4-><-----D5----->          <---------D6-------->

                           125                          150
   DKGSEHTVAGTKYPAELHLVHWNTKYPSFGEAASKPDGLAVVGVFLKIGD
   -><---K6---><-----K7----><---------K8---------><--
   <---------D7--------------*********>

                           175                          200
   ANASLQKVLDAFNDIRAKGKQTSFADFDPSTLLPGCLDYWTYDGSLTTPP
   -K9--->----K10-->  <--------K11-------**********
                <----D8---->  <---D9--->D10><-------

                           225                          250
   LLESVTWIVCKEPISVSCEQMAKFRSLLFSAEGEPECCMVDNYRPPQPLK
   **********><----K12--->><-----------K13----------->
   --D11----------->                          <---------


   GRHVRASFQ
   <--K14-->
   -D12---->
```

Fig. 2. Amino acid sequence of Ice-CA obtained by Edman degradation. Arrows indicates fragments obtained by enzymatic digestion with Asp-N (D) and Lys-C (K). Asterisks indicate not entirely sequenced regions. Peptides from each digestion are numbered according to their order in the sequence.

completely reversed by reducing the enzyme with 0.1 M DTT (Fig 4B, lanes 4–6). S-glutathionylation does not have any effect on the anhydrase activity of Ice-CA since the reduction did not significantly change the catalytic activity (Fig. 4D). Instead, DTT-treated Ice-CA showed higher sensitivity to oxidation by H_2O_2 than the native form. Treatment with 0.045 M H_2O_2 for 15 min decreased the activity of the reduced and native Ice-CA to 46% and 80%, respectively (Fig. 4D).

3.5. Thermodependence and Activation Energy of Ice-CA

The thermodependence of Ice-CA was examined over a temperature range from 0–40°C (Fig. 5A) in saturating substrate conditions ($NaH^{14}CO_3$) and compared to that of a homologous mesophilic enzyme. The specific activity of Ice-CA increased exponentially with temperature up to 24°C, then showed a decay at higher temperatures, and was lost at 40°C. The specific activity of the homologous mesophilic enzyme, isolated from the gills of the temperate teleost European eel (Eel-CA), exponentially increased with temperature up to 35°C, showing a significantly lower catalytic rate

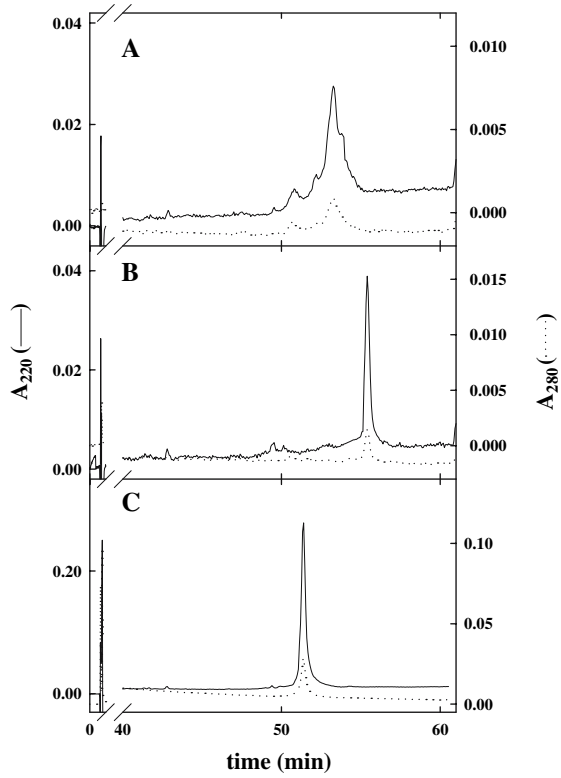

Fig. 3. Reverse-phase HPLC of native (A), DTT treated (B) and denatured and S-pyridylethylated (C) Ice-CA.

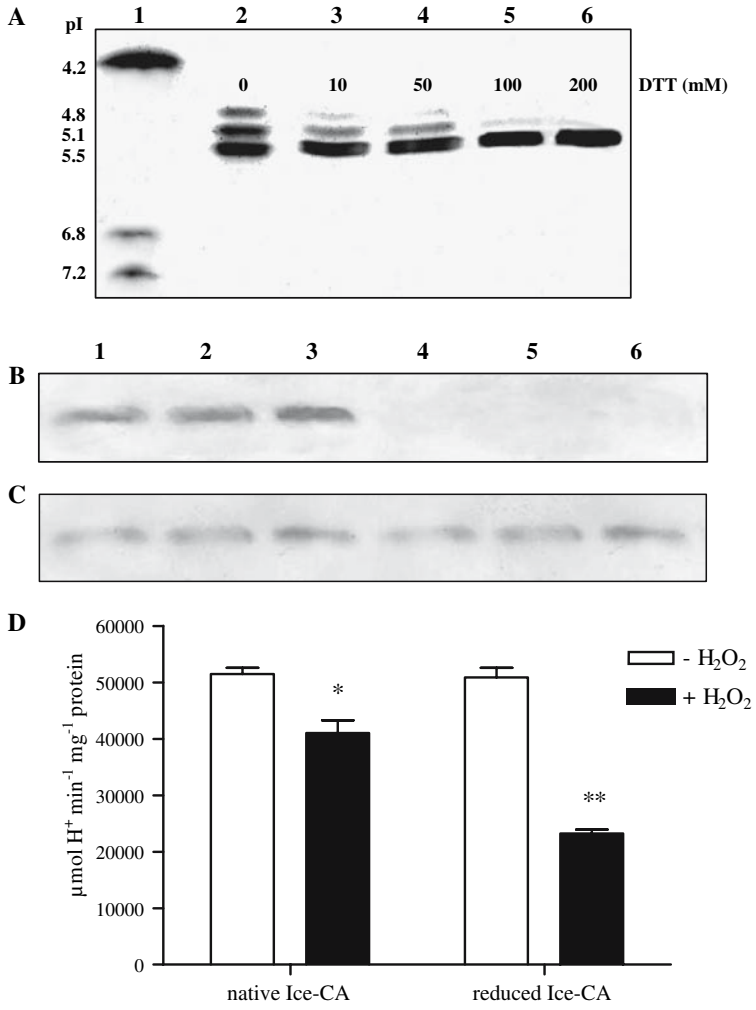

Fig. 4. Post-translational modification of Ice-CA. (A) Isoelectric focusing gel of Ice-CA stained with Coomassie Blue after treatment with increasing concentrations of DTT (Lanes 3–6). The isoelectric point of markers and untreated Ice-CA are reported in the gel (Lanes 1 and 2, respectively). (B) Detection of S-glutathionylation by anti-GSH antibody on Ice-CA before (Lanes 1–3) and after (Lanes 4–6) 100 m*M* DTT treatment. (C) Validation of the loading of Ice-CA to nitrocellulose by Red Ponceau staining. (D) The effect of 45 m*M* H_2O_2 on the specific activity of the native and reduced Ice-CA (*$P < 0.05$; **$P < 0.01$; $N = 5$).

then the cold-adapted isozyme. The Arrhenius plot of the catalytic rate at different temperatures was used to calculate the activation energy (E_a) of the reaction, showing a lower value for the Antarctic isozyme [31.8 ± 6.6 kJ/mol (0–24°C) and 91.9 ± 7.2 kJ/mol (0–35°C) for Ice-CA and Eel-CA, respectively].

3.6. Sequence Analysis

The FASTA homology search demonstrated that the Ice-CA sequence possesses significant similarity to piscine CAs (with amino acid percentage identities ranging from 63 to 86%), with the except of trout CAIV (33%), dogfish CAIV (33%) and zebrafish CAVII (58%) (Table 1). Moreover, Ice-CA most closely resembles cytoplasmic and mitochondrial human CA isoforms, showing 62% identity with Human CAII (Table 1). Sequence identity with human CA isozymes with different sub-cellular distribution was lower then 40% (data not shown).

NJ analysis of vertebrate cytoplasmic CA isozymes (Fig. 6) grouped the Ice-CA sequence within the previously described fish cytoplasmic CA clade, basal to that of other vertebrate CAs (CAI, CAII, CAIII and CAXIII), but appearing after the divergence of CAV and CAVII. Within the fish CA

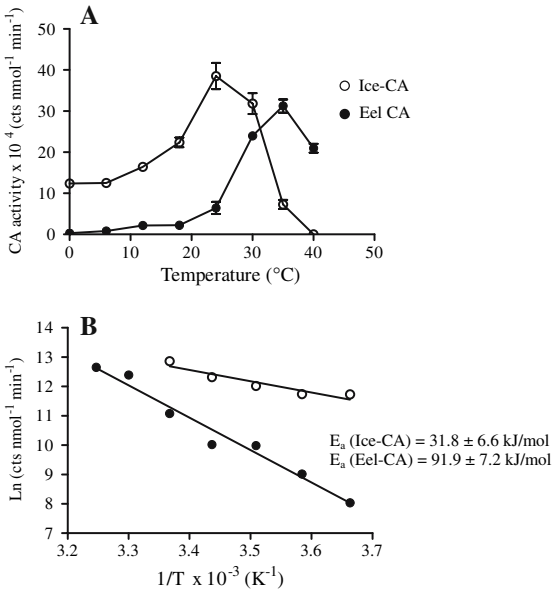

Fig. 5. (A) Thermodependence of the activity of Ice-CA (*o*) and Eel-CA (•). The maximal velocity (V_{max}) of the enzymatic reaction, expressed as cts $nmol^{-1}$ min^{-1}, was measured in saturating substrate condition at 0, 6, 12, 18, 24, 30, 35 and 40°C. (B) Arrhenius plot of the temperature-dependence curves reported in (A), showing different activation energy (E_a) values for Ice-CA and Eel-CA ($P < 0.01$; $N = 3$).

cluster, Ice-CA grouped most closely with the winter flounder enzyme.

Sequence analysis also included comparison of the Ice-CA active site with those of other cytoplasmic fish and human CAs (Table 2). The active-site sequence of Ice-CA is similar to the sequences of cytoplasmic CAs of winter flounder and trout, whereas it differs in 1–4 residues from other fish isoforms.

4. DISCUSSION

Cold adaptation of gill cytosolic carbonic anhydrase of the Antarctic icefish *Chionodraco hamatus* (Ice-CA) results from a shift of the optimal temperature toward low values, an increased overall catalytic efficiency and relatively low E_a (about one third of that measured with temperate Eel-CA). This finding is in accordance with the general observation that E_a of reactions catalyzed by cold-adapted enzymes are lower than those catalyzed by the mesophilic counterparts (Maffia *et al.*, 1993; 2001; Ciardiello *et al.*, 1995; 2000; Feller *et al.*, 1996; Gerday *et al.*, 1997; Lonhienne *et al.*, 2000). Higher catalytic efficiency at low temperature has been correlated with higher molecular flexibility and solvent interactions (Lonhienne *et al.*, 2000; Fields and Somero, 1998; Kim *et al.*, 1999). Both features depend on the combined effect of several structural factors related to cold adaptation (see below). The complete primary structure (259 residues) of Ice-CA displays acetylated Ala at the N-terminus. The active site of Ice-CA is highly similar to those of other cytoplasmic CA isozymes, differing by only two residues from other teleost CA isozymes. Albeit the replacements do occur in the active site, they are not within the specific aminoacid sequence directly involved in the catalysis (Stams and Christianson, 2000), indicating that the critical factors of the enzyme function are entirely conserved.

The comparative analysis between Ice-CA and the available complete sequences of CAs of other fish (including temperate and tropical species) show high identity with few exceptions. In the phylogenetic analysis of cytoplasmic α-CA isozymes, Ice-CA groups with a monophyletic clade of all fish CAs (Lund *et al.*, 2002; Esbaugh and Tufts, 2006a, b; Esbaugh *et al.*, 2004, 2005), with the exception of zebrafish CAVII. The cytoplasmic fish CA group appears to have emerged before the gene duplication event that gave rise to mammalian CAI, II, III and XIII genes. The analyses support previous studies showing that the cytoplasmic fish CA group diverged after the emergence of CAV and CAVII in vertebrates (Esbaugh and Tufts, 2006b; Esbaugh *et al.*, 2004, 2005).

Within the fish CA cluster, Ice-CA groups closely with tilapia CA, trout TCAc, Dace CAII and zebrafish CAII, all sequenced from cDNA obtained from gills or tissues other than erythrocytes, supporting the existence of a general cytoplasmatic CA distinct from a specific erythrocyte isozyme (Esbaugh *et al.*, 2005; Georgalis *et al.*, 2006b). In fact, all the fish erythrocyte isoforms group closely, with the exception of winter flounder RBC CA which shows a higher similarity with non-erythrocytic CAs and, in particular, with Ice-CA (95%). Unfortunately, the absence of a publication with a detailed description of this sequencing does not allow further speculating on this apparent inconsistency. Anyhow, while the sequence of Ice-CA shows a higher number of negative charges (Asp + Glu) than other teleost CAs, the Asp + Glu content in CA of winter flounder, which experiences temperatures ranging between 14°C in the summer and

Table 1. Sequence identity (%) of cytosolic and mitochondrial CAs from man and fish

Species	Human CAXIII	Human CAVII	Human CAVB	Human CAVA	Human CAIII	Human CAII	Human CAI	Dogfish CAIV	Trout CAIV	Zebrafish CAVII	Lamprey CA	Zebrafish CAII	Gar CA	Carp CA	Dace CAII	Trout TCAb	Zebrafish CAHZ	Trout TCAc	Tilapia CA	Winter flounder CA
Ice-CA	60	58	52	53	54	62	59	33	33	58	63	70	74	74	76	77	78	78	81	86
Winter flounder CA	61	57	52	51	53	60	58	34	31	58	62	70	75	71	73	76	74	79	80	
Tilapia CA	60	57	51	52	55	58	57	36	32	58	61	72	74	71	77	74	74	75		
Trout TCAc	59	54	47	47	51	61	57	33	32	57	60	72	75	71	75	76	74			
Zebrafish CAHZ	61	60	50	50	55	63	60	34	33	59	60	72	77	91	71	81				
Trout TCAb	60	58	49	50	51	61	57	32	33	58	58	73	74	78	73					
Dace CAII	63	59	51	50	54	64	60	33	33	59	61	76	74	69						
Carp CA	61	60	51	50	54	62	59	35	32	57	59	69	75							
Gar CA	63	58	50	50	59	65	62	34	32	59	60	70								
Zebrafish CAII	61	58	49	49	53	62	60	34	33	58	58									
Lamprey CA	58	55	51	51	48	62	57	35	35	61										
Zebrafish CAVII	53	69	48	47	48	58	54	36	34											
Trout CAIV	33	33	26	27	33	32	31	42												
Dogfish CAIV	33	39	25	27	30	33	27													
Human CAI	59	50	46	48	53	60														
Human CAII	59	56	52	50	57															
Human CAIII	58	50	44	45																
Human CAVA	46	48	58																	
Human CAVB	47	49																		
Human CAVII	52																			

Amino acid sequences accession nos. were taken from the SWISS-PROT Data Bank and reported in Materials and Methods. Percent identity was calculated with the FASTA3 Program (http://www.ebi.ac.uk/fasta33) using default parameters.

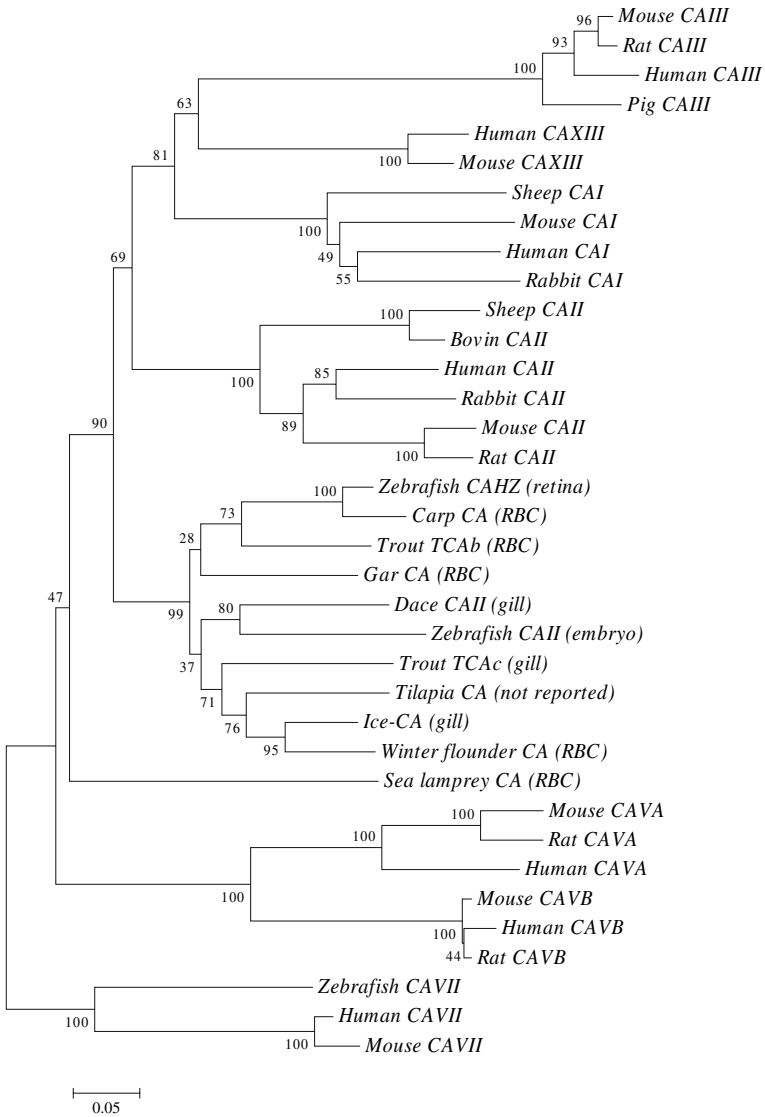

Fig. 6. Phylogenetic tree of Ice-CA and cytosolic and mitochondrial isozymes of fishes and other vertebrates. Alignments were performed using ClustalW, bootstrapped 1000 times and the tree constructed using neighbour-joining analysis with data corrected for multiple hits through Poisson distribution. The tree was ordered using human, mouse and zebrafish CAVII as monophyletic outgroup.

−1.4°C in the winter (Fletcher, 1977), is similar to that of Ice-CA. These negatively charged residues, together with ligated glutathione (see below), account for the isoelectric point of Ice-CA, significantly lower than the theoretical ones calculated (ProtParam on http://www.expasy.org) for other fish CAs, but similar to that of winter flounder erythrocyte (5.22), trout (5.45) and sea lamprey CAs (5.47). Studies on subtilisins isolated from two Antarctic *Bacillus* strains (Davail *et al.*, 1994; Feller *et al.*, 1996) indicate that acidic pI can be somewhat related to the low environmental temperature. Psychrophilic subtilisins have acidic pI = 5.5, unlike their mesophilic homologues (pI = 9), consistent with the higher content of Asp residues, some of which are exposed. Although the adaptation strategies appear unique to each protein, in Ice-CA, similar to Antarctic subtilisins, the high number of negative charges with consequent low pI could be a common feature of cold adaptation. This feature can increase the protein flexibility by decreasing molecular compactness and, in addition, can increase hydrophilicity, facilitating interactions with the solvent.

Table 2. Comparison of the active site sequence of Ice-CA with sequences of cytosolic isozymes from other fish and man

Ice-CA	Y	S	N	N	H	S	F	Q	T	K	Q	H	H	E	H	E	H	V	F	L	V	G	W	Y	L	T	T	P	P	L	S	V	W	V	N	R	aa difference
	*	*				*		*	*	*	*	*	*	*	*	*	*							*		*	*							*		*	*	
					+							z	z				z	~		~	~			~								~	~					
Winter flounder CA	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0
Trout TCAc	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0
Trout TCAb	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1
Zebrafush CAII	·	·	·	·	·	·	·	·	S	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1
Dace CA	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	1
Tilapia CA	·	·	·	·	·	·	V	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	2
CAHZ	·	·	·	·	·	·	·	·	D	R	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	2
Carp CA	·	·	·	·	·	·	·	·	D	R	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	2
Gar CA	·	·	·	·	·	·	·	·	D	R	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	2
Lamprey CA	·	·	·	·	·	·	·	S	E	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	F	·	·	·	·	·	·	3
Zebrafish CAVII	·	·	·	·	·	·	V	V	E	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	4
Human CAI	·	·	·	V	·	·	·	H	N	F	·	·	·	·	·	·	·	A	L	·	·	·	·	·	·	·	H	·	·	Y	·	·	·	I	·	·	9
Human CAII	·	·	·	·	·	A	·	N	E	I	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	5
Human CAIII	·	·	·	·	K	T	C	R	V	R	·	·	·	·	·	·	·	·	·	I	·	·	·	·	F	·	·	·	·	E	C	I	·	L	·	·	12
Human CAVA	W	·	·	T	Y	L	·	·	E	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	I	·	·	7
Human CAVB	·	·	·	·	Y	·	·	L	E	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	S	·	·	·	I	·	·	5
Human CAVII	·	·	·	·	·	·	V	·	D	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	S	·	·	·	·	·	·	3
Human CAXIII	·	·	·	S	·	·	·	N	D	R	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	V	·	·	·	·	·	·	·	·	·	5

Identical amino acids are indicated by dots.
*, putative active site; z, zinc binding ligand; +, proton shuttling associated ligand; ~, substrate associated pocket.

The heterogeneity of purified Ice-Ca is abolished by DTT, indicating post-translational modification of several Cys residues, which may account for the higher molecular mass obtained by mass spectrometry. Ice-CA has six Cys residues and, following reduction with DTT, four moles of free –SH per mole of native enzyme were measured by the Ellman method, suggesting that four of them are exposed on the molecular surface and available for post-translational modification (S-thiolation). S-glutathionylation of Cys residues has been already described in rat CA III, where Cys-181 and Cys-186 can be glutathionylated (Chai *et al*., 1991; Mallis *et al*., 2000, 2002) and in tiger shark erythrocyte CA, where one or more of the eight Cys residues present in the molecule can undergo S-glutathionylation (Maynard and Coleman, 1971; Bergenhem *et al*., 1986). Cys-186, the most reactive Cys of CAIII, is conserved in Ice-CA, as well as Asp-188, Lys-211 and Glu-212, all important for modulation of the susceptibility of CAIII to glutathione binding (Kim and Levine, 2005). Similar to CAIII and tiger shark CA, also Ice-CA is S-glutathionylated, as shown by immunostaining with monoclonal anti-GSH antibody. A possible role of this post-translational modification was investigated by analysing the effects of deglutathionylation on the enzymatic activity. While Ice-CA activity is unaffected by DTT, excluding a correlation between S-glutathionylation of Cys residues and CA capacity, deglutathionylated Ice-CA is more susceptible to H_2O_2 than the native form, suggesting a protective action of S-glutathionylation against oxidative stress. Oxidative stress in cold adapted ectotherms is a still debated issue. Whole organism aerobic metabolism measurements with computerized, high resolution respirometry have shown that polar fish do not show elevated resting aerobic oxygen consumption values, or standard metabolic rate, compared with oxygen consumption values of tropical or temperate fish extrapolated to similar low polar temperatures (Steffensen *et al*., 1994; Drud Jordan *et al*., 2001; Steffensen, 2002). Nevertheless, *in vitro* rates of ROS production by mitochondria from polar and temperate ectotherms can be similar, even when the mitochondria are assayed at habitat temperature (Heise *et al*., 2003; Abele and Puntarulo, 2004) and at least some pathways of energy metabolism are cold adapted in Antarctic fishes (Crockett and Sidell, 1990). These features can be in good part referred to the higher mitochondrial volume density of mitochondria in the cells of polar benthic invertebrates, compared to animals from warmer environments (Johnston, 1981; Dunn, 1988; Johnston *et al*., 1998; Sommer and Pörtner, 2002; Guderley and St-Pierre, 2002). Besides the resting metabolic

rate, several other aspects must be considered in discussing ROS formation in icefish. *C. hamatus* is a demersal, sluggish predator, but capable of short bursts of swimming to catch its preys (Eastman, 1993). Because of the haemoglobinless conditions and the limited oxygen transport capacities, it can very likely experience extreme physiological hypoxic conditions during exercise with release of oxygen radicals during both hypoxia and subsequent re-oxygenation, a phenomenon amplified by the high solubility of oxygen at low temperature (Jamieson *et al*., 1986). Interestingly, the icefishes *Champsocephalus gunnari*, *Chaenocephalus aceratus* and *Pseudochaenichthys georgianus* have a significantly higher superoxide dismutase (SOD) activity in gills as compared to other red-blooded Antarctic fish, but significantly lower amounts of catalase (CAT) and glutathione peroxidase (GPx) (Ansaldo *et al*., 2000). SOD catalyzes glutathione dismutation of superoxide into oxygen and hydrogen peroxide. The latter is then reduced to water by other enzymes such as CAT and GPx. All these characteristics can lead icefish gills to experience high cellular concentration of H_2O_2 which, if not enzymatically decomposed, can be converted to the very short-lived and highly aggressive $^{\bullet}OH$, via the transition metal catalyzed Fenton reaction (Halliwell and Gutteridge, 1985). In this condition, the presence of glutathione ligands on Cys residues can protect the enzymatic capacity of Ice--CA from oxidative impairment during the physiological hypoxic events experienced by the animal. However, further work is needed to confirm this hypothesis, due to as yet insufficient comparative information on glutathionylation of cytosolic protein in fish from different environments, as well as on possible glutathionylation of red-blooded Antarctic ectotherms.

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