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Carbonic Anhydrase III in Liver and Muscle of Male Rats Purification and Properties[1]

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ABSTRACT

Cytosolic carbonic anhydrases CAI, CAII, and CAIII from liver, and CAII, and CAIII from muscle of adult male Sprague-Dawley rats were purified to homogeneity.

CAIII from liver and muscle had the same amino acid composition and were immunochemically similar. Their kinetic properties at 0°C were also similar. $Km_{(CO2)}$ was 4 mM and k_{cat} 3×10^5 s^{-1}. K_i was 0.4 and 0.2 M for acetazolamide and NaCl, respectively. Both CAIIIs ran as single bands on SDS-electrophoresis and high-speed centrifugation, with a mol wt of 29.3 kDa. Their hydrodynamic properties suggest that CAIII is a compact, nearly spherical molecule. It contained 0.9 M zinc per M protein.

In both tissues isoelectric focusing identified neutral and acidic isoforms with *pIs* near 7.0 and 6.3, respectively. These forms were immunologically identical and had the same amino acid composition and mol wts. The acidic forms probably represent subspecies of CAIII in different states of oxidation.

CAIII is the major soluble protein in rat liver and muscle. Its function is probably to protect proteins of these tissues from oxidation catalyzed by iron-containing degradation products of haemoglobin and myoglobin.

Liver CAI and CAII and muscle CAII were identical to CAI and CAII of rat erythrocytes.

INTRODUCTION

Carbonic anhydrases (CA; EC 4.2.1.1) are the products of a gene family that encodes eleven distinct isozymes (CAI–CAXIV) and several additional CA-related proteins. By accelerating the reversible conversion of CO_2 and HCO_3^-, CA facilitates CO_2 and electrolyte transport in various tissues (10).

Among these isozymes, CAIII is unique. It is the most abundant cytosolic protein

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in rat liver, skeletal muscle, and adipocytes, where it amounts to 8, 10 and 24 % of the soluble proteins, respectively (4,16,21). However, CAIII exhibits very low catalytic activity and is resistant towards inhibition by sulfonamides.

In the liver, there are 20–30 times higher amounts of CAIII in the male compared to the female liver (4,21). CAIII therefore exhibits sexual dimorphism, not seen in other rat tissues (21) or in other species (23). In skeletal muscle CAIII increases several-fold after denervation (3), and in adipocytes the levels of CAIII are down-regulated in obesity (16). The reason for the regulation of CAIIII in these rat tissues is not known.

Also, the function of CAIII is largely unknown. However, since CAIII has been highly conserved throughout evolution (10), it should have a fundamental physiological role. It was recently suggested that it serves an antioxidant function (17,18). This is probably related to the fact that CAIII is the most oxidatively modified protein in the rat liver cytosol (1,5,14).

CAIII has been thoroughly investigated in several species (8). However, it has been only partially characterized in the rat (12). Because of its unique properties in this species we have therefore isolated CAIII from rat liver and muscle, and characterized its physico-chemical and kinetic properties in greater detail.

MATERIALS AND METHODS

Preparation of liver and muscle

Twenty adult male Sprague-Dawley rats of 300–350 g were anesthetized with 40 mg × kg^{-1} of pentobarbital intraperitoneally, and perfused *in situ* with a Ringer type of solution via the abdominal aorta. Slices from either liver, 96 g, or mixed hind leg muscles, 416 g, were minced and suspended in two volumes of 0.05 M Tris-buffer of pH 9.3 (standard buffer) and homogenised, while maintaining the pH by adding solid Tris. All solutions throughout the purification contained 0.5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 100,000 × g for 90 min. The particulate fractions were resupended in two volumes of the standard buffer, homogenised and centrifuged at 100,000 g. The supernatants from this centrifugation were pooled with the other supernatants.

To enable correction for blood contamination, the concentration of haemoglobin in the supernatants was measured by a cyan-methaemoglobin method (4).

The protein content of the supernatants and of the pooled chromatographic fractions was assayed by the Lowry method.

Enzyme assays

CA activities were assayed by a changing pH method (24). One enzyme unit (U) is the amount of enzyme that doubles the rate of hydration of CO2. Fractions were assayed in the absence and presence of the CA inhibitor acetazolamide (Diamox®, American Cyanamid Co.) at a concentration of 10 μM, which inhibits all known isozymes of CA, except for CAIII.

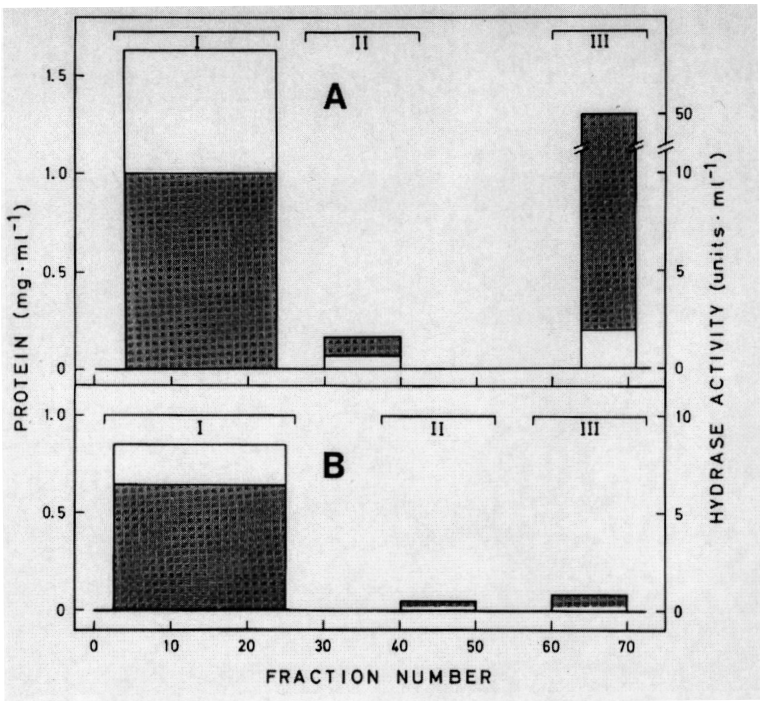

Fig 1. Elution profile from the sulfanilamide Sepharose 6B column.

The column (0.9 x 18 cm) was equilibrated with 50 mM Tris-H_2SO_4 buffer, pH 8.7 and loaded with the pooled fractions of the gel chromatography. Non-retained proteins, including the acetazolamide resistant enzyme activity, were eluted with the same Tris-buffer (step I). The CA isozymes bound to sulfanilamide were liberated first by adding 2 M NaCl in 50 mM sodium phosphate buffer, pH 6.5 (step II), and thereafter by adding 1 M $NaClO_4$ in 50 mM sodium phosphate buffer, pH 5 (step III). Finally, the column was washed extensively with 0.1 M sulfanilamide in the phosphate buffer, pH 5. The flow was 30 ml x h^{-1} during elution, the temperature was 4°C, and the fractions were 2.5 ml. They were pooled, dialysed against the Tris buffer and concentrated. Dark columns are hydrase activity and open columns protein. The non-retained material from the first run (A) was concentrated and rechromatographed on a fresh affinity column (B).

Purification of CAIII

Ammonium sulphate was added to the supernatant fractions to a final concentration of 40% of saturation. The precipitate was removed by low-speed centrifugation and the supernatant fractions were dialysed against the standard buffer for two days. The supernatants were then concentrated by ultrafiltration and applied to a column of Sephadex G-75. Equilibration of the column and elution was done with the standard buffer.

Fractions with CA activity from the gel filtration were then added to a sulfanilamide affinity column (24). CAI and CAII became adsorbed to the column and were then eluted with chloride or perchlorate, respectively, as described in the legend to Fig 1.

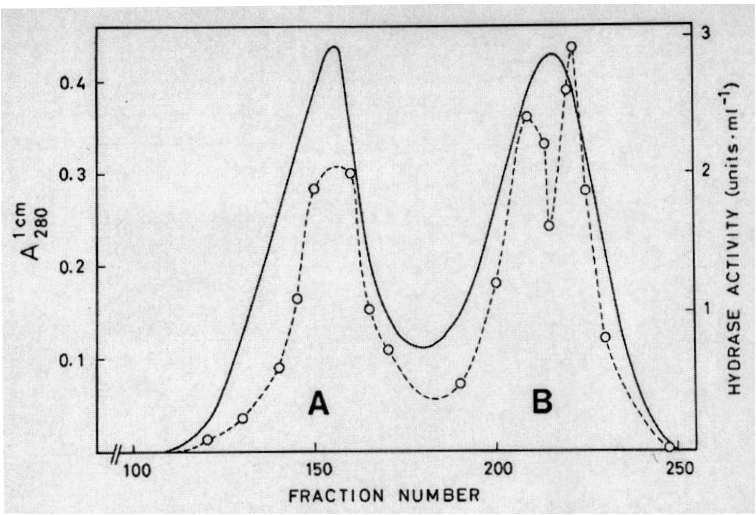

Fig 2. Elution profile from the DEAE Sephadex A50 column.

The column (3.2 x 30 cm) was loaded with 12 ml of the concentrated non-retained material eluted from the sulfanilamide affinity column. The column was equilibrated with 50 mM Tris-H_2SO_4 buffer, pH 8.7. The elution was accomplished with 10 mM Tris-H_2SO_4, pH 8.0, buffer, increasing stepwise to 25 mM (step I) and 100 mM (step II). Flow rate was 42 ml x h^{-1} and fractions were 2.5 ml. The temperature was 4°C. Solid line represents absorbance at 280 nm and broken line CA activity.

Fractions containing CAIII activity, not adsorbed after two repeated runs on the affinity column, were pooled, dialysed against 0.05 M Tris-H_2SO_4 buffer, pH 8.3, and concentrated. The concentrate was added to a DEAE-Sephadex A-50 column, equilibrated with 0.05 M Tris-H_2SO_4 buffer, pH 8.7. Sulphate was used as counter ion. Elution was accomplished as described in the legend to Fig 2.

Table 1. Comparative properties of liver LCAIII, LCAII and LCAI, and muscle MCAIII, of adult male rats.

Kinetic parameters are given with their 95 % confidence limits within brackets.

Property	LCAIII	MCAIII	LCAII	LCAI
SDS, mol wt, kDa	29	29	29	29
Centrifugation, mol wt, kDa	28.91	29.67	28.87	29.19
$A^{1\%}_{280cm}{}^{-1}$	19.6	19.6	17	18
$S^0_{20,W}$ (Svedberg units)	3.0	3.0	2.9	2.3
Isoelectric point (pH)	6.3–7.0	6.3–7.0	6.8	7.2
K_m CO_2, mM	25 (31–19)	19 (22–16)	5.6	12.8
k_{cat}, CO_2 $10^3 \times V/[E_0] \times^{s-1}$	2.1 (2.6–1.6)	1.9 (2.2–1.6)	82	3
Phenylacetate hydrolysis, µmoles×mg^{-1}×min^{-1}	<0.02	<0.02	1.7	0.7
K_i acetazolamide, µM	400	460	0.02	0.07
I_{50}, NaCl, M	0.2	0.2	0.7	n.d.

The muscle fractions with CAIII activity had to undergo a final gel filtration step, using a Sephadex G-100 column, to remove a contaminating protein of 43 kDa ahead of the CA III peak in the chromatogram.

Enzyme concentrations

The isozymes were kept at –20° C until analysed. Their concentrations were calculated from the absorbance, $A^{1\%}_{280}$ (cm^{-1}), values of Table 1. The kinetic parameters and the amount of zinc bound per mole of CA were calculated using the mol wts obtained from the analytical ultracentrifugation (Table 1).

Electrophoretic methods

Isoelectric focusing was done in acrylamide gels at a pH range of 3.5–9.0, using the Multiphor® equipment of LKB-produkter (Bromma, Sweden) and ampholine gels. The *pIs* were determined by using an isoelectric focusing calibration kit.

Polyacrylamide gel electrophoresis (PAGE) was done with a sulphate-borate discontinuous buffer system running at pH 9.2. The mol wts were calculated from relative mobilities.

Amino acid composition

This was analysed by ion exchange chromatography on an automatic amino acid analyser. Protein samples were hydrolyzed in 6 M HCl at 110°C for 24 or 72h. Half-cystine and methionine were measured as cysteic acid and methionine sulfone, respectively, after performic acid oxidation. Values for threonine and serine were obtained by extrapolation to zero hour hydrolysis.

Table 2. Amino acid composition of liver LCAI, LCAII, and LCA III, and muscle MCAIII of male rats.

The values for LCAI and LCAII are means from two preparations. The values for LCAIII and MCAIII are the average ± SD from five analyses of different fractions of peak A and B of the ion chromatography (Fig 2). Values are from hydrolysis at 24 or 72 h.

Amino acid	Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly
MCAIII	7.7±0.9	5.0±0.1	4.9±0.3	9.7±0.7	4.1±0.3	6.3±0.6	10.1±0.6	9.7±1.8	8.4±0.6
LCAIII	7.0±0.3	4.8±0.7	4.8±0.5	9.7±0.5	4.4±0.3	7.1±1.0	9.8±0.9	8.6±0.6	8.4±0.8
LCAII	7.0	4.8	2.8	9.7	4.4	7.5	10.9	7.9	8.5
LCAI	6.8	4.1	2.9	9.9	4.5	2.7	9.5	6.8	10.2
Amino acid	Ala	Val	Met	Ileu	Leu	Tyr	Phe	Trp	Cys
MCAIII	6.2±0.7	4.8±0.6	0.9±0.5	4.0±0.6	8.7±0.3	3.1±0.1	4.5±0.2	1.2±0.7	1.0±0.4
LCAIII	6.1±0.6	5.5±0.3	1.0±0.3	4.0±0.3	8.7±0.4	3.1±0.1	4.6±0.3	2.7	1.4
LCAII	6.0	5.2	0.9	4.1	8.7	3.0	4.6	3.3	1.7
LCAI	7.1	5.5	0.6	3.6	8.7	2.9	3.7	n.d.	1.2

Zn was 0.8; 0.9 mole/mole protein for LCAIII and 1.0 for HCAI.

Analytical ultracentrifugation

Sedimentation velocity experiments were performed in an analytical ultracentrifuge. The sedimentation constants were calculated and corrected to give values in water at 20°C ($S_{20, w}$). Sedimentation equilibrium ultracentrifugation was done by means of Chervenka's long column meniscus depletion technique (6). The mol wt was calculated from the slope of ln C versus r^2. The partial specific volumes were obtained from the amino acid composition.

Immunochemical methods

The antigenicity of the isozymes was tested by immunodiffusion in agarose, using rabbit antiserum against rat erythrocyte CAI and CAII, purified in our laboratory (24).

Zinc analysis

The zinc content of the liver CAIII, and the reference isozyme, human HCAI, was determined by atomic absorption spectrometry.

Kinetic methods

The kinetic parameters were investigated at 0°C by an electrometric method (24). The decrease in pH after the addition of CO_2 to 10 mM sodium phosphate buffer with 25 mM Na_2SO_4, 500 M EDTA, and 12.4 mg × ml^{-1} bovine plasma albumin, was measured with a glass electrode, as a function of time. $[E]_0$ was 0.8, 43 and 145 nM, for CAII, CAI, and CAIII, respectively. The initial pH was 7.0 and the change in pH was less than 0.01 pH units. The rate of hydration was deduced from the rate of generation of protons. The uncatalysed rate constant, k_{CO2}, under these conditions was 0.0021 × s^{-1}. Kinetic parameters were determined with one enzyme buffer solution and seven substrate concentrations between 3.9 and 18 mM CO_2. Inhibition was studied using four concentrations of acetazolamide and three concentrations of CO_2. Inhibitor and enzyme were incubated for 15s.

Esterase activity was measured spectrophotometrically in 5 mM Tris-H_2SO_4 buffer, pH 7.5, at 25°C, using 1 mM p-nitrophenyl acetate as substrate (8).

RESULTS

Purification

The various steps in the separation of the isozymes are illustrated in Fig 1 and 2. They generated homogeneously pure CAI, CAII, and CAIII from the liver, and CAII and CAIII from the muscle.

CAI and CAII. Salt precipitation of the liver supernatants removed 74 % of non-enzymatic proteins. When the remaining soluble material was added to a G-75 Sephadex column, a sharp peak with CA-activity could be eluted.

When this activity was added to the affinity column (Fig 1), 70% of the CA activity was adsorbed. Elution with chloride gave a fraction of 3 mg protein with a spe-

cific activity of 24 U·mg^{-1} (step II, Fig 1). Elution with perchlorate (step III, Fig 1) gave a fraction of 6 mg protein with a specific activity of 252 U·mg^{-1}.

Small amounts of CA protein could also be eluted from the muscle supernatant by perchlorate, but not by chloride.

Less than 10% of the amounts of purified CAI and CAII originated from contaminating blood.

CAIII. About 30% of the CA activity of the liver and muscle was not adsorbed to the affinity column, even after a second run on a fresh column (Fig 1B). This activity was resistant to inhibition by acetazolamide.

It was further purified on an ion-exchange chromatography column. The elution profile for liver CAIII is seen in Fig 2.

Two peaks, A and B, with CA activity were separated. The fractions of peak A contained 12–18 U·mg^{-1} and those of peak B, 6–8 U·mg^{-1}. The CA activity of peak A and B had an I_{50} for acetazolamide of 4 and 3 × 10^{-4} M, respectively. The acetazolamide resistant CA activity of muscle, eluted from the ion-exchange chromatography column, also appeared as two peaks A and B, both with activities of 12 U·mg^{-1}.

By several criteria these CA proteins of liver and muscle were identical to CAIII (Table 1, 2).

Criteria of purity

The CA isozymes appeared monomeric by several criteria. Liver CAI, CAII, and CAIII, and muscle CAIII, were homogenous with respect to sedimentation in the ultracentrifuge. They sedimented as single symmetrical distribution curves throughout the run of 3h. The mol wt distribution curves also indicated a mono-disperse system from the linear relation between ln C versus r^2.

Analysis of the isozymes by SDS-PAGE also gave single bands.

Liver CAI and CAII, and muscle CAII, were immunologically similar to the corresponding erythrocyte isozymes.

Physical properties

Plotting of the sedimentation constants as a function of the protein concentration resulted in straight lines. Extrapolated values for $S_{20,W}$ are given in Table 1.

The mol wts of the centrifugations (Table 1) are the average values of runs at 7 different initial protein concentrations. The mol wt of CAIII was identical to that, 29.25 kDa, previously reported (1) from mass spectrometry.

The hydrodynamic properties suggest that rat CAIII is a compact, nearly spherical molecule.

Isoelectric focusing of the fractions of peak A of the ion-chromatography of liver and muscle CAIII gave three major bands with *pI* values of 7.0–7.2. The fractions of peak B revealed one major band at pH 6.3 and faint bands at pH 7.0. When fractions of peak B of liver and muscle CAIII were run together, they showed a similar picture with one major acidic band at pH 6.3, and minor bands around pH 7.0 (Fig. 3).

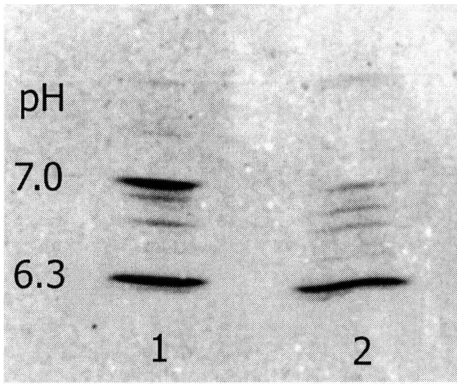

Fig. 3. Isoelectric focusing pattern of CAIII of male rat muscle and liver.

The amount of applied protein was 10 µg. CAIII was taken from peak B of the ion-chromatographic separation of muscle (lane 1) and liver (lane 2) CAIII.

Kinetic Properties

The hydratase activity of liver and muscle CAIII accurately obeyed the Michaelis-Menten equation and gave kinetic parameters similar for liver and muscle. Their Km_{CO2} was high and the turnover numbers, k_{cat}, were low compared to those of CAI and CAII. The activity of CAIII at pH 7.0 and 0°C, expressed as k_{cat}/Km was 0.2 % of that of CAII (Table 1).

CAIII of muscle and liver (Table 1) exhibited the same low p-phenyl acetate hydrolysis activity.

The hydratase activity was inhibited by acetazolamide with I50 and Ki values similar for muscle and liver CAIII, about 20,000 fold higher than for CAII. Chloride was weakly inhibitory against CAIII.

Zinc content and amino acid composition

Human erythrocyte HCAI contained one mole of zinc per mole protein, whereas rat liver CAIII contained less than one mole of the metal (Table 1). Zinc therefore appears to be loosely bound, and some was probably lost during the purification, as previously reported to be the case for CAIII of other species (8).

The amino acid compositions of muscle and liver CAIII were similar (Table 2) and different from those of CAI and CAII, which in turn had the same compositions as erythrocyte CAI and CAII (24).

DISCUSSION

Purification of CAI, CAII and CAIII

CAI and CAII. During the course of the purification of CAIII, we also purified small amounts of CAI and CAII from the liver, and CAII from the leg muscle of the same adult male rats. CAI was not found in the muscle.

CAI and CAII were by all tested criteria identical to CAI and CAII from rat erythrocytes (24).

CAIII. Like others (12, 20), we found that CAIII is the major CA protein in rat liver and muscle, amounting to more than 90% of the isolated soluble CA proteins.

Amino acid composition and sequence of CAIII

The full sequence of rat CAIII has not been determined. However, it has been reported from partial sequencing, that liver and muscle CAIII are either slightly different (5) or identical (2). We found that their amino acid compositions were similar (Table 2) and identical to that recently reported for rat muscle CAIII (1).

Kinetic properties of CAIII

Our data from the rat, and those reported from other species (8,13), show that CAIII differs markedly from other CA isozymes by its low CO_2 hydratase and esterase activity, and the independence of k_{cat} and K_m on pH between 5.0 and 8.2 (20). Another unique property is its resistance to inhibition by sulfonamides and anions. In analogy with findings in human CAIII (15) these kinetic properties in the rat CAIII could be explained by the presence at the active site of a bulky phenylalanine, Phe-196, which reduces the size of the substrate and sulfonamide binding region (9,15), and induces a low pK_a of $ZnOH_2$. The resistance towards inhibition by mono-valent anions, such as chloride, is explained by the small fraction of $ZnOH_2$ available for binding of anions at physiological pH (15).

The lower CA activity of the acidic fractions of peak B of the ion-chromatographic separation (Fig 2) of the liver CAIII, agrees with the report (1), that oxidised acidic forms of CAIII have 1/3 of the hydratase activity of the unmodified form. Oxidation of either Cys-181, Cys-186, or Arg-187 of rat CAIII by S-thiolation or by other means, could be the reason for the lower catalytic activities (22), but this needs further study (8).

The oxidised forms of CAIII also lack phosphatase activity (1). The domain responsible for this activity has not yet been defined (13) but is different from the regions responsible for the hydratase and esterase activities (9).

Charge isoforms of CAIII

Ion-exchange chromatography separated two major fractions (A and B of Fig 2) of CAIII from liver and muscle. The enzymes of these fractions were immunochemically similar and had the same amino acid composition and mol wt (Table 1). However, the fractions contained enzyme proteins with different *pIs*. We believe that these are unmodified and acidic forms of CAIII. Rat liver CAIII has namely been shown to undergo posttranslational modifications *in vivo*, involving oxidative S-thiolation (a mixed disulfide between a cysteine residue and glutathione) (5,14,19), and carbonylation (the introduction of aldehydes and ketones into certain amino acid residues of the enzyme) (1). S-thiolation is a reversible process, and occurs at two reactive sulfhydryl groups (8), localised on residues 181 and 186 (9). Glu-

tathionated CAIII has been found to be the major S-glutathionated protein in rat liver cytosol under oxidative stress.

These modifications produce acidic forms of the protein with *pIs* (5,19), similar to those of our purified fractions of CAIII. The unmodified form has been found to have a *pI* of 7.0 (5).

Similar modifications of CAIII probably occur also in muscle (1) and adipocytes (16).

Function of CAIII

Because CAIII is a very slow catalyst in the CO_2–H_2CO_3 system, there have been speculations about roles for CAIII independent of its catalytic properties (7,16).

Rokutan et al (19) were the first to identify, in rat liver and isolated hepatocytes, a 30 kDa major protein that underwent S-thiolation. This protein was later found (5) to be identical to CAIII. The enzyme is located in perivenous hepatocytes and in slow red muscle fibres, where haemoglobin and myoglobin, respectively, are catabolised, generating iron and iron-binding degradation products. These could participate in the oxidation of amino acids by the so called Fenton system, i.e. the catalysed oxidation of amino acids by H_2O_2+Fe(II) (22). The resulting oxygen radicals can initiate rapid protein S-thiolation and carbonylation. These types of oxidation and reduction of proteins are enhanced in bicarbonate buffers. This would suggest that the hydratase activity of CAIII could enhance its own oxidative modification (22). Its high Km and the independence of catalysis on pH, would secure an effective buffering over a wide physiological range.

Since S-thiolation is a reversible process, it may preserve the biological activity of the protein under conditions in which irreversible protein damage might otherwise occur (19). CAIII would therefore appear to be a major component of the S-thiolation/dethiolation response to oxidative stress (5). Such a stress occurs when the balance between prooxidants and antioxidants in a cell is disturbed in favour of the prooxidants (14). It is therefore interesting that recent data suggest that CAIII indeed may act as an antioxidant (1,5,17,18). This function of CAIII is perhaps the reason for the presence of large amounts of CAIII, particularly in tissues where haemoglobin and myoglobin are degraded.

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