The phosphatase activity of carbonic anhydrase III is reversibly regulated by glutathiolation

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ABSTRACT Carbonic anhydrase isozyme III (CAIII) is unique among the carbonic anhydrases because it demonstrates phosphatase activity. CAIII forms a disulfide link between glutathione and two of its five cysteine residues, a process termed S-glutathiolation. Glutathiolation of CAIII occurs in vivo and is increased during aging and under acute oxidative stress. We show that glutathiolation serves to reversibly regulate the phosphatase activity of CAIII. Glutathiolation of Cys-186 is required for phosphatase activity, while glutathiolation of Cys-181 blocks activity. Phosphotyrosine is the preferred substrate, although phosphoserine and phosphothreonine can also be cleaved. Thus, glutathiolation is a reversible covalent modification that can regulate CAIII, a phosphatase that may function in the cellular response to oxidative stress.

The carbonic anhydrases catalyze the reaction of carbon dioxide and water to form carbonic acid. Seven human isozymes are known (1) and deficiency of isozyme II causes a syndrome of osteopetrosis, renal tubular acidosis, and cerebral calcification (2). Carbonic anhydrase isozyme III (CAIII) is distinguished from the other isozymes by several characteristics, particularly by a lower specific activity (~1% of isozyme II) and by its resistance to acetazolamide, which inhibits isozymes I and II (3-5). CAIII is present in large amounts in certain tissues, comprising ~5% of the soluble protein of male rat liver (6), ~8% of skeletal muscle (7), and up to 25% of adipocytes (8). The physiological function of the enzyme is unknown, but these characteristics suggest that it may not act simply as a carbonic anhydrase.

It has been known for some years that isozyme III is unique among the carbonic anhydrases because it also possesses phosphatase activity when assayed with nitrophenyl phosphate (9). Activity against such a substrate provides presumptive evidence that it could function as a phosphotyrosyl phosphatase. The three-dimensional structure of CAIII was determined to 2.0 Å resolution (5), although the domain responsible for phosphatase activity could not be identified at the time. Subsequently Zhang et al. (10) pointed out that enzymes catalyzing phosphoester hydrolysis have a consensus sequence of C-X3-C-R, and we noted that this sequence occurs in all of the known CAIII sequences (11). In the rat, this sequence begins at residue 181 and includes a cysteine at residue 186, adjacent to the essential arginine: C181-LFPA-C186R187.

In the course of studies on the biology of aging, we purified CAIII from the livers of young and old rats (2 and 18 mo) (11). The specific activity of the carbonic anhydrase was similar for the protein from young and old rats. However, the phosphatase activity was lost in enzyme purified from the older animals, an alteration with potential ramifications for cellular regulation and signal transduction. Mammalian CAIII has been shown to form a disulfide link with glutathione at both Cys181 and Cys186 (12), a process termed glutathiolation. We found that the extent of glutathiolation was doubled in the preparation from the older rats, suggesting that the increase in glutathione content at the most reactive cysteine caused the loss of phosphatase activity.

We investigated this possibility and found that the opposite actually occurs: Phosphatase activity requires glutathiolation of Cys186. The activity can be switched on and off by the reversible glutathiolation of this cysteine residue, providing a novel mechanism for the regulation of phosphatase activity.

MATERIALS AND METHODS

Purification and Assay of CAIII. CAIII was purified from liver of young male rats (Fischer 344) as described (11). The three enzymatic activities of CAIII were measured by published methods: carbonic anhydrase (13), esterase (14), and phosphatase (9). Each assay was performed with and without enzyme, and a blank with all additions except the enzyme was subtracted to calculate the catalyzed rate. Protein concentration was determined by the procedure of Bradford (15) using bovine serum albumin as standard. S-glutathiolation of CAIII was determined by reverse phase chromatography (11) and isoelectric focusing (16). Recombinant human CAIII was produced using a T7 vector kindly provided by P. J. Laipis (17). It was purified with a 50-80% ammonium sulfate cut followed by DEAE chromatography in 50 mM Tris, pH 7.8. CAIII did not bind to the column and was pure by SDS electrophoresis.

The rates of dephosphorylation of phosphoserine, phosphothreonine, and phosphotyrosine were measured by determining the rate of production of inorganic phosphate (18). Assays were performed in a 100-µl volume mixture containing 10 mM L-serine phosphate, L-threonine phosphate, or L-tyrosine phosphate (Sigma) in 100 mM sodium succinate, pH 5.3 at 37°C. The phosphotyrosyl phosphatase activity was monitored by the conversion of the phosphorylated form of a test peptide to the unphosphorylated form. Two synthetic phosphotyrosyl peptides were used as a substrate. The first peptide used was 15 residues (KVEKIGETpYGVVVK) corresponding to residues 6-20 of p34cdc2, a substrate of the src-family tyrosine kinases (Upstate Biotechnology, Lake Placid, NY). The peptide was phosphorylated as described (19) and purified on a C18 reverse phase column [Vydac (Hesperia, CA) model 218TP54] running a linear gradient from 10 to 26% acetonitrile at 0.8% per min, with 0.05% trifluoroacetic acid. The flow rate was 1 ml/min giving retention times for the phosphotyrosyl-peptide and the nonphosphorylated peptide of 17.5 and 20.5 min, respectively. The phosphatase assay was performed in 100 mM sodium succinate buffer, pH 5.3, with 0.5 mM phosphotyrosyl peptide and 40 µM CAIII. The mixture was incubated at room temperature for different times and the unphosphorylated peptide was separated from the phosphotyrosyl peptide, using the same conditions employed to purify the phosphorylated peptide.

Abbreviations: CAIII, carbonic anhydrase isozyme III; S-IAF, S-(iodoacetamido)fluorescein.

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The second phophopeptide was the decapetide, CGDND- pYIipl (Peptide Biotechnology, Gaithersburg, MD), present in the SH_2 domain of phospholipase C_y, a substrate of the growth factor receptors family. The peptide was carboxymethylated on the amino terminal cysteine to prevent cystine formation during the assay. The assay was performed in a 20-μl volume containing 0.5 mM photophosphorylated peptide, 40 μM CAILL, and 100 mM sodium succinate buffer, pH 5.3. The mixture was incubated at room temperature for different times, and the unphotophosphorylated peptide was separated from the phosphorylated peptide (10–25% acetonitrile over 5 min, then 25–40% over 15 min). The retention times for the photophosphorylated-peptide and the standard peptide were 13.1 and 15.7 min, respectively.

Preparation were used, Monoglutathiolated, and Diglutathiolated CAIII. Fully reduced CAILL was obtained by incubating the enzyme at room temperature with 25 mM dithiothreitol for 30 min in 50 mM Tris-HCl, pH 7.5 (16), and buffer exchange through a Sephadex G-25 PD-10 column equilibrated with 50 mM Tris, pH 6.2. Monoglutathiolated CAILL was obtained from the fully reduced protein. It was concentrated to ~1 mg/ml by ultrafiltration (Centricon-10; 10,000 MW cutoff; Amicon), brought to 25 mM oxidized glutathione, incubated 30 min at room temperature, and buffer exchanged on a PD-10 into 50 mM Tris, pH 6.2. Dигlutathiolated protein was also obtained from the fully reduced protein. The solution was made 125 μM in glutathione and 250 μM in diadime [1,1-azobis(N,N′-dimethylformamide)], incubated 10 min at 37°C, and buffer exchanged on a PD-10 (16). All proteins were concentrated to ~1 mg/ml with the Centricon-10.

Labeling of Cysteine Residues with 5-iodoacetic acid) fluorescently labeled CAIII were prepared by incubating the enzyme with 1 mM of 5-IAF (Molecular Probes) dissolved in dimethylformamide previously dried with molecular sieves (20). The buffer was 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and the solutions were made 6 M in guanidine HCl by addition of 1 g of solid guanidine HCl per ml. Reaction was initiated with 0.1 M NH_2OH, the pH adjusted to 7.5, and 5-IAF was added to give 1 mM concentration. Incubation was continued for 1 hr in the dark at room temperature, and reagent was removed by gel filtration on a PD-10 column equilibrated with 6 M guanidine/50 mM Tris-HCl, pH 7.5/5 mM EDTA. The protein was then desalted by reverse-phase HPLC as described above, and the fraction containing CAILL was dried in a Speed-Vac.

Thirty milliliter of 10 mM Tris-HCl (pH 8.0) and 1% (wt/wt) trypsin were added to the dried protein. The mixture was incubated at 37°C for 12 hr when another 1% trypsin was added. The reaction was stopped after 20 hr total incubation time by addition of 1 g of solid guanidine HCl per ml. The digest was stored at ~70°C if not used for immediate analysis. Reverse-phase mapping of the digest was performed, but the very hydrophobic peptide Asp<sup>190</sup>-Lys<sup>133</sup> (containing two cysteine residues) could not be reproducibly recovered despite various experimental manipulations. We therefore used the technique of simultaneous sequencing of the entire tryptic peptide collection using a sequencer equipped with a diode array detector to allow recording of chromatograms at multiple wavelengths (Hewlett-Packard model G1005A with a model 1090 diode array). Recovery of each amino acid in each cycle was determined from the chromatogram of PTH-amino acids, followed by 269 nm. The amount of fluorescein-derivatized cysteine–PTH was determined from the chromatogram at 470 nm.

The sequence of liver CAILL has not yet been reported, so we have used the sequence and numbering for the rat muscle enzyme (Protein Identification Resource, Release 43.00, May 1, 1994), which differs from that used in the report of the crystal structure of CAILL (5). Tryptic digestion should place the five cystines in cycles 2, 11, 14, 16, and 17, and that is where they were found in CAILL pretreated with dithiothreitol to yield the fully deglutathiolated protein. Thus, sequence analysis of the peptide collection can assess the status of all cysteines in CAILL.

RESULTS

CAIII Has Phosphoysrol Phosphatase Activity. The first report establishing the phosphatase activity of CAIII used p-nitrophenyl phosphate as a substrate (9), which today would be taken as presumptive evidence of phosphoysrol phosphatase activity. We compared the phosphatase activity using p-nitrophenyl phosphate, phosphotyrosine, phosphoserine, and phosphothreonine. The activity with phosphotyrosine was 93% that with p-nitrophenyl phosphate (0.14 nmol/min/mg). Phosphoserine and phosphothreonine were substrates but activity was only 25%.

The natural substrate for CAIII's phosphatase activity is not yet known, so we tested two phosphopeptides that are products of tyrosine kinase catalyzed phosphorylation. The first was a 15-residue peptide phosphorylated by src kinase. No dephosphorylation by CAIII was detected. The second peptide was the 10-residue peptide phosphorylated by platelet-derived growth factor receptor. Dephosphorylation was readily detected with an HPLC assay, and the specific activity was 0.04 ± 0.01 nmol per min per mg of protein.

The phosphatase activity of CAIII is known to be inhibited by anions such as molybdate, arsenate, and phosphate, and they have no effect on the carbonic anhydrase activity (9). We tested vanadate, a well-known phosphotyrosyl phosphatase inhibitor, and found that it inactivated the enzyme with an apparent Ki of 2.5 μM. Vanadate had no effect on the carbonic anhydrase activity of CAIII. Phosphatase activity was unaffected by 100 mM NaCl, 100 mM KCl, or 10 mM MnCl<sub>2</sub> but decreased 65% by 1 mM EDTA. MgCl<sub>2</sub> stimulated activity to 170% of the basal value, with 60 μM Mg<sup>2+</sup> yielding half-maximal stimulation.

Glutathiolation Is Required for Phosphatase Activity. CAIII forms disulfide linkages between glutathione and cysteine residues of the protein (16, 21, 22). Isoelectric focussing and reverse phase chromatography are two convenient methods for separation of the native, monogluthathiolated, and diglutathiolated forms of CAIII. In the studies described below, we used both techniques, and the results from the two techniques were in agreement. Glutathiolation is a reversible covalent modification. Exposure of the purified protein to oxidized glutathione leads to glutathiolation of CAIII through disulfide exchange. The extent of glutathiolation is dependent on pH and the concentration of oxidized glutathione (Fig. 1). Exposure to a mixture of reduced glutathione and the oxidizing agent diamide drives the reaction to the diglutathiolated form (16). The fully deglutathiolated form is obtained upon treatment with dithiothreitol, monitored by both HPLC and isoelectric focusing (Fig. 2).

During aging, CAIII loses phosphatase activity and becomes more extensively glutathiolated, suggesting a causal relationship. Deglutathiolation of CAIII purified from old rats did not restore phosphatase activity, nor did it affect carbonic anhydrase activity. However, deglutathiolation of CAIII from young animals virtually abolished phosphatase activity (Fig. 3). Reconversion of this deglutathiolated protein to the monogluthathiolated form restored phosphatase activity to a higher level than seen with the enzyme as originally purified. The higher activity is consistent with a greater extent of glutathiolation after in vitro treatment compared to the original isolate. This increase in activity does not require cycling through the deglutathiolated form by treatment with dithiothreitol. It was also observed when CAIII purified from young animals was treated directly with oxidized glutathione to increase the extent of glutathiolation. None of these treatments affected
were focusing gel different treatments. Treated with either dithiothreitol (a), oxidized glutathione (dotted line), or glutathione/diamide (dashed line). (Inset) Isoelectric focusing gel of 4 μg of CAIII after the same treatments: dithiothreitol (a), oxidized glutathione (b), or glutathione/diamide (c).

the carbonic anhydrase activity of the enzyme, confirming reports (23). While monogluthation is thus required for phosphatase activity, conversion of CAIII to the diglutathiolated form by diamide treatment led to loss of phosphatase activity. Activation by glutathiolation is not limited to the rat enzyme; it was also observed with recombinant human CAIII produced in Escherichia coli (17) (not shown).

CAIII purified from old animals was also readily deglutathiolated and reglutathiolated, but phosphatase activity was not restored, indicating that some other modification was responsible for loss of activity during aging. Given the ready reactivity of the cysteine residues with glutathione, it seemed possible that intermolecular dimerization or aggregation could occur between subunits, with consequent loss of phosphatase activity. This possibility was ruled out when SDS-gel electrophoresis under nonreducing conditions established that the purified protein remained a monomer.

Glutathiolation of Cys186 Is Required for Phosphatase Activity. The known CAIII enzymes are very similar in sequence and all have five cysteine residues. Working with the enzyme from horse, Wendorff et al. (24) reported that two of the five residues readily formed mixed disulfides with Ellman's

![Graph](image1.png)

**Fig. 1.** Effect of pH and concentration of oxidized glutathione on the extent of glutathiolation of CAIII. Fully reduced CAIII purified from 2-month-old animals was incubated in 100 mM sodium phosphate buffer for 15 min at the indicated pH with oxidized glutathione. The samples were analyzed by isoelectric focusing and the results quantitated by densitometry. The upper panel shows the percentage that was monoglutathiolated and the bottom panel the percentage that was diglutathiolated. The difference from 100% is the amount that remained unmodified. The bars give the results at four different concentrations of oxidized glutathione: (from left to right) 2, 10, 25, and 50 mM and are represented by the open, filled, cross-hatched, and stippled bars, respectively.

![Graph](image2.png)

**Fig. 2.** Analysis of the state of glutathiolation of CAIII by reverse-phase HPLC and isoelectric focusing. CAIII was analyzed by reverse-phase chromatography and isoelectric focusing (Inset) after different treatments. Each chromatogram monitored 20 μg of CAIII treated with either dithiothreitol (solid line), oxidized glutathione (dotted line), or glutathione/diamide (dashed line). (Inset) Isoelectric focusing gel of 4 μg of CAIII after the same treatments: dithiothreitol (a), oxidized glutathione (b), or glutathione/diamide (c).

![Graph](image3.png)

**Fig. 3.** Effect of cysteine modifications on the phosphatase activity of CAIII. Phosphatase activity was determined in each sample after the treatment shown on the abscissa. SDS/PAGE of the same proteins was performed under nonreducing conditions and demonstrated that each sample remained monomeric.
FIG. 4. Determination of the free sulfhydryl content at the five cysteine residues of CAIII. A total of 250 μg of CAIII purified from young rat liver was treated with 5-IAF under denaturing conditions, digested with trypsin, and the entire peptide collection subjected to 20 cycles of Edman degradation. Reduced cysteine residues were detected and quantified from the chromatogram at 470 nm. For each cycle, the value obtained with the diithiothreitol-treated enzyme was set to 100%. The recovery of labeled cysteine from the other proteins was compared to this value, with correction for slight differences in recovered protein made by determining recovery of total PTH-amino acids at 269 nm. Solid bars: CAIII purified from young rats, without diithiothreitol treatment. Shaded bars: CAIII treated first with dithiothreitol and then oxidized glutathione to yield a mixture of ~40% monogluthathiolated and 60% ungluthathiolated forms. Open bars: CAIII treated first with dithiothreitol and then with glutathione/diamide to give primarily the digluthathiolated form.

reagent. These would be Cys\(^{181}\) and Cys\(^{186}\) in the rat sequence, and a preliminary report (12) of the crystal structure of the digluthathiolated form confirmed that these residues were derivatized. We wanted to determine which residue must be gluthathiolated to activate the phosphatase and whether there is a difference in the sites of gluthathiolation between young and old enzyme that might account for loss of phosphatase activity. The strategy that proved successful in locating the sites was to alkylate the free sulfhydryl groups with 5-IAF. This procedure leaves the gluthathiolated residues intact while labeling the other cysteines with a chromophore and fluorophore, fluorescein.

Fig. 4 shows that activation of the fully reduced phosphatase by monogluthathiolation introduced a glutathione moiety specifically at Cys\(^{186}\). Similar analysis of protein purified from young and old animals demonstrated gluthathiolation occurred at the same cysteine. In addition, disulfide formation among Cys\(^{181}\), Cys\(^{201}\), and Cys\(^{204}\) involved ~30% and ~40% of the residues in CAIII from young and old liver, respectively, a conclusion confirmed by assay with Ellman’s reagent. As noted above, diamide treatment inactivates the phosphatase and converts the protein to a digluthathiolated form. In this digluthathiolated protein, 5-IAF reactivity was almost completely lost in four of the five cysteines, with only Cys\(^{65}\) unaffected. This finding indicates that while diamide led to the gluthathiolation of Cys\(^{181}\) (12), it also stimulated the formation of intramolecular disulfide bridges. This conclusion also was confirmed by assay with Ellman’s reagent.

DISCUSSION

Gluthathiolation of CAIII is known to occur in vivo (16, 21, 22), and the extent of gluthathiolation increases in response to oxidative stress (23, 25, 26). It has been proposed that formation of the disulfide with glutathione protects the cysteine residue from irreversible oxidation, for example, to the sulfonic acid (26). Subsequently, the protected residue could be degluthathiolated, perhaps by glutaredoxin (12, 16, 27). Such a protective mechanism makes sense and may be important in some situations.

The gluthathiolatable Cys\(^{186}\) only occurs in CAIII and it is invariant among the species reported (Protein Identification Resource). Cysteine is clearly not required for carbonic anhydrase activity but it is essential for phosphatase activity. We suggest that the gluthathiolation of CAIII in response to oxidative stress is the mechanism for activation of its phosphatase activity, directed toward substrates not yet identified.

Gluthathiolation, like many other modifications of proteins, can occur nonenzymatically. However, as has been discovered for so many other modifications, it seems likely that controlled, enzymatic mechanisms for reversible gluthathiolation will be found in the cell (28). Other proteins are known to be susceptible to gluthathiolation and the list is growing (28–30).

CAIII has the motif C-X\(^2\)-R identified as the signature sequence in phosphoester hydrolysis. The motif is found in phosphotyrosine-specific phosphatases, in dual specificity phosphatases, and in low molecular weight acid phosphatases (10). While CAIII can act as a phosphatase toward CAIII it could also function as a dual specific phosphatase. The finding of activity against phosphoserine and phosphothreonine points out the need for further studies, including identification of the natural substrates.

The C-X\(^2\)-R motif appears to form a phosphate binding loop (31), a concept supported by the crystal structure of the Yersinia phosphotyrosine phosphatase (10). An arginine was shown to be important in the phosphatase activity of CAIII by chemical modification (32). The role of the arginine residue is not obvious, but it has been suggested to stabilize the transition state. The cysteine is at the center of a distinctive phosphate binding loop, facilitating formation of the phosphocysteine intermediate that is essential to the catalytic cycle. Gluthathiolation of Cys\(^{181}\) in CAIII mediated by diamide would certainly block phosphatase activity.

Thus, phosphatase activity will be present under conditions that lead to monogluthathiolation but will be absent under conditions that cause removal of that glutathione or cause addition of the second glutathione. CAIII joins a growing list of protein kinases and phosphatases whose activities are dependent on the level of oxidative stress (33–35). Oxidative stress has been shown to increase gluthathiolation, and it is conceivable that the concentration of a specific reactive oxygen species such as hydrogen peroxide is important in regulating the extent of gluthathiolation. The mechanism of activation of the CAIII phosphatase by gluthathiolation is a matter of speculation at present. The preliminary report (12) of the crystal structure of digluthathiolated CAIII noted that (i) both glutathiones are linked to surface exposed cysteines; (ii) the glutathione tripeptides appear to have considerable mobility; and (iii) no substantial conformational changes are induced by gluthathiolation. The addition of glutathione adjacent to the essential arginine might have been expected to interfere with catalytic efficiency, the opposite of what actually occurs. Thus, the additional residues may interact with the catalytic cysteine residue or induce a change in shape not detected yet, or they may complete the binding site for substrates. Activation by micromolar concentrations of Mg\(^{2+}\) but not other divalent cations is curious but not unprecedented (36). The site and structure of the Mg\(^{2+}\)-binding site are unknown, although the α-carboxylate group of the glutamate in glutathione could contribute a ligand.

Reversible covalent modification of proteins is a fundamental mechanism of cellular regulation. Phosphorylation–dephosphorylation is surely the most prevalent example, although many others are well-described (37). The physiological function of CAIII is unknown, but the finding that it can function as a protein phosphatase suggests a role in cell signaling, particularly in response to oxidative stress. We
conclude that reversible glutathiolation of CAIII provides an on-off switch for the regulation of its phosphatase activity.

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