ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 7 & 8, 2005

Forum Original Research Communication

Molecular Determinants of *S*-Glutathionylation of Carbonic Anhydrase 3

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ABSTRACT

Carbonic anhydrase 3 is easily *S*-glutathionylated *in vivo* and *in vitro*. The protein has two surface-exposed cysteine residues that can be modified. We found that Cys186 is more readily glutathionylated than Cys181. We studied a series of site-specific mutants to identify the residues that interact with Cys186 to make its thiol more reactive. We found that Lys211 is responsible for lowering the pK_a of Cys186. We also found that two acidic residues, Asp188 and Glu212, interact with the thiol and actually decrease its reactivity. We speculate that conformational changes that alter the interaction with these three residues provide a mechanistic basis for modulation of the susceptibility of carbonic anhydrase 3 to glutathionylation. *Antioxid. Redox Signal.* 7, 849–854.

INTRODUCTION

The mammalian carbonic anhydrases reversibly hydrate carbon dioxide, thus generating both bicarbonate and hydrogen ions for maintenance of pH homeostasis (28, 32). At least 15 different mammalian proteins with carbonic anhydrase structure are known, 11 of which are catalytically active (18). The existence of multiple isozymes underscores the importance of the reaction in a variety of physiologic functions, including acid–base balance, respiration, urinary acidification, and bone resorption (4). The isozymes vary in developmental expression, tissue distribution, and subcellular location. Carbonic anhydrase isozyme 3 (Car3) has several characteristics that distinguish it from the other isozymes, especially its low specific activity, which is only ~3% that of Car2 (15, 16). Car3 had been thought to possess also intrinsic tyrosine phosphatase activity, but that was subsequently found to be due to a contaminating phosphatase (13).

The enzyme is remarkably rich in skeletal muscle (3) and adipocytes (29), constituting up to 8% and 25% of the soluble fraction of these tissues. *Car3* expression is negligible in preadipocytes, becoming substantial upon differentiation (20), but the mechanism of differentiation-dependent Car3 expression is not understood. Despite its notable abundance in fat and muscle, the function of Car3 is unknown although it has been implicated in fatty acid metabolism (22). Car3 could facilitate rapid conversion of glycolytic intermediates to oxaloacetate and citrate and stimulate their incorporation into fatty acids. However, adipocyte Car3 expression in obese mice is lower than in lean mice (20, 30). Exposure of differentiated mouse adipocytes to insulin decreased Car3 expression by 90%, whereas expression of Car2 was unchanged (21). A knockout mouse lacking Car3 has no unusual phenotypic characteristics; the amount of fat and its distribution are normal (14).

Car3 has two surface-exposed sulfhydryl groups (Cys181 and Cys186),[1] which can conjugate to glutathione through a disulfide link, that is, they can be *S*-glutathionylated (4, 19). Car3 is rapidly glutathionylated *in vivo* and *in vitro* when cells are exposed to oxidative stresses (4, 5), and it is also one of the most carbonylated proteins in rodent liver (1). These observations have led to the suggestion that the enzyme plays a role in the cellular response to oxidative stresses, including reperfusion injury and aging (1, 33). Expression of Car3 in

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[1]Cys181 and Cys186 are the residue numbers of the exposed cysteines in Car3 (24). Some authors prefer to use a numbering sequence derived from a consensus sequence of certain isozymes (9) in which these residues are referred to as Cys183 and Cys188 (10, 24). This scheme is also used in the crystal structure of glutathionylated rat Car3 deposited in the Protein Data Bank as 1FLJ (24). Given the large number of carbonic anhydrase isozymes now known, we have chosen to use the actual residue numbers to avoid confusion.

cells lacking the protein protects them from hydrogen peroxide-induced apoptosis, whereas expression of the closely related Car2 does not (25).

The crystal structures of Car3 from cow and rat have been solved, including the glutathionylated form of the rat enzyme (10, 24). The structures of the unmodified and glutathionylated forms are essentially superimposable; the glutathione moieties appear to have a high degree of mobility. We examined the crystal structures to identify candidate residues that might contribute to the high susceptibility to glutathionylation of Car3. We tested these candidates experimentally by creating a series of site-specific mutants.

MATERIALS AND METHODS

Expression of rat liver Car3 in *E. coli* and its purification and assay were described earlier (13). Site-directed mutagenesis was used to produce mutant proteins as follows. *Nde*I and *Bam*HI restriction sites were added to the 5′ and 3′ ends of the DNA sequence of rat liver *Car3* open reading frame (GenBank accession no. g2708635). Mutagenic oligonucleotides containing the desired mutation, with each complementary to opposite strands of the expression vector, pET17b (Novagen, Madison, WI, U.S.A.), were extended by polymerase chain reaction using *Pfu* DNA polymerase. The synthesized DNA containing the desired mutation was used for transformation of *E. coli* strain BL21(DE3). Bacteria were cultured overnight with shaking at 37°C in Luria broth supplemented with 100 μg/ml ampicillin. Car3 expression was induced by 0.5 m*M* isopropyl thio-D-galactopyranoside when the optical density at 600 nm was between 0.6 and 0.8 AU. After 3 h of induction, the cells were collected by centrifugation, frozen in liquid nitrogen, and stored at −70°C until use. The recombinant Car3 was purified as previously described (13). Reverse-phase HPLC coupled with electrospray mass spectrometry confirmed the purity of the preparations and was performed as described (31). The mass of each protein matched that calculated for its sequence, within 0.6 Da. The C181S and C186S mutants each had a single site-specific mutation. All others had the C181S mutation plus a second site-specific mutation. These were R187A, D188A, D188K, K211A, K211D, E212A, E212K, and E212T.

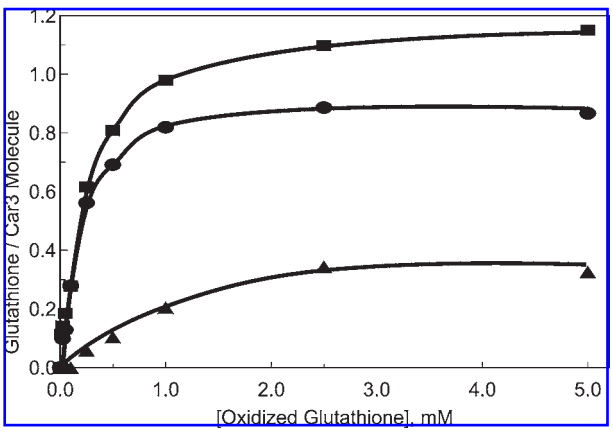

FIG. 1. Glutathionylation of Car3. The proteins were incubated with the indicated concentrations of oxidized glutathione for 1 h at 37°C in phosphate-buffered saline at pH 7.4. The number of glutathiones per protein molecule was determined by mass spectrometry as described in Materials and Methods. ■, wild-type; ●, C181S; ▲, C186S. The experiment was replicated three times.

Glutathionylation was carried out by disulfide exchange with oxidized glutathione (Sigma, St. Louis, MO, U.S.A.). Typically, 5.1 μ*M* Car3 was incubated in 20 μl total volume at 37°C with the indicated concentration of glutathione, added from a stock solution of 100 m*M* oxidized glutathione dissolved in phosphate-buffered saline (150 m*M* NaCl, 8 m*M* Na_2HPO_4, 2 m*M* KH_2PO_4, 3 m*M* KCl, pH 7.4) (31). The reaction was stopped by the addition of 1 μl of glacial acetic acid, giving a final concentration of 5%. Mono- and diglutathionylation was quantitated by reverse-phase HPLC–mass spectrometry (2, 31). Alkylation by iodoacetamide (Sigma) was analyzed with the same system. The buffers used in pH studies were as follows: acetate, pH 5.0; MES, pH 5.5–6.5; HEPES, pH 7.0–7.4; Tris, pH 8.0–9.0; and glycine, pH 10.0.

RESULTS

Car3 can be glutathionylated on two surface-exposed residues, Cys181 and Cys186, both of which are highly surface-exposed in the *x*-ray crystal structures. Our earlier study of Car3 isolated from rat liver showed that Cys186 was more prone to form disulfide bonds than was Cys181 (2); the crystal structure of Car3 revealed that Cys186 would be more accessible to glutathione than Cys181 (24). To quantitate the relative susceptibilities to glutathionylation, we determined the extent of glutathionylation of the wild type and of the C181S and C186S site-specific mutants. Figure 1 shows that Cys186 is more readily modified than is Cys181. The sum of the residues incorporated in the two mutants was very close to that incorporated into the wild type at each concentration of oxidized glutathione, leading to the conclusion that there is no significant positive or negative cooperativity between the two sites (Fig. 2). As Cys186 was more readily modified, subsequent studies focused on that site using the C181S mutant.

It has been suggested that Car3 and other glutathionylated proteins may have binding sites for reduced glutathione that bring the tripeptide in close proximity to the cysteine residue that will be glutathionylated (33). However, examination of the crystal structure of Car3 led the same authors to doubt the existence of a specific binding site for glutathione (24). We reasoned that if there were a binding site, even one formed by an induced fit, then the relative reactivity of oxidized glutathione would be greater than that of a simple alkylating agent. However, Fig. 3 shows that the pH profile was the same for both glutathione and iodoacetamide.

This result was consistent with the view that Cys186 is a reactive residue because of interaction with other amino acids in Car3 that lower the pK_a of its thiol group. We examined the crystal structure of Car3 to identify nearby charged residues that might affect the pK_a of Cys186, using the crystal structure of bovine Car3, whose active-site structure is identical to that of the rat enzyme (10, 24) (Fig. 4 and Table 1). The ϵ-

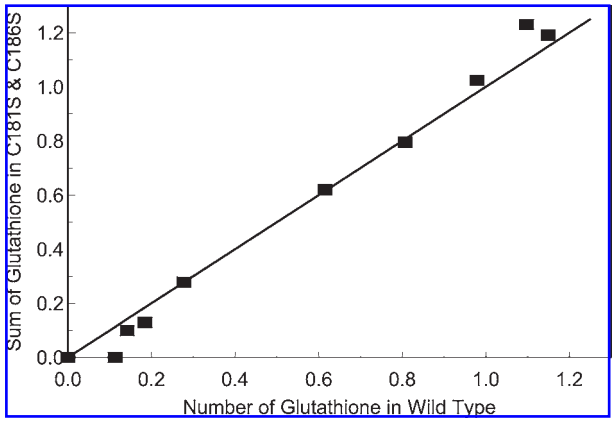

FIG. 2. Lack of cooperativity between the glutathionylated Cys. The data are from the experiment shown in Fig. 1. For each concentration of glutathione, the glutathionylation of the wild-type protein is plotted against the sum of that for Cys181 and Cys186. The line at 45° slope shows the value expected if there were no cooperativity. The experiment was replicated three times.

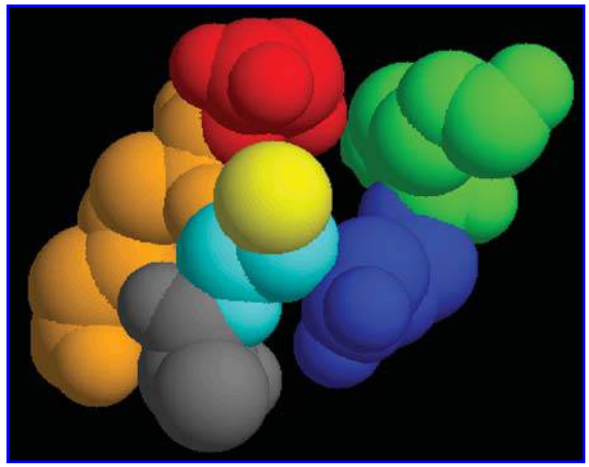

FIG. 4. Residues near Cys186. This space-filling model was constructed from the crystal structure coordinates of the unglutathionylated bovine Car3 (10) with RasMol Windows version 2.7.1 (27). Cys186 is colored cyan except for its sulfur atom, which is yellow. Ala185 is gray, Arg187 orange, Asp188 red, Lys211 green, and Glu212 blue.

amino group of Lys211 faces the sulfur of Cys186 and could promote the ionization of the thiol, although the distance between the sulfur of Cys186 and nitrogen of Lys211 is fairly large at 8.9 Å. Arg187 is the other basic residue near Cys186, but the distance to its guanidino group is 9.2 Å. There are also two acidic residues near Cys186, Asp188 and Glu212, and these might decrease the reactivity of the thiol because their carboxylates are relatively close to the sulfur at 3.6 and 4.6 Å.

To examine the effect of each residue, we constructed site-specific mutants that were purified and assayed for both dehydratase and esterase activities; both specific activities were unchanged by introduction of the mutations. We then compared their reactivity with glutathione. We studied the extent of glutathionylation at varying concentrations of glutathione, at varying pH, and for varying times of incubation. The results of the studies were consistent, so for simplicity we present the results showing the fraction that was glutathionylated by exposure to 100 μ*M* oxidized glutathione at pH 7.4 for 1 h at 37°C (Fig. 5). The wild type and C181S mutant were ~30% monoglutathionylated, whereas the C186S mutant was not glutathionylated. These results are consistent with the greater reactivity of Cys186 compared with Cys181 and also show that, under the conditions used, only Cys186 is glutathionylated.

The other proteins shown in Fig. 5 are double mutants, one of which was always C181S. Changing the basic Arg187 to a neutral alanine had no effect on susceptibility to glutathionylation, indicating that the guanidino group of the arginine does not interact with the thiol of Cys186. However, changing either of the acidic residues, D188 or E212, markedly increased susceptibility to glutathionylation. Conversion to a neutral alanine was as effective as conversion to a basic lysine. Conversely, changing Lys211 to alanine substantially decreased glutathionylation, whereas conversion to an acidic aspartate completely blocked glutathionylation.

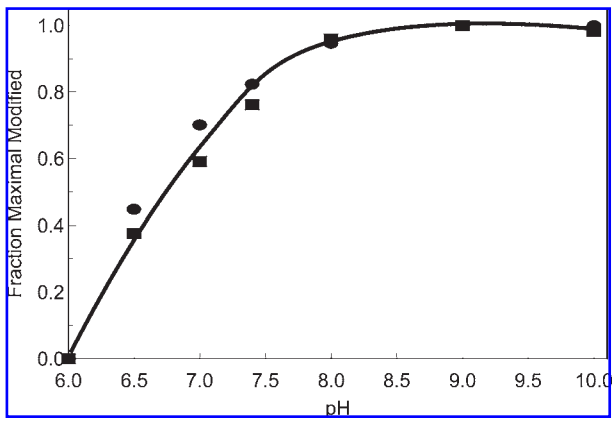

FIG. 3. Reactivity of Cys186 with glutathione or an alkylating agent. The C181S preparation was incubated with either 250 μ*M* oxidized glutathione (■) or 250 μ*M* iodoacetamide (●) for 1 h at 37°C at the indicated pH. The fraction of derivatized protein molecules was determined by mass spectrometry as described in Materials and Methods. The values have been normalized to the maximal fraction modified to allow easy comparison. For iodoacetamide, the maximum was 0.92, and for glutathione it was 0.52. The experiment was replicated four times.

TABLE 1. DISTANCES FROM CYS186 TO NEIGHBORING RESIDUES

Residue	*Atom*	*Distance (Å)*
Arg187	Nitrogen of guanidino	9.2
Asp188	Oxygen of carboxylate	3.6
Lys211	Nitrogen of ϵ-amino	8.9
Glu212	Oxygen of carboxylate	4.6

Distances were measured with RasMol (27) from the sulfur of Cys186 to the listed atom, using the crystal structure of the bovine unglutathionylated protein (10).

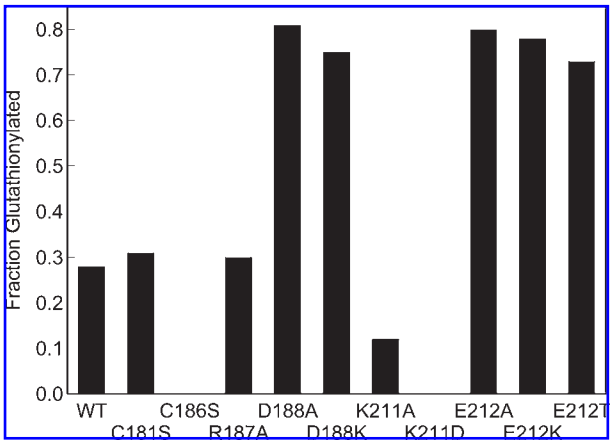

FIG. 5. Susceptibility to glutathionylation at pH 7.4. The proteins were incubated with 100 μ*M* oxidized glutathione for 1 h at 37°C in phosphate-buffered saline at pH 7.4. The fraction of glutathionylated Car3 was determined by mass spectrometry as described in Materials and Methods. The experiment was replicated twice.

Given the propensity of Car3 to glutathionylation *in vivo*, we expected to find basic residues that interact with Cys186 to lower its pK_a. We had not anticipated finding acidic residues that raise the pK_a. We therefore examined the behavior of the Asp188 and Glu212 mutants in more detail. We assessed the reactivity of Cys186 by assaying the fraction of Car3 that was glutathionylated at varying pH, following the approach previously used to characterize residues affecting the reactivity of a surface-exposed cysteine in the human immunodeficiency virus protease (8). The lower the pH at which disulfide exchange occurs, the more reactive the cysteine has been rendered. Figure 6 confirms the modulating effect of Asp188 and further demonstrates that mutation of Asp188 has a greater effect than mutation of Glu212. The site-specific mutants can be compared by determining the pH at which half-maximal glutathionylation occurred. For the reference C181S, this was ~6.9, confirming that Cys186 is a "reactive" cysteine. However, the acidic residues Asp188 and Glu212 clearly decrease the reactivity. Exposure of D188A to 50 μ*M* glutathione led to glutathionylation of 88% of the molecules, compared with only 19% with Asp188 present (Fig. 6). The pH at half-maximal glutathionylation is ~5.7 for D188A compared with 6.9 with Asp188 present.

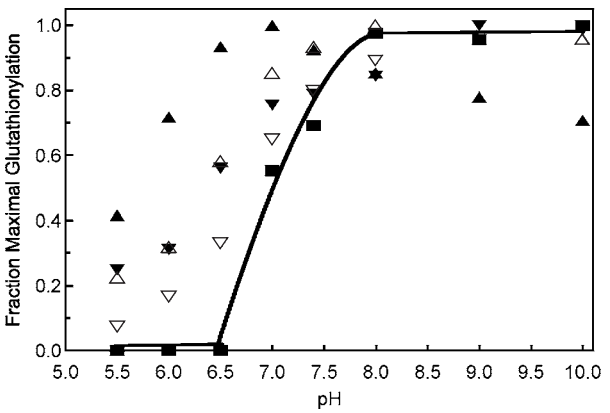

FIG. 6. pH dependence of glutathionylation for selected site-specific mutants. The proteins were incubated with 50 μ*M* oxidized glutathione for 1 h at 37°C at the indicated pH. For ease in visualization, the extent of glutathionylation was plotted as the fraction of the maximal value for each mutant, which generally increased with the reactivity of Cys186. The maxima were as follows: ■, C181S, 0.19; ▼, D188A/C181S, 0.88; ▲, D188K/C181S, 0.61; ▽, E212A/C181S, 0.66; △, E212K/C181S, 0.81. The experiment was replicated twice.

DISCUSSION

Car3 is the most highly glutathionylated protein identified to date. Although both surface-exposed Cys181 and Cys186 are susceptible to glutathionylation, Cys186 is more readily modified. Oxidation of a thiol requires its ionization to the thiolate. The pK_a of free cysteine is ~8.5, the same as for cysteine in the tripeptide glutathione. Hence, at physiological pH, a typical cysteine residue is almost completely protonated. However, within the three-dimensional structure of a protein, the pK_a of specific cysteine residues can be decreased, usually by interaction with basic residues. The cysteine is thus ionized to the thiolate and becomes an "active cysteine." The lowering of the pK_a is particularly important for cysteine residues that function at the active site of enzymes, including phosphotyrosine protein phosphatases (7), dehydrogenases (11), kinases (23), peroxiredoxins (26), and proteases, including the caspases (34). Reactive cysteines are susceptible to oxidation, usually generating the sulfenic acid (S-OH). The sulfenic acid derivative has been demonstrated at the active site of a number of cysteine-dependent enzymes (6). If the sulfenic acid is near another cysteine residue, a disulfide link will likely form. Alternatively, if the sulfenic acid is not near another cysteine but is solvent-accessible, then a low-molecular-weight thiol compound can form the disulfide. In the case of the surface-exposed Cys186 and Cys181 of Car3, a sulfenic acid can be readily attacked by glutathione to yield the mixed disulfide. Reduced glutathione is rather ineffective in removing glutathione from Car3 (4). Reactive cysteines also readily undergo disulfide exchange so that Car3 could also be glutathionylated if the intracellular concentration of oxidized glutathione increases. Thus, even though cellular levels of reduced glutathione are much higher than those of the oxidized form (17), a modest increase in the latter may lead to glutathionylation of Car3. The relative importance *in vivo* of the sulfenic and the disulfide exchange pathways is not known.

The crystal structures of Car1, Car2, Car3, and Car5 are available, and all are very similar to each other, especially in their active sites. Car1 and Car2 have a serine residue in place of Cys186, preventing glutathionylation. However, the mitochondrial Car5 has three surface-exposed cysteine residues, including those corresponding to Cys181 and Cys186 in Car3 (12). We expect that mitochondrial Car5, like cytosolic Car3, will be particularly susceptible to glutathionylation.

Knowing that Cys186 of Car3 was readily glutathionylated both *in vitro* and *in vivo*, we expected to identify several basic residues that serve to lower the pK_a of the thiol. Lys211 appears to be primarily responsible for the lowering of the pK_a,

despite the relatively long distance from its ϵ-amino group in the crystal structure (Table 1). Arg187 has no apparent effect on the pK_a. Most notable in our study is the finding that Asp188 and Glu212 are positioned so that they markedly decrease the reactivity of Cys186. A conformational change that increased the distance between Cys186 and these two acidic residues would greatly increase the reactivity of the thiol. Thus, the movement of Asp188 or Glu212 could serve to regulate the susceptibility of Car3 to glutathionylation.

ABBREVIATIONS

Car, carbonic anhydrase; Car3, carbonic anhydrase isozyme 3.

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Received for publication July 2, 2004; accepted January 19, 2005.

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