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The pK_a Value and Accessibility of Cysteine Residues Are Key Determinants for Protein Substrate Discrimination by Glutaredoxin

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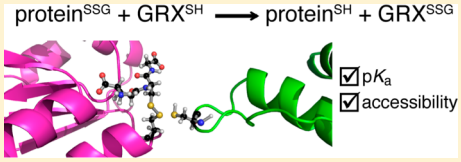
ABSTRACT: The enzyme glutaredoxin catalyzes glutathione exchange, but little is known about its interaction with protein substrates. Very different proteins are substrates in vitro, and the enzyme seems to have low requirements for specific protein interactions. Here we present a systematic investigation of the interaction between human glutaredoxin 1 and glutathionylated variants of a single model protein. Thus, single cysteine variants of acyl-coenzyme A binding protein were produced creating a set of substrates in the same protein background. The rate constants for deglutathionylation differ by more than 2 orders of magnitude between the best ($k_1 = 1.75 \times 10^5$ M^{-1} s^{-1}) and the worst substrate ($k_1 = 4 \times 10^2$ M^{-1} s^{-1}). The pK_a values of the substrate cysteine residues were determined by NMR spectroscopy and found to vary from 8.2 to 9.9. Rates of glutaredoxin 1-catalyzed deglutathionylation were assessed with respect to substrate cysteine pK_a values, cysteine residue accessibility, local stability, and backbone dynamics. Good substrates are characterized by a combination of high accessibility of the glutathionylated site and low pK_a of the cysteine residue.



Protein function and enzyme activity are frequently regulated through post-translational modification of specific residues in response to changes of the cellular environment. It has been suggested that modification of specific cysteinyl residues with glutathione through a disulfide bond could be a means of redox regulation.[1,2] This process is affected by the oxidoreductase glutaredoxin, which catalyzes reversible protein (de)glutathionylation.[3] The catalysis takes place through two successive thiol−disulfide exchange reactions involving a protein substrate, glutathione, and a stable glutathionylated enzyme intermediate (Figure 1).[4,5] During catalysis, the active site cysteine of glutaredoxin gets glutathionylated. The binding site for the covalently attached glutathione is characterized by specific interactions between the ligand and glutaredoxin.[6] The specific interaction with glutathione distinguishes glutaredoxin from other oxidoreductases with thioredoxin fold. Glutaredoxins are found with either one or two cysteinyl residues in the active site allowing for two different reaction mechanisms to take place.[3] In contrast, the activity of thioredoxins is dependent on having two cysteinyl residues in the active site. A mixed disulfide between the protein substrate and thioredoxin is formed, and subsequent reduction of this bond requires the second cysteinyl residue.[7] The function of glutaredoxin in deglutathionylation reactions is only dependent on having the N-proximal cysteine in the active site, and dicysteinyl glutaredoxins may be mutated to remove the C-proximal cysteine without loss of activity.[6] The specificity toward glutathione has been investigated in several studies.[8,9] van der Waals interactions with the γ-glutamate part of glutathione are important for optimal interaction,[9] together with ionic interactions with the N- and C-terminal of glutathione. Glutaredoxin has an unique specificity for glutathione compared to other oxidoreductases with thioredoxin fold. This has been associated with a conserved valine residue (Val70 in human GRX 1) preceding a conserved *cis*-proline.[10]

A binding site for GSH (for the reduction of the mixed intermediate) or the protein part of the substrates has not been identified in contrast to the site of covalently bound glutathione. A number of very diverse proteins have been identified as substrates for glutaredoxin in in vitro studies. Among these are both transcription factors and enzymes, for example, p65-NFκB, HIV protease, glyceraldehyde-phosphate dehydrogenase, bovine serum albumin, and actin.[4,11−13] There are reports on glutaredoxin discrimination in deglutathionylation assays between cysteine residues in HIV protease and *Chlamydomonas reinhardtii* isocitrate lyase.[11,14] The HIV protease is a functional dimer with two cysteine residues (Cys67 and Cys95) that are not required for function but are still conserved among viral isolates.[11] Both cysteine residues are substrates for glutaredoxin, with glutathionylated Cys95 being a far better substrate than Cys67.[11] Cys95 is located in the dimer interface, and glutathionylation at this position apparently inhibits dimer formation and, as a consequence, protease activity.[11] Cys67 is solvent exposed and glutathionylation increases the protease activity, presumably by protecting the enzyme against autoprotolysis.[15] These findings suggested a role of glutathionylation in the regulation of protease activity.

Two different functions of glutaredoxin have been suggested. The first is in a general response to oxidative stress where glutaredoxin may catalyze protein glutathionylation and hereby protect cysteinyl residues against irreversible oxidation. Upon restoration of normal redox conditions, glutaredoxin may

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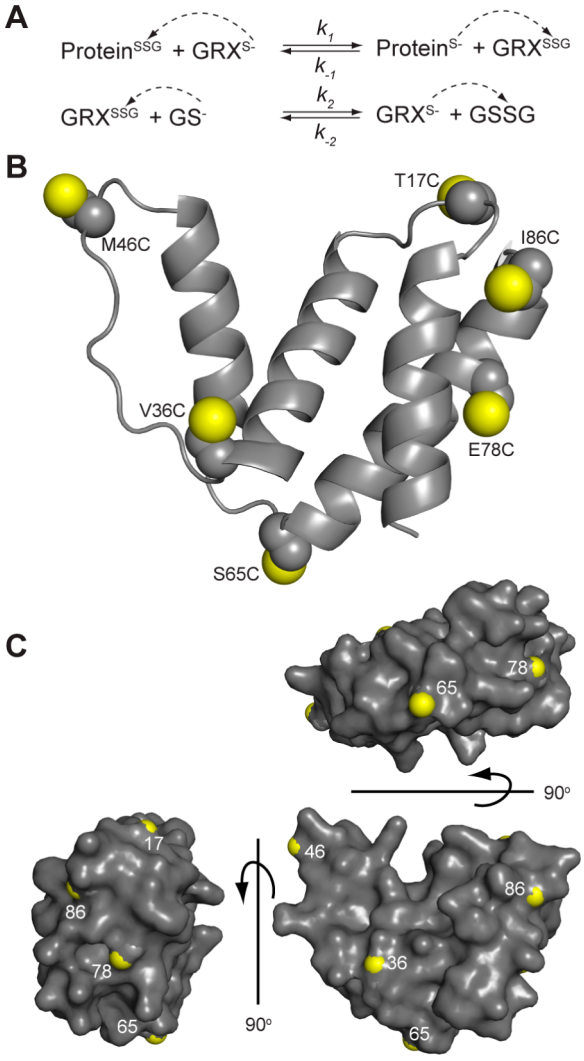

Figure 1. Glutaredoxin catalysis of protein deglutathionylation. (A) Glutaredoxin (GRX) catalyzes reversible glutathionylation of proteins with glutathione as the second substrate. The reaction follows two nucleophilic displacements involving a stable glutathionylated glutaredoxin intermediate (GRX^{SSG}). (B) The structure of the four helix bundle protein ACBP is displayed in cartoon (1NTI) with the six sites individually mutated to cysteine shown in space fill with yellow sulfur atoms. (C) Surface structure of ACBP showing the surface accessibility of the six introduced cysteines with sulfur atoms shown in yellow. Protein structures were drawn using Pymol (Schrödinger, LLC).

catalyze deglutathionylation[16] and could in this way be involved in maintaining redox homeostasis.[17] In effect, the catalytic action of glutaredoxin would equilibrate protein glutathionylation with the glutathione redox poise according to Figure 1a. Exposing mammalian cells to the oxidizing reagent diamide results in massive formation of disulfide bonds, both in protein and glutathione and as mixed species. Restoration of cellular protein glutathionylation levels takes place with a half-life of about 15 min, while the GSH/GSSG ratio is reestablished in less than 2−3 min.[18] This may reflect that glutaredoxin activity is necessary for equilibration with glutathione and that glutaredoxin activity is the rate-limiting factor for overall cellular protein redox recovery after massive oxidative stress.

The second suggested function of glutaredoxin is in redox signaling by catalysis of specific protein deglutathionylation/glutathionylation in response to changes in the cellular redox status.[19] The latter scenario may imply that glutaredoxin should have specificity for certain proteins over others, whereas the former implies a relaxed specificity profile. However, it could also be hypothesized that spontaneous glutathionylation of specific regulatory cysteine residues, prone to oxidation under mildly altered redox conditions, could take place. Upon restoration of normal redox conditions, glutaredoxin could deglutathionylate these sites in a nonspecific manner. This scenario of glutathionylation in redox signaling does not imply a specificity of glutaredoxin for some proteins over others and is therefore not in conflict with a more general role of glutaredoxin as a rescue reductase.

To gain a basic understanding of what makes a good protein substrate for glutaredoxin, we set out to compare different cysteine variants, in the same protein background, as substrates for glutaredoxin. The 10 kDa acyl coenzyme A binding protein (ACBP), of which both structural and biophysical properties are well characterized,[20−23] was used as a model substrate for the analysis. Six variants were studied, where the cysteines were placed on the surface of ACBP, but in very different local context. In this way, we could address which local factors are important for the interaction between glutaredoxin and protein substrates. Using cysteines placed in six different environments rather than point mutations around a single cysteine allows us to sample a much broader structural space by an incomplete factorial design. In the present study, we used a variant of glutaredoxin, where the four noncatalytic cysteine residues were substituted for serine (hGRX^{4CS}). Potential glutathionylation of other sites in the enzyme and consequently formation of a heterogeneous sample was thus prevented. This variant has the same efficiency as the wild type enzyme in catalysis of protein deglutathionylation.[6]

Our results show that the key characteristic of a good protein substrate for glutaredoxin is exposure of the glutathione moiety and hereby accessibility of the protein sulfur atom. The enzymatic activity was found to correlate with the degree of exposed surface area and cysteinyl pK_a, while backbone flexibility appears less important.

MATERIAL AND METHODS

Protein Expression and Purification. Human glutaredoxin 1 with the four noncatalytic cysteine residues replaced by serine (hGRX^{4CS}) was expressed and purified as described.[5] The ACBP variants were expressed and purified as described[24] with the addition that all buffers contained 0.5 mM β-mercaptoethanol to keep the cysteine residues of the ACBP variants reduced. Glutathionylated ACBP variants were prepared by incubation with 5 mM DTT for 1 h at room temperature followed by addition of oxidized glutathione (GSSG) to 100 mM. The sample was left overnight at room temperature. Glutathionylated ACBP (ACBP^{SSG}) was purified by anionic exchange chromatography on an 8 mL Mono Q column and eluted from the column by a 0−0.5 M NaCl gradient in 20 mM Tris-HCl pH 8.0 over 30 column volumes. Fractions containing ACBP^{SSG} were acidified to 0.5% TFA and further purified by RP-HPLC (Zorbax C18, Agilent Technologies) with a gradient of 20−70% acetonitrile in 0.1% TFA. ACBP containing fractions were pooled and lyophilized. The purity and identity of ACBP^{SSG} and ACBP^{SH} samples were confirmed by analytic RP-HPLC, see below, and MALDI-TOF mass spectrometry (Bruker Daltonics). ACBP concentrations

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were determined using the calculated extinction coefficient of 16 800 M^{-1} cm^{-1}.

Kinetic Assay. Kinetic assays were performed in 10 mM sodium phosphate pH 7.0 at 298 K. Fresh 10 times concentrated GSH stock was prepared in Milli Q water and titrated to pH 7.0 with 10 mM NaOH. Exact concentrations were determined by reaction with DTNB, and a 10.0 mM stock was prepared. Reactions contained 1 mM GSH, 1 μM glutaredoxin, and varying $ACBP^{SSG}$ concentrations (5, 10, 20, 40, 80, and 160 μM). Reactions were initiated by addition of $ACBP^{SSG}$ to minimize uncatalyzed deglutathionylation of $ACBP^{SSG}$. Samples were extracted at different time points and immediately quenched by addition of 3.5% TFA (1:1) mixture. The degree of deglutathionylation was determined by reverse phase-HPLC using a C18 column (Grace Vydac 4.6 mm × 150 mm) with a gradient from 30% to 70% acetonitrile in 0.05% TFA at a flow of 1 mL/min over 12 column volumes. ACBP was detected by tryptophan fluorescence (excitation at 280 nm and emission at 340 nm).

Kinetic data were analyzed by calculating the relative deglutathionylation of ACBP ($ACBP^{SH}/ACBP_{total}$) and fitted using KinTek Explorer[25] by numerical integration of the rate equations describing a two-step model (Figure 1a). The rate constants of the lower reaction in Figure 1a were not optimized in the fits but kept constant at $k_2 = 4.3 \times 10^4$ M^{-1} s^{-1} and $k_{-2} = 1.24 \times 10^6$ M^{-1} s^{-1}, as previously determined under identical conditions.[5]

Determination of Equilibrium Constants. For each variant, 11 samples of 10 μM ACBP were incubated in 10 mM sodium phosphate pH 7.0 with GSSG/GSH at concentrations of respectively 10:0, 10:2, 10:4, 10:6, 10:8, 10:10, 8:10, 6:10, 4:10, 2:10, 0:10 mM for 3 h at room temperature. After incubation, the samples were acidified and analyzed by reverse phase HPLC as described above. The equilibrium constant for the direct deglutathionylation of ACBP, K_{eq}, were obtained by fitting $[ACBP^{SH}]/[ACBP_{total}]$ obtained from the integrated peak areas to eq 1:[26]

$$\frac{[ACBP^{SH}]}{[ACBP_{total}]} = \frac{[GSH]/[GSSG]}{[GSH]/[GSSG] + K_{eq}} \quad (1)$$

pH Titrations. For each ACBP variant, an NMR sample of 1 mM protein in 50 mM NaCl, 5 mM DTT, and 1 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was prepared as previously described.[27] A series of standard ^{15}N-HSQC and ^{13}C-HSQC spectra were recorded at 298 K on a Varian Inova 750 MHz NMR spectrometer equipped with a triple resonance probe. Between each spectrum, pH in the sample was increased in steps of approximately 0.25 units by the addition of NaOH. pH was measured before (to control the titration) and after recoding the NMR spectra using a standard pH electrode (Hamilton), and the latter measurement was used as the pH of the sample. The pH of the sample after each NMR measurement gives the correct pH of the sample, as the NMR tube was not cleaned in between each titration point to minimize sample loss.

NMR data were transformed with nmrPipe[28] and analyzed with CcpNmr.[29] The spectra were referenced to DSS. pK_a for the cysteine residues were extracted from the pH dependence of the chemical shifts, δ(pH), by fitting to eq 2, where δ_{offset} is the chemical shift of the protonated state, and $\Delta\delta$ is the change in chemical shift upon deprotonation.[30] A linear term was included to compensate for chemical shift changes induced by the increase in ionic strength upon pH titration.

$$\delta(\mathrm{pH}) = \frac{\Delta\delta}{1 + 10^{\mathrm{p}K_a - \mathrm{pH}}} + \delta_{offset} + \alpha\mathrm{pH} \quad (2)$$

Solvent Accessibility Surface in ACBP. The solvent accessible surface area of ACBP was calculated form the solution structure determined by NMR (PDB code: 1NTI) using a probe size of 1.4 Å^2 and averaged over the 20 structures in the NMR ensemble.[31]

Model-Free Analysis. Relaxation data for wt ACBP have been published.[22] From these data, the internal motions in ACBP were analyzed with the Lipari-Szarbo model-free formalism using the software FAST-Modelfree.[32]

RESULTS

Protein Substrate Discrimination by Glutaredoxin. To investigate which factors characterize a good substrate for $hGRX^{4CS}$, six single cysteine variants of ACBP were compared as substrates for glutaredoxin in a deglutathionylation assay. The positions of the introduced cysteine residues were chosen to be surface exposed (Figure 1B,C). Except for E78C, these ACBP variants have previously been chemically modified with a paramagnetic spin-label and were found to readily react with a thiol-specific reagent.[23] The six positions are distributed over the structure with two positions in loop regions (T17C and M46C), one just following an α-helix (V36C), one preceding an α-helix (S65C), one in the middle of an α-helix (E78C), and one at the C-terminus (I86C) (Figure 1). To determine the ability of glutaredoxin to deglutathionylate the different substrates, all six ACBP cysteine variants were glutathionylated by incubation with a large excess of GSSG. Glutathionylated ACBP ($ACBP^{SSG}$) was separated from glutathione and remaining reduced ACBP ($ACBP^{SH}$) by anionic exchange chromatography and reverse phase HPLC. A deglutathionylation assay of glutaredoxin activity was developed using reverse phase HPLC to separate $ACBP^{SH}$ from $ACBP^{SSG}$. Fluorescence detection provided high sensitivity, and glutathionylation of ACBP did not alter the fluorescence properties of ACBP under the acidic conditions used. The method was validated by analysis of mixtures of known amounts of reduced and glutathinoylated ACBP. Other assays of deglutathionylation activity exist;[4,8,33] however, the present assay allows for small reaction volumes to be used, avoids use of radioactive isotopes, and prevents the coupling to other enzymes, ensuring that other reactions do not become rate limiting. All assays were performed with 1 mM GSH and 1 μM $hGRX^{4CS}$ and with varying concentration of glutathionylated ACBP ($ACBP^{SSG}$) at pH 7.0 and 298 K. To follow the kinetics of deglutathionylation of $ACBP^{SSG}$, samples over the full reaction course were analyzed as described above (Figure 2A).

The kinetic data were fitted by numerical integration of the rate equations corresponding to the kinetic two-step model described in Figure 1a. The fits were constrained by the known values of k_2 and k_{-2} for the reaction between $hGRX^{4CS}$ and glutathione[5] and by the value of K_{eq} listed in Table 1. To a first approximation, all the data sets fit well to the kinetic two-step model (Figure 2B) and yield well determined rate constants (Table 1). More complex reaction schemes cannot be excluded from our data, but the simple model with no observable intermediates allows us to compare the physicochemical and structural properties of the substrates with a simple measure of hGRX reactivity.

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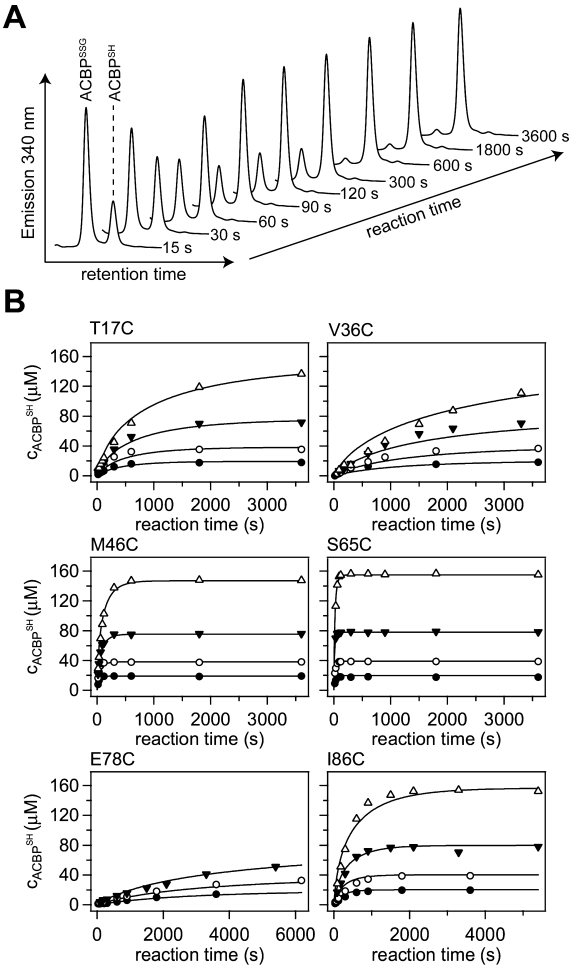

Figure 2. Protein substrate deglutathionylation kinetics. (A) HPLC separation profiles. The deglutathionylation assay was performed by sampling at different times by acid quenching of the reaction. The degree of deglutathionylation was determined by separation of $ACBP^{SSG}$ from $ACBP^{SH}$ with reverse phase HPLC by detection of tryptophan fluorescence. (B) Kinetic data of $hGRX^{4CS}$ activity with ACBP cysteine variants as substrates. Reactions contained 1 mM GSH, 1 μM $hGRX^{4CS}$, and 10 mM sodium phosphate pH 7.0 at 298 K and 160 μM $ACBP^{SSG}$ (open triangle); 80 μM $ACBP^{SSG}$ (filled triangle); 40 μM $ACBP^{SSG}$ (open circle); 20 μM $ACBP^{SSG}$ (filled circle). Solid lines are fits of the experimental data to the kinetic model in Figure 1a.

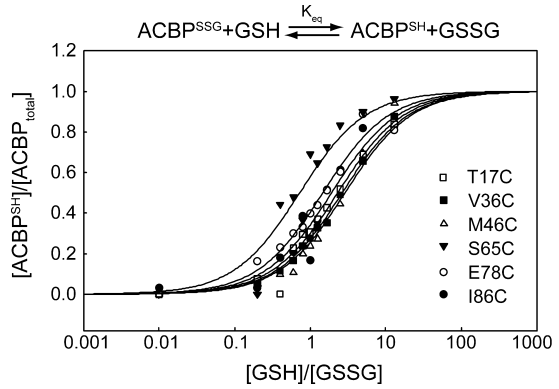

Figure 3. Redox titration of the ACBP cysteine variants with glutathione. 10 μM ACBP was incubated with varying ratios of GSH/GSSH in 10 mM sodium phosphate pH 7.0 at 298 K. The ratio between $ACBP^{SH}$ and $ACBP^{SSG}$ were determined by RP-HPLC. ACBP T17C (open squares), ACBP V36C (filled squares), ACBP M46C (open triangles), ACBP S65C (filled triangles), ACBP E78C (open circles), and ACBP I86C (filled circles). Solid lines are fits of the experimental data to eq 1.

In addition to the kinetic data, the equilibrium constant for the direct deglutathionylation of $ACBP^{SSG}$ by GSH, K_{eq}, was determined in a separate set of experiments (Figure 3 and Table 1). K_{eq} for the six ACBP variants fall in a narrow range between 0.7 (for S65C) and 2.9 (for M46C). The disulfide in glutathionylated ACBP-S65C is thus less stable than the disulfide in GSSG. For the remaining ACBP variants, the disulfide in the glutathionylated protein is more stable than in GSSG. There is no correlation between stability (K_{eq}) and the deglutathionylation rate constant (k_1). S65C and M46D are thus the two substrates deglutathionylated fastest but represent the least and the most stable glutathionylated variants of ACBP, respectively. The missing linear free energy relationship reflects that multiple parameters change between the different ACBP variants.

Whereas K_{eq} for glutathionylation for the ACBP variants is rather similar, the catalyzed rate of deglutathionylation (k_1) differs by more than 2 orders of magnitude between the six glutathionylation sites. For all six substrates, k_1 is lower than the rate constant for reaction of $hGRX^{4CS}$ with oxidized glutathione (k_{-2} = 1.24 × 10^6 M^{-1} s^{-1}). For the fastest reacting ACBP variant, k_1 is 1/7 of k_{-2}, whereas for the slowest reacting ACBP variant k_1 is 1/3100 of k_{-2}. The difference in reaction rates is thus much larger among the ACBP variants than between the best ACBP variant and GSSG.

Substrate Cysteinyl pK_a. The rate of thiol disulfide exchange reactions is strongly dependent on pH.[34,35] This dependency originates from the involvement of the thiolate anion as both nucleophile and leaving group in the reaction. The pK_a value of cysteinyl may therefore be important for determining the characteristics of a substrate. We determined the pK_a values of the single cysteine residue in each ACBP variant by following changes in NMR chemical shifts as a function of pH (Figure 4). The most reliable determination of

Table 1. Characteristics of ACBP Variants

variant	k_1 [10^3 M^{-1} s^{-1}]	k_{-1} [10^3 M^{-1} s^{-1}]	K_{eq} [a]	pK_a	exposure (%) [b]	S^{2c}	HX [d]
T17C	2.7 ± 0.9	0.21 ± 0.07	2.3 ± 0.2	9.8 ± 0.1	58	0.84 ± 0.01	no
V36C	1.0 ± 0.2	0.09 ± 0.02	2.7 ± 0.1	9.5 ± 0.1	51	0.67 ± 0.03	no
M46C	29 ± 8	2.7 ± 0.8	2.9 ± 0.3	8.2 ± 0.1	98	0.88 ± 0.01	no
S65C	175 ± 8	4.3 ± 1.2	0.7 ± 0.1	9.0 ± 0.1	66	0.95 ± 0.01	no
E78C	0.4 ± 0.1	0.020 ± 0.006	1.5 ± 0.1	9.6 ± 0.1	56	0.95 ± 0.02	128 × 10^3
I86C	5 ± 3	0.4 ± 0.2	1.9 ± 0.2	9.9 ± 0.2	74	0.83 ± 0.01	19

[a]Equilibrium constant for glutathionylation of ACBP (eq 1). [b]Exposure of the side chain in the structure of wild type ACBP (PDB id 1NTI) relative to an extended structure. [c]Generalized model free order parameter for NH. [d]Hydrogen exchange protection factor.[21]

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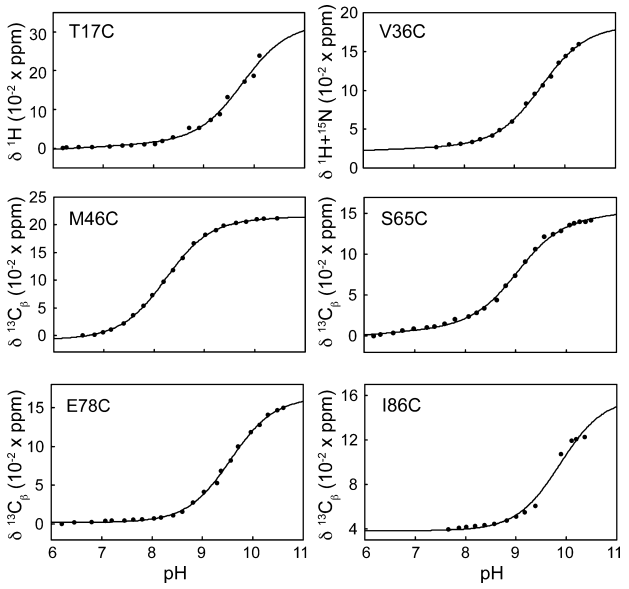

Figure 4. Determination of cysteine pK_a values. Chemical shift changes as a function of pH was followed at 298 K. All samples contained 1 mM ^{13}C,^{15}N-ACBP, 50 mM NaCl, 5 mM DTT, and 1 mM DSS. Solid lines are fits of eq 2 to the experimental data.

cysteine pK_a values by NMR is obtained from the change in chemical shift of Cβ. For C46, C65, C78, and C86, the absolute pH-induced changes of the Cβ chemical shift are larger than 0.1 ppm and were readily fit to eq 2. The changes in Cβ chemical shifts for C17 and C36 are on the other hand very small and cannot be used to determine pK_a. Therefore, the titration of backbone amides was followed instead. Backbone amide chemical shifts for a given amino acid residue may be influenced by the titration of other amino acids, making pK_a values from these titration curves more prone to error.[36] However, in the ACBP cysteine-variants, only the SH-group of the introduced cysteine titrates between pH 7 and 10. The pK_a values of the six ACBP variants range from 8.2 to 9.9 (Table 1) and rank as C46 < C65 < C36 = C78 < C17 = C86. M46C and S65C that have the lowest pK_a values are also the two ACBP variants that are deglutathionylated fastest.

Order Parameters for ACBP. On the basis of published NMR relaxation data,[22] we calculated the generalized order parameter S^2 that is a convenient measure of pico- to nanosecond dynamics in the proteins. The data support isotropic diffusion and a rotational correlation time of 6.2 ns. This is as expected from calculations of the hydrodynamic properties of ACBP with the program HydroNMR.[37] The calculated S^2 for ACBP are generally high (Figure 5D) and may be slightly overestimated due to very high published NOE values.[22] Still, the qualitative conclusions about the flexibility of ACBP we draw from the S^2 are consistent with the conclusions drawn in the original paper.

DISCUSSION

Substrate Characteristics. We have analyzed the properties of the six glutathionylation sites located at different positions in ACBP to determine what structural and physicochemical characteristics may affect the catalytic reduction by hGRX. In addition to the characterization of the reaction kinetics and the pK_a values of the cysteine residues in ACBP, we have estimated the accessible surface area (Table 1,

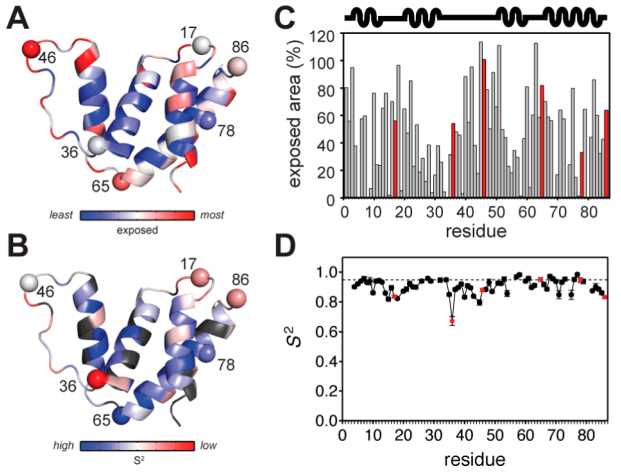

Figure 5. Local characteristics of cysteine sites in ACBP. (A, B) ACBP (1NTI) with the position of inserted cysteine residues shown by spheres. Color gradient runs from blue over white to red. (A) Colored according to relative exposed side chain surface area. Side chains with less than 10% exposed surface are blue and side chain with 100% exposed surface are red. Exposed areas are calculated using a probe size of 1.4 Å^2. (B) Colored according to the S^2 order parameter which is a measure of the nanosecond−picosecond backbone flexibility and calculated from data recorded at pH 6.3 and 298 K reported by Rischel et al.[22] The most flexible residues with S^2 less than 0.75 are red, and the least dynamic residues with S^2 above 0.95 are blue. Data are not available for gray residues due to spectral overlap or presence of proline residues. (C) Bar charts of % exposed side chain surface area of each residue in ACBP (1NTI). (D) S^2 order parameters. (C, D) Data points for positions where cysteine is introduced are red. On top is a diagram of ACBP secondary structure shown.

Figures 5A, 5C and 6) and the nanosecond−picosecond dynamics of the polypeptide backbone from published data

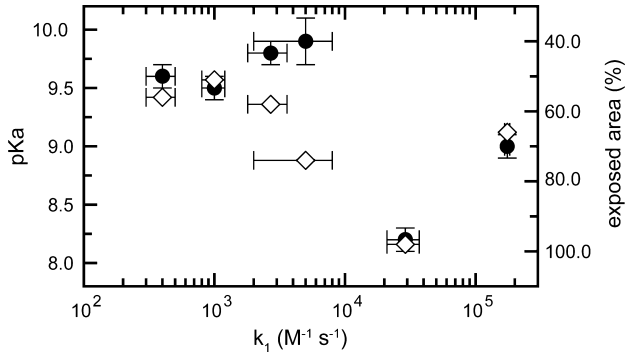

Figure 6. Correlation of the rate of deglutathionylation with pK_a and exposed surface area. pK_a value (filled circles) and side chain exposure (open diamonds) for each variant as a function of the second-order rate constant, k_1 (note reversed y-axis for exposure). All values are listed in Table 1.

(Figure 5B and 5D). We also compared our kinetic data to published data on the local structural stability measured by hydrogen/deuterium exchange.[20,21]

Glutathionylated S65C is the best substrate and characterized by having the second lowest pK_a value. Position 65 in ACBP is placed in a region where the backbone amide hydrogens are not protected from exchange with the solvent by stable hydrogen bonds. However, the high S^2 order parameter demonstrates that the polypeptide backbone structure is as well-ordered as the

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neighboring secondary structure elements (Figure 5B). The cysteine of ACBP M46C (the second best substrate) has the lowest pK_a value, the side chain is more exposed than at position 65, and it is placed in the most dynamic part (residue 35−50) of ACBP with depressed S^2 order parameters and no backbone hydrogen bonds to protect the amide hydrogen from exchange (Figure 5).

The two variants of ACBP deglutathionylated at intermediate rates, ACBP I86C and T17C, have the two highest pK_a values (Figure 6). The pK_a value of C86 is the highest of the six ACBP variants; the side chain at position 86 is more exposed than at position 17 and is located in a region of the protein with slightly higher protection from hydrogen exchange but also lower S^2. The two poorest substrates are V36C and E78C that both have intermediate pK_a values (Figure 6). ACBP E78C is the poorest of all six substrates. The residue at position 78 is slightly more exposed than at position 36. Position 78 has the most stable local environment of the sites tested[21] and is located in an area with high S^2. In contrast, position 36 has the lowest S^2 and no protection from hydrogen exchange. The backbone at position 36 thus appears disordered, while the side chain is still highly buried.

Comparing the pK_a values of the cysteines, side chain accessibility and protection factor of the backbone amide shows that the two best substrates are those with the lowest pK_a values and the least local stability and which also have highly exposed side chains. The finding that the two best substrates have the lowest pK_a value fits well with theory.[34] The decreased pK_a value stabilizes the thiolate anion and hereby makes ACBP a better leaving group in the thiol−disulfide exchange reaction. However, the results show that other parameters (e.g., electrostatics and thermodynamics) must be important as well. Thus, M46C has the lowest pK_a value and a much more exposed side chain than S65C, but S65C is the better substrate (Figure 6).

The intrinsic stability of the glutathionylated species, as determined by K_{eq}, could be suspected to affect the deglutathionylation rate. Redox properties for glutathionylation have typically inferred an equilibrium constant of around 1 for generic sites.[34] However, this study and others[38] suggest that the K_{eq} for surface-exposed sites is probably likely often between 2 and 3. In relation to the difference in deglutathionylation rates, the differences seen in K_{eq} are, however, minor and unlikely to be influential for substrate discrimination. Noteworthy, the disulfide bond of glutathionylated ACBP S65C differs from the other substrates by being less stable than GSSG.

The overall analysis shows that a low pK_a value is the main criteria for a glutathionylated cysteine to be a good substrate for glutaredoxin but also that the effect may be modulated by the accessibility of the side chain. It is expected that there will be a certain limit above which the side chain accessibility no longer will influence the deglutathionylation process. The current data set, however, is too limited to determine this threshold. In contrast, there seems to be no correlation between how good substrates the ACBP^{Cys} variants are and the flexibility of the backbone as evaluated by the order parameter S^2 (Figure 5B,D). This is in contrast to protein kinases and proteases where backbone flexibility has been demonstrated to be important.[39] It also differs from the observation that many proteins undergo post-translational modification in disordered regions.[40,41] The reason for the difference is most likely that glutaredoxin mainly interacts with glutathione and not with the backbone of the protein substrate, whereas, for example, proteases have an extended binding site for the protein substrate, which makes flexibility of the substrate backbone a necessity for binding.

Interaction between Glutaredoxin and Protein Substrates. Our results show that all six glutathionylated ACBP^{Cys} variants are substrates for glutaredoxin. Consequently, the interaction of glutaredoxin with proteins cannot be dependent on a large specific interaction site. This is in line with the previous studies of glutaredoxin substrates.[4,11−13,42]

Simple docking by hand of ACBP (1NTI) with glutathionylated hGRX^{4CS} (1B4Q) reveals that contact between the side chain of position 65 in ACBP and the sulfur atoms in hGRX is possible without any other contact area between the proteins (Figure 7). This fact does of course not exclude additional contacts but shows that a large interaction site is unlikely to exist.

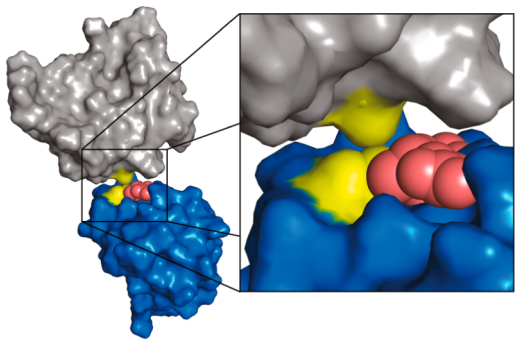

Figure 7. Accessibility of cysteinyl residues. Schematic presentation by hand-docking of ACBP S65C on the hGRX^{4CS}−glutathione complex[6] showing that hGRX^{4CS} does not need to interact directly with the protein substrate. ACBP is displayed in gray with position 65 shown in yellow, hGRX^{4CS} is colored blue with Cys23 in yellow, and glutathione is shown in pink spheres albeit with the sulfur atom in yellow. Contact between the ACBP and glutathione is possible without any contacts between the glutaredoxin surface and the ACBP surface. Figure is prepared in Pymol (Schrödinger, LLC).

Reports on variation in catalytic efficiency toward specific substrates between different glutaredoxin variants exist, but whether this is due to any specific glutaredoxin-protein interactions remains unclear.[43,44]

■ CONCLUDING REMARKS

The proposed redox regulation by glutaredoxin-catalyzed glutathionylation rests on the implicit assumption that glutaredoxin possesses a certain protein specificity. The present study, however, shows that the main factor in determining whether a glutathionylated cysteine residue will be a good substrate for glutaredoxin is the cysteine pK_a and the accessibility of the cysteinyl residue, as the interaction seems to rely mainly on contacts between glutaredoxin and glutathione rather than between glutaredoxin and the protein substrate. To this end, it is important to recognize that these factors are likely also rate-determining for spontaneous disulfide formation upon exposure to high concentrations of GSSG and for the reaction of GSH with sulfenic acid intermediates.[45] Consequently, glutathionylation sites of potential importance for regulation are more likely to be some that are not good substrates for glutaredoxin but are affected by other mechanisms.

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ABBREVIATIONS

ACBP, acyl coenzyme A binding protein; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; hGRX, human glutaredoxin

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