Traditionally, quantification of protein-ligand affinity is performed using kinetic or equilibrium measurements. However, if the binding reaction proceeds via a stable covalent complex, these approaches are often limited. By exploiting the fact that the conformational stabilization of a protein is altered upon ligand binding due to specific interactions, and using an array of selectively chosen ligand analogs, one can quantify the contribution individual interactions have on specificity. We have used ligand-induced stability as a basis to dissect the interaction between glutaredoxin-3 (Grx3) and one of its native substrates, the tripeptide glutathione. Taking advantage of the fact that Grx3 can be trapped in a covalent mixed disulfide to glutathione or to selected synthetic glutathione analogs as part of the natural catalytic cycle, individual contributions to binding of specific molecular groups can be quantified by changes in ligand-induced stability. These changes in conformational stability are interpreted in terms of interaction energies (i.e. specificity) of the particular groups present on the ligand analog. Our results illustrate that although Grx3 recognizes glutathione predominantly through independent and additive ionic interactions at the N- and C-terminal of glutathione, van der Waals interactions from the unique γ-glutamate moiety of glutathione also play an important role. This study places us closer to understanding the complex task of accommodating multiple substrate specificities in proteins of the thioredoxin superfamily and underscores the general applicability of ligand-induced stability to probe substrate specificity.

In the era of structural genomics, a large number of biomolecules will have their tertiary structure determined. However, to fully appreciate the potential of these structures, it is fundamental to determine how these molecules interact with other molecules. Therefore, when exploring the structural basis of observed biological activity it is of great importance to investigate the details of intermolecular interactions. One way of attaining a deeper insight into these interactions is by quantifying ligand affinity, usually performed by kinetic or equilibrium measurements of the binding reaction. Specific interactions between a ligand and a protein can be investigated further by modifying specific residues within the protein using site-directed mutagenesis. The relative binding constants of a wild-type and a mutant protein to a particular ligand then provide information about specificity. However, protein mutagenesis sometimes results in secondary effects as intrinsic properties of the protein may be altered, making subsequent analysis complex. Furthermore, if the interaction between two molecules involves a relatively stable covalent intermediate, standard methods of measuring affinity are often of limited value.

Several studies have demonstrated that the free energy of non-covalent binding of a ligand to a protein results in a change in conformational stabilization with respect to the non-bound form (1–4). This observation also applies to cases where a covalent intermediate between a protein and a ligand is natural or artificial (5–7). As suggested by Kleanthous et al. (5), it should be feasible to utilize this ligand-induced stabilization more thoroughly and quantify the contribution individual interactions have on protein-ligand specificity. To our knowledge, none of these ligand-induced stabilizations have been exploited to dissect protein-ligand specificity. By quantifying the effect a natural ligand and a set of specifically designed ligand analogs (in which the ligand as opposed to the protein is altered) have on the conformational stability of a protein, specific molecular interactions can be separated using a thermodynamic cycle (Fig. 1). Using this approach, many shortcomings of conventional binding experiments are circumvented. Conformational stability can be quantified by determining the free energy differences between the folded protein–ligand complex and the unfolded complex (\(\Delta G^{\text{Unfold}}\)) (8). This can be achieved by the use of standard temperature or solute (e.g. guanidinium chloride or urea) induced unfolding experiments monitored by a suitable spectroscopic probe like absorbance, fluorescence, or circular dichroism (CD) spectroscopy.

In this study we use ligand-induced stability changes to probe *Escherichia coli* glutaredoxin-3 (Grx3) substrate specificity using a C14S/C65Y mutant form of the protein in concert with its natural substrate glutathione (GSH, γ-Glu-Cys-Gly) and a set of synthetic glutathione analogs. The glutaredoxins are ubiquitous enzymes present in most organisms from virus and plants to humans (9) and are characterized by having a common tertiary structure, the thioredoxin fold (10). The activities of the

1. To whom correspondence should be addressed: Hälsövägen 7, S-141 57, Huddinge, Sweden. Tel: 46-0-86083302; Fax: 46-0-86089290; E-mail: kurt.berndt@ki.se.

2. The abbreviations used are: \(\Delta G^{\text{Unfold}}\), free energy of protein unfolding; Grx3, *E. coli* glutaredoxin-3; \(\Delta G^f\), free energy of folding (conformational stability); \(\Delta G_s\), free energy difference between non-bound and ligand-bound form of a protein; \(\Delta G_s\), difference between two ligand-bound forms of a protein; Grx3-SG, Grx3-glutathione-mixed disulfide.
glutaredoxins are based on the reversible oxidation of an active site CXXC disulfide motif, in which only one or both active site cysteines are used (11). Despite having some overlapping substrate preferences, the glutaredoxins display considerable variations in specificity, allowing them to participate in a large number of biological processes including reversible protein S-glutathionylation due to oxidative stress or redox signaling (12), and DNA synthesis by supplying ribonucleotide reductase with electrons (13). The general enzymatic mechanism proceeds via a covalent intermediary glutaredoxin-substrate mixed disulfide complex.

Grx3 from *E. coli* displays extraordinary specificity for reducing protein-glutathione (SG) complexes (14, 15), also referred to as glutathione-mixed disulfide complexes. The catalytic reaction proceeds naturally via nucleophilic attack of the N-terminal cysteine thiolate on the sulfur of the glutathione participating in the mixed disulfide, achieving deglutathionylation of the substrate by forming a transiently stable Grx-SG complex (16). This so-called monothiol reaction does not require the C-terminal active site cysteine (17). On the other hand, this cysteine is required for intramolecular disulfide formation with concurrent release of glutathione and forms an obligatory part of the dithiol mechanism.

Because of the formation of the covalent Grx-SG mixed disulfide complex, standard equilibrium techniques for determining affinity are not optimal. Furthermore, while the Grx-SG mixed disulfide is transiently stable, the presence of the C-terminal cysteine creates the possibility of self-deglutathionylation. By mutating the C-terminal active site cysteine of glutaredoxin into a non-cysteine residue, it is possible to interrupt the catalytic mechanism and trap the Grx-SG mixed disulfide, forming a stable, covalent 1:1 complex. Using this active site mutation, a few examples of Grx-SG mixed disulfide structures have been determined (18–22) and highlight the importance of electrostatics in the interactions between glutaredoxins and their substrates.

To elucidate the importance of these observed interactions, we have quantified the contribution individual molecular groups in the natural substrate glutathione have on Grx3 ligand-induced stability. This has been achieved by comparing the conformational stabilities of the Grx3(C14S/C65Y) mutant in complex with glutathione and a selected set of synthetic analogs having various degrees of glutathione resemblance. The active site C14S mutation has been used successfully to trap intermediate mixed disulfides in *E. coli* Grx1 and Grx3 for structural studies (18, 20). The C14S mutations used throughout this study do not appear to affect the conformation of the proteins as judged by far-UV circular dichroism (CD) spectra which are essentially indistinguishable from the proteins not carrying this mutation.³

The C65Y variant of Grx3 has proved useful in preventing unwanted intermolecular disulfides (16, 20, 24). This mutant form of Grx3 has been shown to have an activity similar to the wild-type protein both in vitro (20) and in vivo.³

The differential stabilities (ΔΔG_f) afforded by mixed disulfide formation between the Grx3(C14S/C65Y) mutant and the various analogs are interpreted as a measure of interaction energies (i.e., specificity). The conformational stability of each mixed disulfide complex was determined by standard urea unfolding experiments monitored by CD spectroscopy at 222 nm. Our results demonstrate the usefulness of ligand-induced stability to quantify the contribution individual interactions have on specificity, and provide further details about substrate specificity of Grx3.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Mutant Grx3 was prepared from *E. coli* BL21(DE3) cells transformed with a pET-24D-plasmid containing the gene for Grx3(C14S/C65Y) (16). The protein was expressed and purified as follows. Cells were grown at 37 °C in LB media, supplemented with kanamycin, until A_600 had reached 0.6. Induction was initiated by the addition of isopropyl-1-thio-β-d-galactopyranoside (0.4 mM final concentration), and cells were harvested after 4 h of expression. The resulting cell pellet was subjected to three freeze-thaw cycles and thereafter dissolved in 20 mM Tris HCl, 1 mM EDTA buffer at pH 8.0. Following centrifugation, the supernatant was

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³ T. H. Elgán, A. G. Planson, J. Beckwith, P. Güntert, and K. D. Berndt, submitted manuscript.
reduced by dithiothreitol and loaded onto a DE52 anion column (Whatman) and eluted with a 1 M NaCl, 20 mM Tris-HCl, 1 mM EDTA buffer at pH 8.0, using a gradient from 0 to 30% (v/v). Fractions containing Grx3(C14S/C65Y) were pooled and concentrated by pressure dialysis using a membrane with a 3-kDa cutoff (Amicon YM3, Millipore). The resulting sample was further purified using size-exclusion chromatography (Sephadex G-50, GE Healthcare) equilibrated with 50 mM potassium phosphate, 50 μM EDTA buffer at pH 7.0. Grx3(C14S/C65Y)-containing fractions were pooled and concentrated as above.

Expression and purification of Grx1(C14S) was performed as for the wild-type protein and has been described elsewhere (25). Characterization of the proteins was performed by RP HPLC (Chemstation 1100, Agilent Technologies) using a C18 semi-preparative column (218TPB510 10 cm × 250 mm, Vydac) and ESI-MS (Finnigan MAT LCQ). Protein concentrations were determined by UV absorbance at 280 nm using a theoretical extinction coefficient of 5120 M⁻¹ cm⁻¹ for reduced Grx3(C14S/C65Y), 5240 M⁻¹ cm⁻¹ for reduced Grx1(C14S) and 10930 M⁻¹ cm⁻¹ for Grx3(C14S/C65Y) mixed disulfides, 10810 M⁻¹ cm⁻¹ for reduced Grx1(C14S) and 10930 M⁻¹ cm⁻¹ for Grx1(C14S) mixed disulfides (26).

**Peptide Synthesis**—The Fmoc-Gly-OH, Fmoc-Cys(trt)-OH, Fmoc-Glu(οtBu)-OH, and Fmoc-γ-aminobutyric acid amino acids, the 2-chlorotrityl chloride and ring amide MBHA resins, and all reagents were purchased from Novabiochem; solvents were from Perseptive Biosystems. Peptides were synthesized manually in 1-mmol batches using standard Fmoc solid phase peptide chemistry (27) employing PyBOP/HOBt as a coupling reagent in a 50-ml reaction vessel (Chemglass Inc.). Peptide C-terminal acids were obtained by the use of a 2-chlorotrityl chloride resin while peptide amides were synthesized by using a ring amide MBHA resin. Following Fmoc-deprotection, all peptides were cleaved from their respective resins using trifluoroacetic acid–dichloromethane–water–triisopropylsilane (82:5:5:7.5 vol%) and recovered by water extraction and subsequent lyophilization using a speed-vac (SC100, Savant). The γ-carboxybutaryl-Cys-Gly and butaryl-Cys-Gly peptide disulfides were prepared by adding a 15-fold molar excess glutaric acid anhydride (Merck) and butyric anhydride (Merck), respectively, to cysteynylglycine disulfide (Bachem) in methanol/triethylamine (10-fold molar excess). The reactions were followed by RP HPLC and allowed to progress until there was no cysteynylglycine disulfide detected (<4 h). The acetyl(Ac)-Cys-NH₂ disulfide was prepared by adding a 10-fold molar excess acetic anhydride (Merck) to cysteynlamidic disulfide (Bachem) dissolved in acetonitrile/triethylamine (10-fold molar excess). All crude peptides were purified by semi-preparative RP-HPLC and analyzed by ESI-MS.

**Protein-Peptide Mixed Disulfide Preparation**—Glutathione disulfide (GSSG, Sigma), 2-hydroxyethyl disulfide (Aldrich), cystine (Fluka) and acetylcysteine disulfide were purchased and used as is. The Fmoc-synthesized peptides in their reduced forms were dissolved in deionized water adjusted to neutral pH and left for 1 day at 4 °C to facilitate oxidation and subsequent homodimerization. Thereafter the oxidized peptides were lyophilized and dissolved in 50 mM potassium phosphate, 50 μM EDTA buffer at pH 7.0. Solubility difficulties were encountered with the Ac-Cys-NH₂ disulfide. However, the addition of urea to a final concentration of 1 M (not sufficient to denature the protein) facilitated solvation. Prior to mixed disulfide formation, the mutant protein was reduced using a 100-fold molar excess dithiothreitol, which was removed after 30 min by gel filtration (PD-10, GE Healthcare). Mixed disulfide formation was achieved by incubating reduced mutant protein (typically at a concentration of ~0.2 mM) overnight at 4 °C with 50-fold molar excess substrate disulfide. Excess substrate was removed by gel filtration as above. Quantitative mixed disulfide formation was confirmed by RP-HPLC and ESI-MS as above.

**Free Energies of Unfolded State Mixed Disulfides**—Stock solutions of GSSG, (Ac-Cys)₂, and (Cys-NH₂)₂ were prepared. The concentration of each compound was determined by quantitative amino acid composition analysis using ortho-phthalaldehyde (OPA) as a derivatization agent following acid hydrolysis (28). Concentrations were calculated based on the total amount of cystine in each sample. Equimolar mixtures of GSSG/(Ac-Cys)₂, GSSG/(Cys-NH₂)₂, and (Ac-Cys-OH)₂/Cys₂ (each peptide at 1.5 mM, 100-fold molar excess relative protein) were prepared in a 50 mM potassium phosphate, 50 μM EDTA solution at pH 7.0 containing urea (7.9 M final concentration). The experiment was initiated by the addition of Grx3(C14S/C65Y) to a final concentration of 15 μM and left to equilibrate at room temperature in a sealed tube purged with nitrogen gas. Samples were taken after 3, 22, and 26 h and quenched by the addition of 50 μl 1 M phosphoric acid to 100 μl of protein/peptide solution. All samples were analyzed by analytical RP-HPLC using a 4.6 × 250 mm C8 column (208TP54, Vydac). The samples were eluted using a gradient from 29 to 38% (v/v) acetonitrile in water containing 0.1% trifluoroacetic acid for 30 min and monitored at 214 nm. The resulting chromatograms were integrated using the multipeak fitting routine in the Igor Pro software package (WaveMetrics).

**CD Spectroscopy**—Far-UV CD spectra of native Grx3(C14S/C65Y) and Grx3(C14S/C65Y) mixed disulfide complexes were measured at 25 °C using a 50 mM potassium phosphate, 50 μM EDTA buffer at pH 7.0 and a protein concentrations of ~17 μM. Data were collected in a 0.2-cm quartz cuvette (Hellma) using an Aviv 202 spectropolarimeter, collecting ellipticity between 260 and 190 nm every 1.0 nm using a bandwidth of 1.5 nm for 10 s.

The far-UV CD measurements at various urea concentrations were performed as above with the exception that a 2.0-nm bandwidth was used. Five different urea concentrations were selected for each sample based on their corresponding urea unfolding curve.

Conformational stabilities of the mutant in its reduced form and various mixed disulfide forms were measured by urea (ICN Biomedicals) unfolding experiments monitored by CD spectroscopy at 222 nm. Measurements were performed in the same buffer as above and protein concentrations ranged from 3.3 to 5.5 μM. Semi-automated solute titrations were performed using a 2-syringe pipetting robot (Hamilton, Microlab 500). Urea concentration was increased in 0.1 M steps from 0 to 8 M directly in the stirred 1-cm path length cuvette. Ellipticity at 222 nm was measured for 30 s following a 7-min equilibration time using a 1.5 nm bandwidth. Temperature was maintained at 25 °C for all experiments. Reversibility was confirmed for the reduced form.
**Quantifying Grx3 Substrate Specificity**

of Grx3(C14S/C65Y) by reverse titration with the resulting refolding curve essentially superimposable onto the unfolding curve. All other mixed disulfide complexes were assumed to undergo the same reversibility.

Assuming that unfolding of a protein or protein mixed disulfide in the presence of a solute is a reversible two-state process between the folded (f) and unfolded (u) forms, an equation (Equation 1) for the experimental observable ($y_{\text{obs}}$) can be derived as previously described (29, 30).

$$y_{\text{obs}} = \frac{(y_f + m_{\text{f}}[D]) + (y_u + m_{\text{u}}[D]) \cdot \exp\left(\frac{m_{\text{trans}}[D] + \Delta G_{\text{H}_{2}\text{O}}^{\text{Unfold}}}{RT}\right)}{1 + \exp\left(\frac{m_{\text{trans}}[D] + \Delta G_{\text{H}_{2}\text{O}}^{\text{Unfold}}}{RT}\right)}$$

(Eq. 1)

Here $R$ is the gas constant and $T$ is temperature in Kelvin; $m_{\text{trans}}$ is the solute dependence of the measured free energy changes of unfolding $\Delta G_{\text{H}_{2}\text{O}}^{\text{Unfold}}$ in the presence of denaturant, assuming the linear extrapolation model in the transition region ($\Delta G_{\text{H}_{2}\text{O}}^{\text{Unfold}} = m_{\text{trans}}[D] + \Delta G_{\text{H}_{2}\text{O}}^{\text{Unfold}}$) with $\Delta G_{\text{H}_{2}\text{O}}^{\text{Unfold}}$ being the value of $\Delta G_{\text{H}_{2}\text{O}}^{\text{Unfold}}$ in the absence of denaturant ($[D] = 0$); the parameters $y_f$ and $y_u$ refer to measured values of the fully folded and unfolded forms, respectively. To account for observed linear spectral changes with respect to increasing solute concentration ($[D]$), parameters describing the linear pre- and post-transition dependence ($m_f$ and $m_u$, respectively) are also included. Raw data were fitted with the inverse of the standard deviation of each measured CD value and fit to Equation 1 using non-linear least-square regression analysis within the Igor Pro software package. Fitted coefficients are reported ± 1 S.D. Values describing the conformational stability are reported in terms of the folding reaction using the following relationship $\Delta G_{\text{H}_{2}\text{O}}^{\text{Fold}} = -\Delta G_{\text{H}_{2}\text{O}}^{\text{Unfold}}$. Derived values (i.e. $\Delta \Delta G$ and [Urea]$_{1/2}$) are reported with errors propagated accordingly.

**RESULTS AND DISCUSSION**

**Synthetic Glutathione Analogs and Grx3-mixed Disulfide Preparation**—In an attempt to quantify the importance of individual interactions between Grx3 and glutathione there on specificity, a set of synthetic glutathione analogs were designed based on commercially available compounds for Fmoc peptide synthesis. The selected analogs were synthesized and purified to apparent homogeneity as judged by RP-HPLC and ESI-MS (data not shown). The molecular weights of each trapped and purified ligand-Grx3(C14S/C65Y) mixed disulfide were determined by analytical RP HPLC and ESI-MS (data not shown) and used as evidence of a 1:1 complex.

**Unfolded State Free Energies of Grx3-ligand Mixed Disulfides**—In order for the thermodynamic linkage in Fig. 1 to be true, it is crucial that the differences in unfolded state free energies between the different Grx3 mixed disulfides are known. When comparing glutathione with the synthetic analogs used in this study (Fig. 2) it is obvious that the incremental differences between the analogs, should only make a negligible contribution to the corresponding complex when unfolded. The difference in free energies of the unfolded state mixed disulfides are therefore expected to be similar whereas unfolded oxidized and reduced active site -CXXC- containing proteins differ by ~1 kcal mol$^{-1}$ (16, 25) due to the entropic penalty of forming a cross-link in the polypeptide chain (31). The assumption of the similarity of free energies of the unfolded state mixed disulfides can be tested by exposing unfolded Grx3 to two ligand peptides at equimolar concentration. If the two corresponding unfolded Grx3 mixed disulfides are present at an equimolar ratio at thermodynamic equilibrium, their unfolded state energies are equal. Three peptides were selected representing the extremes in terms of the impact they have on Grx3(C14S/C65Y) stability (Table 1). Mixtures under denaturing conditions were set up where mutant Grx3 was allowed to react with two peptides at equimolar concentrations. All samples had reached equilibrium after 22 h and the resulting mixed disulfide species were quantified by RP HPLC (Fig. 3). Equilibrium was judged by the fact that longer equilibration resulted in identical peak intensities. Since the retention times for the glutathione and Cys-NH$_2$ mixed disulfides with Grx3(C14S/C65Y) were identical, it was only possible to assay these two peptides (in their homodimeric forms) separately with Ac-Cys disulfide. As seen in Fig. 3, the peak areas of the mutant Grx3-(Ac-Cys) mixed disulfides are slightly larger than 50%. This is due to the fact that these peaks also include corresponding Grx3(C14S/C65Y) N-terminal methionine-containing mixed disulfide isoforms (<7%) with glutathione and Cys-NH$_2$. Heterogeneity with respect to the N-terminal methionine is a consequence of incomplete processing in the expression system. However, all peaks can properly be quantified using the multipeak fitting routine in the Igor Pro software and the similarity of the resulting peak areas (<6% difference) demonstrate that Grx3(C14S/C65Y) in the unfolded state does not significantly discriminate between the tested peptides. Therefore it can be concluded that the free energies of the unfolded state mixed disulfides of these analogs are degenerate, and we assume the same is true for the other analogs used in this study.

**Conformational Stability Measurements**—To probe the overall conformation of non-bound Grx3(C14S/C65Y) and ligand-bound mixed disulfides, far-UV CD wavelength scans were collected for the non-bound mutant Grx3 and the corresponding Cys-NH$_2$, β-mercaptoethanol, Ac-Cys, and glutathione-mixed disulfides (data not shown). The close correspondence of the resulting spectra underscores the similarity of secondary structural content between the native state and ligand-bound mixed disulfides. Based on this similarity, we assume in the following analysis that the three-dimensional structures of the protein part of the mixed disulfide complexes are indistinguishable.

The conformational stability of each mixed disulfide complex was measured by standard urea induced unfolding experiments monitored by CD spectroscopy at 222 nm. Analysis of the raw data were performed using non-linear regression to a reversible, two-state model. To ensure a reversible two-state process, far-UV CD wavelength scans (data not shown) of the non-bound and the corresponding Cys-NH$_2$, β-mercaptoethanol, Ac-Cys, and glutathione-mixed disulfides were recorded at different urea concentrations to monitor the unfolding reaction. The presence of an isodichroic point at ~205 nm is a
strong indication that the transition between folded and unfolded mixed disulfide complexes is indeed a two-state process.

To a first approximation, the midpoints of the unfolding curves, $[\text{Urea}]_{1/2}$, are shifted to the left or right depending on whether or not the ligands have a negative or positive effect on stabilization of the complex relative the non-bound Grx3(C14S/C65Y) (Fig. 4). However it is incorrect to consider the conformational stability of a protein as high solely because of a high $[\text{Urea}]_{1/2}$, but rather one must also take into account the slope ($m_{\text{trans}}$ or $m$-value) obtained from the urea dependence of the measured free energies of unfolding within the transition region of the unfolding curve. This explains the observation that the Grx3(C14S/C65Y)-SG complex, which has a higher stability to urea-induced unfolding, as compared with the Grx3(C14S/C65Y)-($\gamma$-Glu-Cys) complex ($[\text{Urea}]_{1/2} = 5.44 \pm 0.16$ versus $5.10 \pm 0.12 \text{ M}$, respectively) is not necessarily the complex with the highest conformational stability (the latter being $0.44 \pm 0.14 \text{ kcal mol}^{-1}$ more stable). This behavior is due to the lower $m$-value of the Grx3(C14S/C65Y)-SG complex ($-0.96 \pm 0.02$ versus $-1.11 \pm 0.02 \text{ kcal mol}^{-1} \text{ M}^{-1}$, Table 1).

In determining the conformational stability, we have attempted to maximize data quality while at the same time conserving sample and minimizing instrument usage. To achieve this compromise, we have used single measurements for each of the complexes reported here, complemented with randomly chosen duplicates for about 25% of the samples. In all cases, data from the duplicate samples yielded parameters that were not significantly different from those reported.

**Binding Effects of the Natural and Synthetic Ligands**—The parameters obtained from the non-linear regression of the raw urea unfolding data to Equation 1 for all mixed disulfide complexes are shown in Table 1. Clearly, glutathione and the different synthetic ligand analogs (Figs. 2 and 4, Table 1) have quite diverse impacts on the stability of Grx3(C14S/C65Y), underscoring the importance certain molecular groups play within the glutathione molecule. Moreover, it is noticeable that the synthetic ligands have upon mixed disulfide formation with Grx3(C14S/C65Y) varies from causing extreme instability, as in the case for the cysteine-mixed disulfide (reliable data could not be measured), to having a very stabilizing effect as for the $\gamma$-Glu-Cys mixed disulfide ($\Delta G^\text{fold}_{H_2O} = -5.66 \pm 0.09 \text{ kcal mol}^{-1}$).

**Implications for Grx3 Substrate Specificity**—Analysis of the solution structure of the mixed disulfide between Grx3(C14S/C65Y) and glutathione (20) reveals six charged surface residues providing the non-covalent electrostatic interactions that
Quantifying Grx3 Substrate Specificity

TABLE 1

| Mixed disulfide complex | \( \Delta G_{\text{fold}}^{\text{old}} \) | \( \Delta G_{\text{fold}}^{\text{new}} \) | [Urea]_{1/2} | \( m_{\text{trans}}^{\text{d}} \) |
|-------------------------|-----------------|-----------------|--------------|-----------------|
| Grx3(C14S/C65Y)-ligand mixed disulfides | | | | |
| Cys | n.a. | n.a. | 1.38 ± 0.07 | n.a. |
| β-Mercaptoethanol | -2.13 ± 0.09 | 2.57 ± 0.12 | 2.06 ± 0.04 | n.a. |
| α-Glu-Cys | 0.17 ± 0.05 | 1.63 ± 0.09 | 1.49 ± 0.02 | n.a. |
| Ac-Cys-NH₂ | -3.24 ± 0.06 | 1.46 ± 0.10 | 1.49 ± 0.02 | n.a. |
| α-Glu-Cys-Gly | -3.74 ± 0.05 | 0.96 ± 0.09 | 0.91 ± 0.02 | n.a. |
| butaryl-Cys-Gly | -4.48 ± 0.07 | 0.22 ± 0.11 | 1.42 ± 0.02 | n.a. |
| γ-Aminobutyl-Cys-Gly | -4.62 ± 0.12 | 0.08 ± 0.14 | 1.13 ± 0.03 | n.a. |
| Grx3(C14S/C65Y) | -4.70 ± 0.08 | 3.85 ± 0.09 | 1.22 ± 0.02 | n.a. |
| Ac-Cys | -4.78 ± 0.08 | -0.08 ± 0.11 | 3.44 ± 0.08 | 0.91 ± 0.02 |
| γ-Glu-Cys-NH₂ | -4.95 ± 0.10 | -0.25 ± 0.13 | 4.54 ± 0.12 | 1.23 ± 0.03 |
| γ-Butylthiol-Cys-Gly | -5.06 ± 0.11 | -0.36 ± 0.14 | 4.13 ± 0.11 | 1.40 ± 0.02 |
| glutathione (GSH) | -5.20 ± 0.10 | -0.50 ± 0.13 | 5.42 ± 0.15 | 0.96 ± 0.02 |
| γ-Glu-Cys | -5.66 ± 0.09 | -0.96 ± 0.12 | 5.10 ± 0.12 | 1.11 ± 0.02 |

| Grx1(C14S)-ligand mixed disulfides | | | | |
| α-Glu-Cys-Gly | -1.49 ± 0.15 | 2.13 ± 0.17 | 2.17 ± 0.12 | -0.35 ± 0.04 |
| Grx1(C14S) | -3.62 ± 0.07 | 3.90 ± 0.07 | 1.25 ± 0.02 | 0.91 ± 0.04 |
| Glutathione (GSH) | -3.63 ± 0.14 | -0.01 ± 0.16 | 3.99 ± 0.23 | -0.91 ± 0.04 |

\( \Delta G_{\text{fold}}^{\text{old}} \) is the conformational stability of the folded state in the absence of urea.

\( \Delta G_{\text{fold}}^{\text{new}} \) indicates the folding free energy contribution of substrate binding relative to the non-bound Grx3(C14S/C65Y) as noted by a dash.

\( [\text{Urea}]_{1/2} \) is the midpoint of the urea unfolding curve.

\( m_{\text{trans}}^{\text{d}} \) is the slope of the urea dependence of the measured free energies within the transition region of the unfolding curve.

The Grx3(C14S/C65Y) - (Cys) mixed disulfide was too unstable for determination of reliable data (not applicable, n.a.).

The differences are not significantly different from zero.

The Grx3(C14S/C65Y) mixed disulfide was too unstable for determination of reliable data (not applicable, n.a.).

The conformational change in the absence of urea is less than in the absence of the two glutathione isoforms (Cys-Gly mixed disulfides) and hence more important for the stability of the complex (\( \Delta G_{2} = -2.13 ± 0.11 \) glutathione versus Cys-Gly mixed disulfides) and hence more important for specificity than the \( CH_{2}COO^- \) moiety of the terminal Gly (\( \Delta G_{2} = -0.25 ± 0.14 \) kcal mol⁻¹, glutathione versus γ-Glu-Cys-NH₂ mixed disulfides), an observation also reported by Peltoniemi et al. (34). The importance of the N-terminal interaction is highlighted when considering that the difference in Grx3(C14S/C65Y) stabilization between peptide C-terminal acids and C-terminal amides in the presence of N-terminal α- or γ-Glu (e.g. \( \Delta G_{2} \) between γ-Glu-Cys and γ-Glu-Cys-NH₂ is 0.71 ± 0.13 kcal mol⁻¹) is less than in the absence of the two glutamate isoforms (e.g. \( \Delta G_{2} \) between Ac-Cys and Ac-Cys-NH₂ is 1.43 ± 0.09 kcal mol⁻¹). Furthermore, the significance of the unique linkage afforded by the γ-Glu is also seen in reference to the analogs containing α-Glu, where the relocation of the positively charged amino group of glutathione decreased the stability of the α-Glu-Cys-Gly mixed disulfide.

FIGURE 3. Determining the free energies of the unfolded state mixed disulfides. Unfolded Grx3(C14S/C65Y) was allowed to react with two peptide disulfides, at equimolar ratios, solid and dashed lines represent the glutathione:Ac-Cys and Cys-NH₂:Ac-Cys mixtures, respectively. The resulting Grx3(C14S/C65Y) mixed disulfides with glutathione (▪), Cys-NH₂ (▲), and Ac-Cys (▲) are formed at approximately equimolar ratios (see text for details), indicating that the unfolded state free energies of these two representative mixed disulfides are essentially indistinguishable. An asterisk indicates a mixed disulfide between Ac-Cys and an N-terminal Met variant of Grx3(C14S/C65Y).

A dipeptide ligand lacking the N-terminal γ-Glu of glutathione makes contact with the ring of Tyr-13 and the methyl group of Thr-51 while the glutathione glycine Cα protons and cysteine Cβ protons are close to the Thr-51 methyl group and the Val-52 side chain, respectively. By assuming that the synthetic ligands are coordinated in a similar fashion as the natural glutathione substrate, the differences in conformational stabilities between the artificial and natural complexes (Table 1) can be interpreted in terms of a structural model.

Serves to orient the natural glutathione substrate (Fig. 5). Two backbone-to-backbone hydrogen bonds are observed between the cysteine of glutathione and Val52 in the cis-Pro loop forming a short anti-parallel β bridge between the protein and substrate. The multifunctionality of this binding theme is illustrated by the diverse substrate specificities present in the entire thioredoxin superfamily (20). Natural substrates can even be accommodated in different orientations (parallel or antiparallel) preserving the β strand-like hydrogen bonds as has been revealed in the related human thioredoxin (32, 33). Furthermore, glutathione is in close contact with the Grx3(C14S/C65Y) binding site at numerous points, underscoring the importance of van der Waals interactions. For instance, the ethylene linker of γ-Glu of glutathione makes contact with the ring of Tyr-13 and the methyl group of Thr-51 while the glutathione glycine Cα protons and cysteine Cβ protons are close to the Thr-51 methyl group and the Val-52 side chain, respectively. By assuming that the synthetic ligands are coordinated in a similar fashion as the natural glutathione substrate, the differences in conformational stabilities between the artificial and natural complexes (Table 1) can be interpreted in terms of a structural model.
by 1.46 ± 0.11 kcal mol$^{-1}$, probably as a consequence of repulsive effects.

As pointed out earlier, the ethylene linker of the glutathione $\gamma$-Glu group makes van der Waals contacts to Tyr-13 and Thr-51 of Grx3(C14S/C65Y). These interaction energies can be quantified by the relative stabilities of the corresponding Grx3(C14S/C65Y) complexes with the Cys-Gly dipeptide ($\Delta G_{\text{Fold}}^{\text{Cys-Gly}} = -3.07 \pm 0.05$ kcal mol$^{-1}$) and butaryl-Cys-Gly ($\Delta G_{\text{Fold}}^{\text{Butaryl-Cys-Gly}} = -4.48 \pm 0.07$ kcal mol$^{-1}$). The difference ($\Delta \Delta G_{\text{f}}$) points out that the interactions made by the ethylene linker are worth $-1.41 \pm 0.09$ kcal mol$^{-1}$ in stability.

In addition to the aliphatic linker, $\gamma$-Glu has the $\alpha$-carboxylate and $\alpha$-amino groups, each involved in electrostatic interactions with the protein (Fig. 5). Quantifying the specific contribution of the former group requires the comparison ($\Delta \Delta G_{\text{f}}$) between the glutathione and $\gamma$-aminobutaryl-Cys-Gly complexes with Grx3(C14S/C65Y) ($\Delta G_{\text{Fold}}^{\text{Butaryl-Cys-Gly}} = -3.35 \pm 0.04$ kcal mol$^{-1}$) and $-3.62 \pm 0.12$ kcal mol$^{-1}$, respectively) which amounts to $0.58 \pm 0.16$ kcal mol$^{-1}$. Although the difference between the Grx3(C14S/C65Y) complexes with glutathione and $\gamma$-carboxybutaryl-Cys-Gly ($\Delta G_{\text{Fold}}^{\text{GH2O}} = 0.15 \pm 0.11$ kcal mol$^{-1}$) is not significant ($\Delta \Delta G_{\text{f}} = 0.14 \pm 0.15$ kcal mol$^{-1}$), it appears as if the $\alpha$-amino group does not participate in favorable interactions. This finding is surprising since the charged amino group is in close contact with negative charges on the Grx3(C14S/C65Y) surface supplied by Asp-67 and/or Asp-66 (Fig. 5). It then appears as if the $\gamma$-configuration of the charged amino group contributes to specificity by allowing for van der Waals interactions involving the ethylene linker, thereby preventing repulsive effects as seen in the Grx3(C14S/C65Y) mixed disulfides with $\alpha$-analogs, rather than engaging in favorable interactions with mutant Grx3.

As discussed above, a number of ligand C-terminal acids were found to be considerably more stable than their C-terminal amide counterparts. This behavior may be explained by the largely positive binding surface on the protein supplied by the flexible surface residues Arg-40 and Lys-8. The flexibility of these two side chains could likely be responsible for the increased stability of the $\gamma$-Glu-Cys mixed disulfide with Grx3(C14S/C65Y), as it was found to be $0.46 \pm 0.13$ kcal mol$^{-1}$ more stable than the glutathione one.

Lack of complementary charges on the ligands Ac-Cys-$\text{NH}_2$ and $\beta$-mercaptoethanol result in poor stability of the corresponding complexes, even if, as in the case of Ac-Cys-$\text{NH}_2$, the two glutathione-like intermolecular backbone-to-backbone hydrogen bonds, seen in the structures of mixed-disulfide complexes, can form. The relative stabilities of these two mixed disulfides ($\Delta G_{\text{Fold}}^{\text{Ac-Cys-NH}_2} = -3.35 \pm 0.04$ and $-2.13 \pm 0.09$ kcal mol$^{-1}$, respectively) allow an estimate of the interaction energy afforded by these two hydrogen bonds which amounts to a value of $\Delta \Delta G_{\text{f}} = -1.22 \pm 0.10$ kcal mol$^{-1}$. In the case of the smallest peptide ligands, Cys and Cys-$\text{NH}_2$, the charged $\alpha$-amino groups may inhibit the backbone-to-backbone hydrogen bond formation, thus causing the observed instability of their corresponding Grx3(C14S/C65Y) mixed disulfides. The incapability of the cysteine ligand to stabilize the corresponding Grx3(C14S/C65Y)-Cys mixed disulfide helps to explain the observed inability of human glutaredoxin-1 to catalyze the reduction of protein-Cys mixed disulfides (14).

**Ligand-induced Stability Correlates with Enzymatic Activity**—To test the generality of this approach, stability measurements on the C14S variant of *Escherichia coli* glutaredoxin-1 (Grx1) together with glutathione and the isofrom of glutathione ($\alpha$-Glu-Cys-$\text{GH}_2$) were performed. Despite being structurally very similar, Grx1 and Grx3 have measurably different activities. Compared with Grx3, Grx1 is more efficient at reducing ribonucleotide reductase and less efficient at reducing glutathione-mixed disulfides (20, 35). As can be seen in Fig. 4 and Table 1, glutathione does not have a significant impact on Grx1(C14S) stability as the mixed disulfide has the same amount of conformational stability as the non-bound form ($\Delta G_{\text{Fold}}^{\text{GH}_2O} = -3.63 \pm 0.14$ versus $-3.62 \pm 0.07$ kcal mol$^{-1}$). On the other hand, a 25-residue peptide from the B1 subunit of ribonucleotide
reductase, which has been shown to bind in a specific manner to Grx1(C14S) (21), did have a stabilizing effect upon mixed disulfide formation with Grx1(C14S) but not with Grx3(C14S/C65Y). This observation may be explained by the fact that the surface charge distribution of Grx1 differs from Grx3 as the Grx1 counterparts to the Grx3 active site residues Arg-49, Thr-51, and Asp-66 are missing (18, 20). The inability of Grx1(C14S) to make the same amount of favorable interactions with glutathione as Grx3(C14S/C65Y) provides a structural rationale for the observed 2-fold lower apparent specificity constant of Grx1, which then has the consequence that (v) not only charged but also the burial of the apolar ethylene linker and van der Waals interactions also are important for Grx3 substrate specificity. These results demonstrate that, at least to a first approximation, the interactions are independent and additive. Summing up the contributions of the individual interactions from the γ-Glu part of glutathione (the ethylene moiety, the charged carboxylate and amino group, ΔΔG2 = −1.41 ± 0.09, −0.58 ± 0.16, and −0.14 ± 0.15 kcal mol\(^{-1}\), respectively, see above) gives a value of −2.13 ± 0.24 kcal mol\(^{-1}\), which statistically indistinguishable from the difference between glutathione and Cys-Gly mixed disulfides (ΔΔG2 = −2.13 ± 0.11 kcal mol\(^{-1}\)). The sum of all individual interactions including the ones from the γ-Glu, the main chain hydrogen bonds (ΔΔG2 = −1.22 ± 0.10 kcal mol\(^{-1}\)), and the Gly part (ΔΔG2 = −0.25 ± 0.14 kcal mol\(^{-1}\)) equals −3.60 ± 0.30 kcal mol\(^{-1}\). This number compares closely to the value obtained when adding all interactions simultaneously as given by the difference between the Grx3(C14S/C65Y)-SG and the β-mercaptoethanol complexes (ΔΔG2 = −3.07 ± 0.13 kcal mol\(^{-1}\), Table 1). The choice of β-mercaptoethanol mixed disulfide as the reference for a minimal disulfide complex free from additional interactions (apart from the covalent attachment) is perhaps not ideal, with the hydroxyl group likely forming repulsive effects. Summarizing our findings, we have presented evidence that (i) the sequence nonspecific backbone-to-backbone hydrogen bonds between the ligand and the protein contributes −1.22 ± 0.10 kcal mol\(^{-1}\) to stability, (ii) the interactions provided by the unique γ-Glu component is more important to specificity than the C-terminal Gly component as the latter one formed a less stable complex (by 1.88 ± 0.11 kcal mol\(^{-1}\)) with Grx3(C14S/C65Y), (iii) it appears as if the stabilizing effect exerted by the carboxylate group of the γ-Glu is −0.58 ± 0.16 kcal mol\(^{-1}\), while the effect of the charged amino group is negligible. However, the configuration of the charged γ-Glu amino group seems to be crucial due to the prevention of repulsive effects, (iv) the aliphatic linker of the γ-Glu component also contributes to the stability by −1.41 ± 0.09 kcal mol\(^{-1}\), which then has
didate for a reference tested, as other complexes (e.g. using methylmercaptan) did not yield interpretable data.

Given the coherence of these results, if our experience with Grx3(C14S/C65Y) is not an exception, exploiting ligand-induced stability holds great promise in investigating ligand specificity of various other covalent complexes. In particular if applied to other members of the thioredoxin superfamily, which display a surprising combination of specificities and promiscuities with the hope of more clearly defining the targets for these important molecules. It will also be interesting to see whether this approach will be as useful in analyzing non-covalent complexes, a topic currently under study in our laboratory.

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Quantifying Grx3 Substrate Specificity