The structurally related glutathione S-transferase isoforms GSTA1-1 and GSTA4-4 differ greatly in their relative catalytic promiscuity. GSTA1-1 is a highly promiscuous detoxification enzyme. In contrast, GSTA4-4 exhibits selectivity for congeners of the lipid peroxidation product 4-hydroxynonenal. The contribution of protein dynamics to promiscuity has not been studied. Therefore, hydrogen/deuterium exchange mass spectrometry (H/DX) and fluorescence lifetime distribution analysis were performed with glutathione S-transferases A1-1 and A4-4. Differences in local dynamics of the C-terminal helix were evident as expected on the basis of previous studies. However, H/DX demonstrated significantly greater solvent accessibility throughout most of the GSTA1-1 sequence compared with GSTA4-4. A Phe-111/Tyr-217 aromatic-aromatic interaction in A4-4, which is not present in A1-1, was hypothesized to increase core packing. “Swap” mutants that eliminate this interaction from A4-4 or incorporates it into A1-1 yield H/DX behavior that is intermediate between the wild type templates. In addition, the single Trp-21 residue of each isoform was exploited to probe the conformational heterogeneity at the intrasubunit domain-domain interface. Excited state fluorescence lifetime distribution analysis indicates that this core residue is more conformationally heterogeneous in GSTA1-1 than in GSTA4-4, and this correlates with greater stability toward urea denaturation for GSTA4-4. The fluorescence distribution and urea sensitivity of the mutant proteins were intermediate between the wild type templates. The results suggest that the differences in protein dynamics of these homologs are global. The results suggest also the possible importance of extensive conformational plasticity to achieve high levels of functional promiscuity, possibly at the cost of stability.

The functional promiscuity of some enzymes and proteins has attracted great attention recently (1–12). In contrast to the well established interactions that lead to substrate specificity for most enzymes, the molecular basis of promiscuity is not established. Specificity clearly is achieved in nature via optimally located hydrogen bonds, electrostatic bonds, and hydrophobic interactions. However, promiscuity now appears also to be a common trait among native and mutant enzymes, and promiscuous variants may be evolutionary intermediates that further mutate to expand the functional repertoire of the genome (3, 6, 8, 11). It is widely assumed that functional promiscuity correlates with structural flexibility, which allows for recognition of diverse structures at minimal energetic cost (13–18). However, a detailed understanding of the relationship between functional promiscuity, stability, and protein structural plasticity has not yet been achieved.

Detoxification enzymes are among the most promiscuous enzymes known (19, 20). Speculatively, detoxification enzymes lie near the limits of substrate promiscuity attainable within the constraints of a defined protein fold. Among detoxification enzymes, the cytosolic glutathione S-transferases (GSTs) are a structurally well defined canonical family that exhibit varying degrees of promiscuity and thus provide a model for the evolution of promiscuity or specificity (21, 22). Cytosolic human GSTs comprise several classes, designated as A, M, P, O, or T, distinguished by sequence and substrate selectivity. Each of these GSTs catalyzes the conjugation of the tripeptide GSH with electrophilic toxins. Elegant studies by others have considered substrate specificity among GSTs (23–25) and of the role of dynamics in a few cases (26, 27). However, no explicit comparison of promiscuity and flexibility within the GST canonical fold has been described.

The A-class GSTs play critical roles in detoxification of oxidative stress products. The A1-1 and A2-2 isoforms are highly substrate-promiscuous with catalytic activity toward many structurally unrelated toxins (23–25, 28, 29) (see Fig. 1 for examples). In contrast, the A4-4 GST appears to have evolved to metabolize lipid peroxidation products with relevance in oxidative stress-related diseases (30–38) (Fig. 1). GSTA4-4 has much higher catalytic activity toward 4-hydroxynonenal and isoprostanes than GSTA1-1, but it has relatively low activity toward the “classic” GST substrate CDNB and other A1-1 substrates (23–25). A comparison of $V_{\text{max}}/K_m$ values for several typical substrates is provided in Fig. 1. GSTA1-1 and GSTA4-4 represent structural homologs with very different promiscuity profiles.

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that global flexibility beyond the immediate binding environment of ligands facilitates functional promiscuity, as observed in detoxification enzymes. We also have redesigned the dynamics of these two isoforms by rationally altering their conformational dynamics with only two mutations in each. The dynamic-based redesign yields a more promiscuous GSTA4-4.

EXPERIMENTAL PROCEDURES

Protein Expression, Mutagenesis, and Purification—The “swap” mutants were constructed using overlap extension PCR. For the GSTA1-1 V111F/R217Y mutant, the sequence of the primer containing the NdeI restriction site and the start codon was 5’-GAG-GTCATATGGCAGAAGCC-CAAGCTC-3’; for the V111F mutation, 5’-GATCCTCTTCT-GCCCCTTGTCCACCTGAGG-3’ and its reverse complement; for the R217Y mutation and the Sall restriction site, 5’-GATGAT-GGTCGACATCTAAACCTGA-AAATTCTTGATATGCTTCTTC-3’. For the GSTA4-4 F111V/R217Y mutant, the sequence of the primer containing the NdeI restriction site and the start codon was 5’-CATATG-GCAGCAAGGCCCCAGCTCACTTCCC-3’; for the F111V mutation, 5’-GGAACTGCTTATCATGCATCCTG-GCCCTTCTGTCCACCTGAGG-3’ and its reverse complement; for the W21F/R217Y mutant, the sequence of the primer containing the NdeI restriction site and the start codon was 5’-GAGGTGATATGGCAGAAGCCACCTCTTCCC-3’; for the W121F mutation, 5’-GGAACTGCTTATCATGCATCCTG-GCCCTTCTGTCCACCTGAGG-3’ and its reverse complement; for the F222W mutation, 5’-GAGGTGATATGGCAGAAGCCACCTCTTCCC-3’; for the F222W mutation, 5’-GGAACTGCTTATCATGCATCCTG-GCCCTTCTGTCCACCTGAGG-3’ and its reverse complement; for the F222W mutation, 5’-GAGGTGATATGGCAGAAGCCACCTCTTCCC-3’; for the F222W mutation, 5’-GGAACTGCTTATCATGCATCCTG-GCCCTTCTGTCCACCTGAGG-3’ and its reverse complement; for the F222W mutation, 5’-GAGGTGATATGGCAGAAGCCACCTCTTCCC-3’; for the F222W mutation.

These differences in substrate promiscuity have been largely attributed to differences in catalytic residues and local dynamics within the active site. Specifically, GSTA1-1 has highly disordered C-terminal residues (residues 212–222) that are crystallographically undefined in the apoform (39). These residues form a “loose” helix in the presence of GSH (40) but not a GSH analog (41). Perhaps most interestingly, the C-terminal helix is highly ordered in the presence of GSH product conjugates, but the detailed location depends on the structure of the ligand (39–48). In contrast, GSTA4-4 has an analogous C-terminal helix that is well defined and located along one side of the narrow active site that readily accommodates lipid peroxidation products (24). Moreover, the location of this helix is presumed to be insensitive to ligands. In short, known active site structural features contribute to the relative specificity of GSTA4-4 compared with GSTA1-1. An elegant redesign of A1-1 based on static crystal structures included replacement of 15 amino acids and resulted in a 20-fold increase in activity toward HNE (25).

As part of our interest in understanding the molecular basis of the promiscuity, we have applied multiple experimental probes of flexibility and MD simulations to compare GSTA1-1 and A4-4. Here we report striking differences in the global solvent exchange properties of GSTA1-1 and A4-4, and differences in the apparent flexibility in the protein core and at residues far from the active site. We propose Y217R mutant, the sequence of the primer containing the NdeI restriction site and the start codon was 5’-CATATG-GCAGCAAGGCCCCAGCTCACTTCCC-3’; for the F111V mutation, 5’-GGAACTGCTTATCATGCATCCTG-GCCCTTCTGTCCACCTGAGG-3’ and its reverse complement; for the Y217R mutation and the Sall restriction site, 5’-GATGAT-GGTCGACATCTAAACCTGA-AAATTCTTGATATGCTTCTTC-3’. For the GSTA4-4 F111V/R217Y mutant, the sequence of the primer containing the NdeI restriction site and the start codon was 5’-GAGGTGATATGGCAGAAGCCACCTCTTCCC-3’; for the F111V mutation, 5’-GGAACTGCTTATCATGCATCCTG-GCCCTTCTGTCCACCTGAGG-3’ and its reverse complement; for the W21F/R217Y mutant, the sequence of the primer containing the NdeI restriction site and the start codon was 5’-GAGGTGATATGGCAGAAGCCACCTCTTCCC-3’; for the W121F mutation, 5’-GGAACTGCTTATCATGCATCCTG-GCCCTTCTGTCCACCTGAGG-3’ and its reverse complement; for the F222W mutation, 5’-GAGGTGATATGGCAGAAGCCACCTCTTCCC-3’; for the F222W mutation, 5’-GGAACTGCTTATCATGCATCCTG-GCCCTTCTGTCCACCTGAGG-3’ and its reverse complement; for the F222W mutation, 5’-GAGGTGATATGGCAGAAGCCACCTCTTCCC-3’; for the F222W mutation.

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Glutathione S-Transferase Dynamics

Urea Denaturation—The denaturation experiments were performed as described by others (49, 50). GST A1-1 or A4-4 (final protein concentration of 2.3 \( \mu \text{M} \)) were unfolded in 0–8 M urea for 4 h at room temperature (25 °C) in 10 mM potassium phosphate buffer, pH 6.5. The fluorescence spectra were measured on an SLM-8100 fluorometer at 25 °C using excitation and emission path lengths of 10 and 2 mm, respectively. The band pass was 4 nm. Excitation was at 295 nm for Trp-21, and emission was monitored at 325 nm (native) and 355 nm (unfolded). The standard free energy difference between the native and denatured conformations was calculated by the equation, \( \Delta G_{\text{app}} = -RT \ln(U/F) \), where \( U \) and \( F \) represent the concentrations of protein in the denatured and native state, respectively, at a particular urea concentration. The free energy of unfolding in the absence of urea, \( \Delta G_{\text{app}}(\text{H}_2\text{O}) \), was determined by fitting the data to a two-state unfolding model for each protein except the A4-4 F111V/Y217R mutant, which was fit to a three-state model. For dimeric proteins like GSTs, where \( \Delta G_{\text{app}}(\text{H}_2\text{O}) \) is concentration-dependent, \( m = (-RT \ln[\text{protein}] + \Delta G_{\text{app}}(\text{H}_2\text{O}))/[\text{urea}]_{1/2} \), where the [urea]_{1/2} is the urea concentration at which the protein is half-denatured. The \( m \) value reflects the sensitivity of the transition region to denaturant concentration.

Hydrogen/Deuterium Exchange—Deuterium exchange experiments were performed on the free native enzymes, with well established experimental methods and data analysis (51–54). Time-dependent deuterium exchange was initiated by a 20-fold dilution of the enzyme with 10 mM Tris-DCI/D_2O buffer (pD 7) at 5 °C. The protein/D_2O solution was incubated at 5 °C for varying times (from 10 s to 40 min). At each time point, the exchange was quenched by mixing aliquots of the incubation mixture with an equal volume of ice-cold 50 mM potassium phosphate-sodium citrate buffer (pH 2.2) to drop the pH to 2.5. The exchange was quenched by mixing aliquots of the incubation mixture with an equal volume of ice-cold 50 mM potassium phosphate buffer, pH 6.5. The fluorescence spectra were measured on an SLM-8100 fluorometer at 25 °C using excitation and emission path lengths of 10 and 2 mm, respectively. The band pass was 4 nm. Excitation was at 295 nm for Trp-21, and emission was monitored at 325 nm (native) and 355 nm (unfolded). The standard free energy difference between the native and denatured conformations was calculated by the equation, \( \Delta G_{\text{app}} = -RT \ln(U/F) \), where \( U \) and \( F \) represent the concentrations of protein in the denatured and native state, respectively, at a particular urea concentration. The free energy of unfolding in the absence of urea, \( \Delta G_{\text{app}}(\text{H}_2\text{O}) \), was determined by fitting the data to a two-state unfolding model for each protein except the A4-4 F111V/Y217R mutant, which was fit to a three-state model. For dimeric proteins like GSTs, where \( \Delta G_{\text{app}}(\text{H}_2\text{O}) \) is concentration-dependent, \( m = (-RT \ln[\text{protein}] + \Delta G_{\text{app}}(\text{H}_2\text{O}))/[\text{urea}]_{1/2} \), where the [urea]_{1/2} is the urea concentration at which the protein is half-denatured. The \( m \) value reflects the sensitivity of the transition region to denaturant concentration.

Electrospray Ionization Mass Spectrometry—Electrospray mass spectra were recorded on a quadrupole/time-of-flight mass spectrometer (Micromass, Manchester, UK). Instrument settings were as follows: electrospray voltage, 3.8 kV; extraction cone, 1 V; cone voltage, 65 V; source temperature, 100 °C. Data acquisition was carried out from 400 to 1700 \( m/z \) using a scan time of 2.4 s. Samples were injected on a perfusion chromatography column (0.5-mm inner diameter \( \times 5 \) cm) packed in house with 30-\( \mu \text{m} \) POROS R2 particles (PerSeptive Biosystems, Framingham, MA). To minimize back-exchange, the deuterated pepsin-digested samples were eluted using isocratic chromatographic conditions, the mobile phase containing 20% solvent A (5% of acetonitrile/isopropanol alcohol = 2:1, 0.1% trifluoroacetic acid) and 80% solvent B (90% of acetonitrile/isopropanol alcohol = 2:1 mixture, 0.1% trifluoroacetic acid) at a flow rate of 4 \( \mu \text{l} \)/min, with a total elution time of \( \sim \)5 min. Sequencing and assignment of the nondeuterated pepsin-digested samples were performed using the method described previously (55). The deuterium incorporation was analyzed using HX-Express software (56) and adjusted for back-exchange (57, 58). Two data sets were averaged for each experiment.

Kinetic Analysis of Exchange—Because two sequentially different proteins (53% sequence identity) were compared in our studies, the percentage exchange (Equation 1) was used instead of the absolute number of deuterons exchanged into each peptide. The exchange progress curve for each peptide was fitted to the sum of first-order rate terms (Equation 2) using OriginPro 7.0 (OriginLab) and by methods described by others to account for intrinsic exchange (57).

\[
P = D/N\% \quad \text{(Eq. 1)}
\]

\[
D = N(1 - P_i \sum \exp(-k_i t)) \quad \text{(Eq. 2)}
\]

\( D \) represents the absolute number of deuterons exchanged after adjustment of back-exchange. \( N \) is the total number of exchangeable amide hydrogens in the sequence. \( P_i \) is the percentage exchange at a given rate constant \( k_i \), during the time allowed for exchange \( t \). For most of the peptides, the total exchangeable amide hydrogens can be divided into three groups, \( P_1 \) (fast), \( P_2 \) (intermediate), and \( P_3 \) (slow), with the exchange rate constants \( k_1 \) (>6 min\(^{-1}\)), \( k_2 \) (0.01–6 min\(^{-1}\)), and \( k_3 \) (<0.01 min\(^{-1}\)), respectively. Those amides that exchange before the first 10 s time point (burst phase or fast exchange) could not be fitted and are reported as percentage of \( P_1 \) only with an estimated \( k_1 \) of >6 min\(^{-1}\).

Data were also analyzed to account for possible sequence-dependent rates of exchange. Specifically, the intrinsic rates of exchange of the peptides in each protein were calculated by previously established methods (57).

Fluorescence Measurement and Analysis—Fluorescence lifetime measurements were conducted using a Spex Fluorolog Tau3 frequency domain spectrophotometer with a 450-watt xenon arc lamp (Horiba Jobin Yvon, Edison, NJ). For each experiment, 20–25 frequencies were chosen, ranging from 1 to 260 MHz. Ten replicate measurements were obtained at each frequency, with an integration time of 60 s for each measurement. The excitation monochromator was set at 295 nm, and the emission signals were collected through a Schott WG 305-nm high pass cut-off filter. The iris opening and slit widths were adjusted to maximize signal intensity while retaining appropriate modulation values. The lifetime reference standard was \( p \)-terphenyl dissolved in ethanol, with the concentration adjusted to achieve even signal strength between reference and sample. Data were collected with the protein concentration at 50 \( \mu \text{M} \), and the S-hexyl-GSH final concentration was 2 mm. All samples were in 100 mM potassium phosphate buffer, pH 6.5, except for samples with S-hexyl-GSH, where 1% ethanol was included. All experiments were performed at 12 °C. Decay data were fit using the Globals software package developed at the Laboratory for Fluorescence Dynamics at the University of Illinois at Urbana-Champaign. Fluorescence intensity (\( I_s \)) can be described with the equation,
A ribbon diagram of GSTA4-4 highlighting structural features emphasized in this work. Left, view is along the 2-fold axis of symmetry, which is perpendicular to the plane of the page. Right, view has the 2-fold axis vertical within the page. The location of the native Trp-21 and the engineered Trp-222 are shown. The C-terminal helix is colored dark blue. Each monomer contains two domains: the N-terminal one-third (magenta) and the C-terminal domain (cyan). Trp-21 (red) is located at the interdomain interface in α-helix 1 of domain I. The graphic was generated from a GSTA4-4 crystal structure (Protein Data Bank code 1GUM) using DS ViewerPro 6.0 software.

Glutathione S-Transferase Dynamics

**Catalytic Studies**—Enzymatic activity assays with CDNB and HNE were performed using previously described methods (24, 25). For the CDNB activity assay, product formation was monitored at 340 nm with 0–1.5 mM CDNB in 5% ethanol. For the HNE activity assay, substrate depletion was monitored at 224 nm in 1% ethanol. Due to low aqueous HNE solubility, high molar absorptivity, and the back-ground reaction rate, the catalytic efficiency (k_{cat}/K_m) was determined with replicates of <10 μM HNE rather than the full steady-state kinetic assay. All reactions were in 100 mM potassium phosphate buffer, pH 6.5, were initiated with 1 mM GSH, and were monitored using an Agilent 8453 diode array spectrophotometer. Kinetic constants were determined by nonlinear regression using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA). For CDNB, the reaction velocities were determined by fitting to a straight line, and for HNE, velocities were determined by fitting to a one-phase exponential depletion curve. For CDNB, the curve of velocity versus substrate concentration was fit to the standard Michaelis-Menten equation.

**Molecular Dynamics Simulations of GST**—MD simulations were performed on the x-ray crystal structure coordinates of GST A1-1 (Protein Data Bank code 1PKZ), A4-4 (Protein Data Bank code 1GUM), and mutants using the MD software package, GROMACS (Groningen Machine for Chemical Simulation). To make mutants of the GST x-ray crystal structures, the mutate feature of Deep View 3.7 was used. All protein structures were immersed in a simulated water box with 120-Å sides and ~70,000–80,000 waters, which was twice the length of the longest diagonal of the protein or ~65 Å. In preparation for the molecular dynamics simulations, the structures were energy-minimized by the method of steepest descent to remove Van der Waals contacts between overlapping waters and the amino acids of the protein. Then molecular dynamics simulations were run with Berendsen temperature and pressure coupling (also known as “bath”) at a simulated temperature of 300 K using the GROMOS 53a6 force field and periodic boundary conditions in all directions. Electrostatics of the system was measured using the particle mesh Ewald method. MD simulations were performed using IBM P655+ supercomputers at the Arctic Regional Supercomputer Center (Fairbanks, AK) or at the San Diego Supercomputer Center. During the MD simulation, the protein structures were initially position-restrained for 50 ps to allow the waters to fill in the cavities. Then molecular dynamics simulations were continued for >8 ns. Equilibration of the protein backbone of each of the structures was reached at ~2 ns.

**RESULTS**

**Structure of A-class GSTs**—The three-dimensional structure of GSTA4-4 is shown in Fig. 2. The backbone traces of GSTA1-1 and A4-4 are nearly superimposable with an average root mean square deviation of 0.91 Å (24). GSTA1-1 is shown in subsequent figures. As noted above, based on several experimental methods, the C-terminal helix of GSTA1-1 is highly disordered in the absence of ligand and becomes localized in the presence of various ligands (39–48). Moreover, the final location of the C terminus is ligand-dependent. In contrast, the
C-terminal helix of GSTA4-4 is well ordered even for the apoenzyme, and it does not undergo significant ligand-dependent reorganization. Also shown in Fig. 2 is the location of the native single tryptophan, Trp-21, in each subunit of each isof orm, and the location of the C-terminal residue, which is modeled as a Trp-222. The W21F/F222W mutant has been used previously as a probe of the C-terminal dynamics of GSTA1-1 (41, 43). Trp-21 and the engineered C-terminal Trp-222 were exploited in separate proteins as fluorescence probes in the current studies as well. It should be noted that the graphic in Fig. 2 is intended to show in a single model the locations of each Trp discussed here. However, experiments described here contained either Trp-21 or Trp-222 but not both.

**H/D Exchange Mass Spectrometry**—To compare the dynamics of GSTA1-1 and GSTA4-4, H/DX mass spectrometry was performed with each enzyme in the absence of ligands. In H/DX, the adjusted deuterium content of the individual peptides obtained upon proteolysis reflects their relative solvent accessibility in the folded structure, prior to proteolysis (51–54). For GSTA1-1, the peptides recovered and analyzed accounted for 85% of the entire sequence, and for A4-4, 75% of the peptides were recovered. The results will be discussed with reference to two time regimes: exchange that occurs faster than 10 s, or the “fast exchange” time regime, and exchange that occurs after 10 s of exposure to deuterium, which is the time regime typically analyzed in H/DX experiments. Discussion of the second time regime is deferred until site-directed mutants are examined.

Results for the first time regime of wild type A1-1 and A4-4 are summarized in Fig. 3, and they reflect the exchange at the lower limit of time resolution in the experiment, 10 s. No detailed rate constants for exchange can be extracted for this regime. This regime is referred to as the “burst” or the fast exchange regime. This distinction between “fast exchange” and subsequent time regimes has been used in H/D exchange of other proteins, including GSTs (59, 60). In this work, the rapid exchange data show that most regions of GSTA1-1 had a higher percentage of deuterium uptake than the corresponding regions of A4-4, indicating greater solvent exposure in general for A1-1. However, for some peptides, the fast exchange was similar. For example, the comparable deuterium levels of the dimer interface region (residues 73–94) and the α-helix-5b (residues 137–147) between the two proteins demonstrate similar solvent accessibility at these loci for both enzymes. One interesting exception to the general trend is at the α4–α5 helix-turn-helix (residues 106–123 or 106–124, shown below in Fig. 5). This corresponds to what we have termed the “tower region” near the subunit-subunit interface. For this peptide, GSTA4-4 exhibits moderately greater fast exchange. The peptide immediately following, residues 124–136, yields much greater fast exchange for the A1-1 protein. Fig. 3B depicts both proteins color-coded according the extent of exchange within 10 s. In Fig. 3B, the majority of the GSTA1-1 structure is orange or red, corresponding to >35% exchange within 10 s. In contrast, most of the GSTA4-4 structure is blue.

**FIGURE 3.** Summary of fast exchange H/DX data, superimposed on the structures of GSTA1-1 and A4-4. A, the percentage exchange during the fast exchange period (10 s) is shown for GSTA1-1 (black) and A4-4 (red) along the linear sequence of each protein. B, the percentage exchange is superimposed on the three-dimensional models of each protein, with the color code shown in the key. GSTA1-1 exhibits much greater exchange within 10 s than A4-4.
or green, corresponding to <25% exchange, with the exception of a few isolated red peptides.

As already noted, the second time regime includes events after the 10-s burst phase. Results for this time regime will be discussed in detail after describing mutant proteins used to probe these differences.

*Swap* Mutants of A1-1 and A4-4—The entire C-terminal region, residues 185–222, including the C-terminal helix and the hinge leading to it (residues 185–208), are much more mobile in the A1-1 isoform in at least one of the time regimes. In order to explore the extent to which the C-terminal dynamics are coupled to other parts of the protein and to its function, we engineered the C-terminal dynamics by mutagenesis on the basis of the following strategy.

The proteins are 53% identical in amino acid sequence. Sequence alignment reveals that the differences are distributed throughout, but there is a region with a very high density of sequence differences in the α4–α5 tower region, as already noted by others (24). Therefore, we focused on this region. Comparison of the α4–α5 helix-turn-helix “tower” region of A1-1 and A4-4 revealed a possible structural basis for the different dynamics. A4-4 includes an intimate edge-to-face aromatic-aromatic interaction between Phe-111 in the tower region and Tyr-217 in the C-terminal helix as well as other hydrophobic interactions (Fig. 4). A1-1 has no comparable interaction. Instead, A1-1 has Arg-217 and Val-211. Hypothetically, the aromatic interactions in A4-4 result in tighter packing of the C terminus with the “tower” while simultaneously exposing part of the tower for fast exchange. Notably, the tower/C-terminal interaction in A4-4 also contributes to the orientation of the C terminus, so both elements are preorganized for HNE binding. This region of the protein has already been recognized as a key determinant of substrate selectivity, but no explicit consideration of dynamics was included (25).

Based on this, we created a “swap” double mutant of each isoform. The mutant F111V/Y217R was made from the A4-4 templates, and V111F/R217Y was constructed for A1-1. Hypothetically, these mutants would destroy or incorporate the tower region-C-terminal interactions for the A4-4 and A1-1 isoforms, respectively.

Each purified mutant was characterized by H/DX. The comparison of the data for individual peptides with corresponding fits to Equation 2 is shown in Fig. 5. Note that differences in the fast exchange time regime are evident at the 10 s time point (first time point in each plot), which displaces the curve to higher values when fast exchange is greater. As already noted above, the curve either starts out higher for A1-1 (black) compared with A4-4 (red), or it ends up higher for most peptides. A notable exception is the 106–123 peptide that includes the Phe-111 in A4-4. For the wild type proteins, this region shows slower H/DX in the fast time regime (Fig. 5). In general, the results for several peptides are shown in Fig. 5, and the results for the remaining peptides are in the supplemental materials (Fig. S1). Interestingly, the effects of this engineered aromatic-aromatic interaction are transmitted throughout other parts of the protein; much of the sequence of the A1-1 swap mutant exhibits slower H/DX in the fast time regime (Fig. 5). In general, the results in Fig. 5 for peptides throughout the protein indicate that the mutations either have no effect or increase exchange or decrease exchange for the A4-4 and A1-1 mutant, respectively.

One peptide not recovered for the A1-1 V111F/R217Y swap mutant is the strand leading to the C terminus (residues 184–215; Fig. 5, bottom row, center). Apparently, this peptide is degraded extremely efficiently, and no peptides corresponding...
to this region were observed. This is counterintuitive, in as much as the less dynamic strand would be expected to be more resistant to proteolysis. However, MD simulations suggest a possible explanation for this. The 9-ns MD simulation indicates that, as a result of the engineered interaction between the C-terminal helix and the tower region, the hinge region (residues 184–212) adopts a new conformation that makes it fully extended into solution and more highly accessible. Thus, the C-terminal helix is less dynamic, but the hinge region leading to it is more solvated. We speculate that it is so rapidly proteolyzed as to not be observed (supplemental materials, Fig. S2). A full analysis of the MD simulations will be reported elsewhere.

The fits of the data in Fig. 5 are summarized in Table S1 (supplemental materials), which compares wild type A1-1 and the corresponding swap mutant, or Table S2 (supplemental materials), which includes the recovered kinetic parameters for wild type A4-4 and its corresponding swap mutant. These fits of the data represent a model in which the exchange of deuterium into each peptide is described by a sum of exponential rate processes (Equation 2).

In principle, the differences in sequences of the proteins could contribute to differences in exchange kinetics. However, we have examined the intrinsic sequence-dependent H/D exchange rates of the peptic peptides for both proteins using the HXPep program (courtesy of Z. Zhang) (57). The differences in sequence-dependent exchange rates for comparable peptides were very modest. The difference in the recovered parameters, with and without this correction, were negligible for all peptides. In short, the differences observed for GSTA1-1 and A4-4 are not due to the differences in amino acid sequence but rather reflect the dynamic properties of the folded structures.

The proteins were also analyzed with molecular dynamics simulations. A detailed analysis of all four proteins will be presented elsewhere, but several key points are noteworthy as support for the mass spectrometry results: 1) the average root mean square deviation for all times between 3 and 9 ns of the main
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FIGURE 6. Fluorescence lifetime distribution analysis. Top left, the phase angle (circles) and modulation ratio (squares) are shown for the GSTA1-1 (black) and A4-4 (gray) at multiple modulation frequencies. The solid lines are the fits to the model for a Gaussian distribution of lifetimes and a discrete lifetime component with only the distribution component shown, and the lower panel shows the weighted residuals. Top right, the recovered distributions of the long lifetime component for GSTA1-1 (black) and A4-4 (gray). The recovered parameters are summarized in Table 1. Trp-21 of GSTA1-1 is much more heterogeneous than Trp-21 of A4-4, based on the width of the distribution. Lower left, phase angles (circles) and modulation ratios (squares) for the A1-1 swap mutant (black) and the A4-4 swap mutant (gray) with weighted residuals below. The solid lines are fits of the data to a model with a discrete component and a distributed component. Lower right, the recovered distributions of the lifetimes for A1-1 swap mutant (black) and A4-4 swap mutant (gray). The extent of conformational heterogeneity for both swap mutants is intermediate between the wild type proteins. The recovered parameters are shown in Table 1.

GSTA1-1 has been exploited as a spectroscopic probe for folding studies and ligand-dependent conformational changes (44, 49, 50), but it has not been used to measure conformational heterogeneity per se, and no fluorescence studies with GSTA4-4 have been reported.

Each protein was studied by phase modulation fluorescence spectroscopy, using 22–23 modulation frequencies. The phase angles and modulation ratios are compared directly in Fig. 6 for the wild type proteins. There is a clear shift in the frequency response of GSTA4-4 to higher frequencies compared with GSTA1-1. The data were fit to several distribution models for excited state decay, including unimodal and bimodal Gaussian distributions and Gaussian distributions with discrete components. The analysis of fluorescence decay parameters as distributions has been described in detail (61–64). In general, except for very short lived decay components (<1 ns), the width of the Gaussian reflects the width of the potential energy well for conformational substates. In other words, the conformational heterogeneity of the ensemble at the time of excitation is related to the width of the distribution if multiple conformational substates are not equilibrating faster than the excited state lifetime (ns).

GSTA1-1 and lowest for GSTA4-4, with the mutants exhibiting intermediate root mean square deviations after 2 ns for the A4-4 mutant and after 6 ns for the A1-1 mutant (supplemental materials, Fig. S3); 2) the highest regions of root mean square fluctuation for each protein (not shown) are the “tower region” (residues 106–123), the C-terminal hinge (residues 185–207), the C-terminal helix (residues 209–222), and the N terminus (residues 1–12), as observed by H/DX; 3) for the A1-1 V111F/R217Y mutant, the engineered Phe and Tyr side chains form, and maintain, an aromatic interaction nearly identical to that observed in A4-4, at all times after 3 ns (supplemental materials, Fig. S4). Together, these results are in good agreement with the H/DX, and they indicate that the aromatic interactions in the A1-1 mutant may form a stable interaction.

Time-resolved Fluorescence Spectroscopy of Trp-21 in the Domain-Domain Interface—A region of the proteins that was not recovered for any of the proteins in the H/DX experiments includes residues 16–31 of the α-helix 1 and 161–165 of the α-helix 6, which together form the interface of the two domains within each monomer. In order to obtain some information about the solution dynamics of this region, we exploited the single Trp residue in each protein, Trp-21. Trp-21 lies at the intrasubunit domain-domain interface (Fig. 2). Trp-21 of width of the distribution if multiple conformational substates are not equilibrating faster than the excited state lifetime (ns). For both of the specific cases here, the data fit best to a Gaussian distribution with a discrete short lifetime component, as is often observed for Trp residues in proteins (65). However, the dominant decay component of Trp-21 of GSTA1-1 is markedly more heterogeneous than Trp-21 in GSTA4-4. The decay parameters recovered from the fits are summarized in Table 1. A simple measure of heterogeneity of such an ensemble is the width at half-height normalized to the height of the Gaussian peak. That ratio, reported as the width in Table 1, is 1.27 for the GSTA1-1 and 0.27 for GSTA4-4. The results suggest a significant increase in the conformational heterogeneity of Trp-21 in GSTA1-1 compared with GSTA4-4. Apparently, the greater dynamics for GSTA1-1 observed by H/DX includes the local region surrounding Trp-21.

The swap, or “tower,” mutants were also characterized by fluorescence distribution analysis. The phase modulation data for these mutants are shown also in Fig. 6. The recovered widths of the distribution for the A1-1 V111F/R217Y mutant was 0.457, and for the A4-4 F111I/Y217R mutant, it was 0.346 (Table 1). Thus, the width of each mutant distribution lies precisely between the two wild type proteins, suggesting that the Phe-111/Tyr-217 interaction of the A4-4 protein contributes to
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TABLE 1
Excited state fluorescence decay parameters
Upper limits for the S.E. values are ±0.003 for the SAS with wild type (WT) proteins and ±0.01 for W21F/F222W; for the center parameters, the upper limit of the S.E. is ±0.025 for the wild type proteins and ±0.176 for W21F/F222W.

| Parameter          | GST A1-1 WT | GST A4-4 WT | GSTA1-1 V111F/R217Y | GSTA4-4 F111V/Y217R | GSTA1-1 W21F/F222W | GSTA1-1 W21F/F222W + S-hexyl-GSH |
|--------------------|-------------|-------------|---------------------|---------------------|-------------------|----------------------------------|
| SAS1               | 0.881       | 0.593       | 0.694               | 0.700               | 0.69              | 0.81                             |
| Center" (ns)       | 3.621       | 4.032       | 4.431               | 4.00                | 6.49              | 10.09                            |
| Width" (ns)        | 1.271       | 0.267       | 0.457               | 0.346               | 2.016             | 0.001                            |
| SAS2"             | 0.119       | 0.407       | 0.306               | 0.300               | 0.31              | 0.19                             |
| Lifetime" (ns)     | 0.539       | 0.410       | 1.045               | 0.471               | 0.945             | 1.662                            |
| χ²                 | 1.889       | 0.272       | 0.733               | 3.407               | 1.679             | 7.769                            |

* The fractional intensity of the distributed component of the fluorescent lifetime.
* The median value of the distribution.
* At half the maximal height.
* The fractional intensity of the discrete component.
* For the discrete component.

the C-terminal/tower packing and thus to the dynamics of the former. Collectively, the Trp-21 analyses suggest that the core of the A1-1 is more heterogeneous than A4-4 and that the Phe-111/Tyr-217 interaction contributes to global dynamics in addition to the C-terminal status.

To validate this conclusion based on distribution analysis, the engineered W21F/F222W mutant of GSTA1-1 was used as a positive control, wherein GSH product conjugates, such as S-hexyl-GSH, are known to cause the dynamic C-terminal helix to “freeze.” The fluorescence lifetime distribution analysis was performed with apo-W21F/F222W and in the presence of 20 μM S-hexyl-GSH. The phase modulation data are shown in the supplemental materials (Fig. S5) along with the fits of the data to a distribution model. The recovered parameters are also summarized in Table 1. The engineered Trp-222 exhibits an extremely broad Gaussian distribution centered at 6.3 ns and a minor short lived component with a very narrow distribution. In fact, as expected, Trp-222 is even more heterogeneous than the Trp-21. Upon the addition of S-hexyl-GSH this broad distribution is eliminated in favor of a longer lifetime component that is nearly homogeneous. In fact, fits of these data to a sum of discrete exponential terms yield a fit that is nearly as good, with no significant change in the average lifetime values. In effect, the analysis of the F222W mutant demonstrates the behavior expected from many previous studies and validates the use of distributions as a measure of conformational heterogeneity with GSTA1-1.

Urea Denaturation Detected by Tryptophan Fluorescence—Hypothetically, a more conformationally heterogeneous protein would be more sensitive to denaturants. To test this possibility, equilibrium unfolding experiments using intrinsic Trp fluorescence were performed as an additional probe of the conformational heterogeneity of the local Trp-21 region. Trp-21 has been used previously for this purpose, and detailed studies of the equilibrium unfolding of GSTA1-1 are available (50, 66). No equilibrium unfolding studies have been reported for GSTA4-4. The fraction of “unfolded” A1-1, A4-4, and each of the swap mutants at different concentrations of urea was measured and plotted (Fig. 7). Each of the proteins starts to unfold in the presence of ~3 M urea, but the unfolding curve is shifted to higher urea concentrations for GST A4-4 compared with A1-1 ([urea]½ = 4.8 M versus 5.6 M). The data were fit to a two-state unfolding model for each of the proteins except the A4-4 swap mutant (Fig. 7), and the recovered parameters are summarized in Table 2. The A4-4 swap mutant exhibited three-state unfolding with the parameters recovered in Table 2. The results for A1-1 are in excellent agreement with the previous findings (50, 66). In addition to the greater stability of A4-4 toward denaturant relative to A1-1, it is striking that the swap mutants are each intermediate between the two template proteins. Also, it is obvious that the A4-4 swap mutant is less stable than the A4-4 wild type, and it populates to a measurable extent an unfolding intermediate. These studies are not intended to determine the global unfolding mechanism of the protein but rather to probe the local structure of Trp-21. Based on the extrapolated ΔG for

FIGURE 7. Equilibrium urea denaturation of GSTA1-1, GSTA4-4, and swap mutants. Trp-21 fluorescence was used to probe the local denaturation of Trp-21. GSTA1-1 (black filled squares, black line) is more sensitive to urea denaturation than GSTA4-4 (gray filled circles, gray line). The A1-1 swap mutant (open squares, dashed line) is slightly more stable to urea than A1-1, whereas the A4-4 mutant (open circles, dashed line) is markedly more sensitive.

TABLE 2
Urea denaturation of A1-1, A4-4, and swap mutants

| Protein           | [Urea]½ | ΔG_app(H2O) | m       | χ²       |
|-------------------|---------|-------------|---------|----------|
| A1-1 wild type    | 4.8     | 9.8         | 1.9     | 1.3 × 10⁻³ |
| A4-4 wild type    | 5.5     | 7.9         | 1.3     | 6.5 × 10⁻⁴ |
| A1-1 V111F/R217Y  | 5.0     | 12.7        | 2.4     | 1.2 × 10⁻³ |
| A4-4 F111V/Y217R  | 2.9     | 8.9         | 1.2     | 2.8 × 10⁻⁴ |
local unfolding at 0 M urea, there is little difference in the overall stability of the local fold, although this region in GSTA1-1 appears very slightly more stable than GSTA4-4. Possibly, this reflects a more favorable entropic contribution for A1-1 versus A4-4 due to greater side chain, backbone, and solvent mobility. Furthermore the m value for A1-1 is slightly larger, suggesting a larger change in solvent-accessible surface area for A1-1. This is curious, in as much as the tighter structure is GSTA4-4, based on the H/DX and fluorescence lifetime distribution, and would yield a larger m value if this two-state model were rigorously correct. However, it must be emphasized that these parameters are based on a two-state model that assumes no residual structure at 8 M urea, which may not be the case. In fact, the urea denaturation curve for GSTA4-4 suggests that complete exposure of Trp-21 may not have been achieved. The important result here is that the local structure around Trp-21 in GSTA1-1 is more sensitive to denaturant, as expected for a less tightly packed protein, and the stability is controlled in part by the C-terminal/tower packing, as indicated by the mutants.

Catalytic Studies—In order to examine the catalytic function of the mutants, their $k_{cat}/K_m$ values were determined for two substrates: CDNB, a general marker for GST activity, and HNE, the substrate for which A4-4 shows high selectivity. The results are summarized in Table 3. Interestingly, the A4-4 mutant is more promiscuous than the wild type A4-4, to the extent that its selectivity for HNE is markedly reduced, with a ~10-fold decrease in activity for HNE concomitant with a ~10-fold increase for the promiscuous substrate CDNB. Also, the A1-1 mutant exhibits a modest increase in activity toward HNE. Because this mutant does not include the catalytically important residue Tyr-212 that stabilizes the HNE enolate, a great increase in catalytic activity was not expected. Presumably, the modest increase reflects binding contributions. Thus, to the extent that it is possible to measure "promiscuity," these results suggest that protein dynamics in several regions of the protein affect the relative promiscuity of these isoforms.

**DISCUSSION**

The GST isoforms A1-1 and A4-4 represent benchmarks for high catalytic promiscuity or selectivity within a single canonical protein fold. These studies aimed to determine whether dynamics of the two proteins, other than the C-terminal dynamics, could contribute to these differences. The parameters used to assess flexibility were H/D exchange rates, MD simulation, fluorescence lifetime distribution, and sensitivity to urea denaturation.

A striking result of these studies is that the flexibility of wild type GSTA1-1 is greater than GSTA4-4 throughout many segments of the protein; the differences in flexibility are not local-

**TABLE 3**

| Protein                  | $k_{cat}/K_m$ (CDNB) | $k_{cat}/K_m$ (HNE) |
|--------------------------|----------------------|---------------------|
| A1-1 wild type           | 130 ± 7              | 13 ± 4              |
| A1-1 V111F/R217Y         | 56 ± 21              | 21 ± 3              |
| A4-4 wild type           | 45 ± 12              | 1400 ± 300          |
| A4-4 F111V/Y217R         | 60 ± 30              | 110 ± 50            |

The catalytic studies reveal a gap in our current knowledge of promiscuous enzymology. Without any available method for quantifying promiscuity, the comparison of catalytic data for a small number of substrates cannot reveal whether there is any causal role for global conformational heterogeneity in "promiscuity." Despite the lack of any method to quantify "promiscuity," the results do suggest that the C-terminal dynamics of A1-1 may be facilitated by flexibility in other regions. Although this does not prove that global flexibility is a requirement for promiscuity, based on our results, it is likely that extreme functional promiscuity is most easily achieved by distributing flexibility throughout an entire protein scaffold rather than limiting it to the active site.

These results also have implications for several fields that have recently appreciated the importance of functional promiscuity. For example, it has been appreciated that single mutations in substrate-specific enzymes can lead to functional promiscuity without much loss of native function. After gene duplication, the "promiscuous" mutant can then serve as a template for subsequent mutations that yield new enzymatic activities. In essence, promiscuous templates may be important evolutionary intermediates in the functional expansion of the existing protein pool. Protein engineers have embraced this model, because it suggests the possibility that promiscuous templates would be ideal starting points for in vitro evolution strategies to obtain novel catalysts (9, 11, 12). Our results support this to the extent that the highly promiscuous A1-1 was easily made more specific for HNE by only two mutations that targeted regions of the protein for which conformational heterogeneity is high and coupled to other parts of the structure. Furthermore, this increase in selectivity for HNE was accomplished with no significant detrimental effects on stability toward denaturants. However, the analogous double mutant of highly specific A4-4 does have decreased substrate selectivity but at the cost of a large reduction in activity toward its cognate substrate HNE and also with a significant decrease in stability. The results emphasize that useful promiscuous evolutionary intermediates that include increased flexibility compared with...
the native protein must balance functional promiscuity with decreased stability.

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