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Equilibrium Unfolding of Class π Glutathione S-transferase

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SUMMARY: The equilibrium unfolding transition of class π glutathione S-transferase, a homodimeric protein, from porcine lung was monitored by spectroscopic methods (fluorescence emission and ultraviolet absorption), and by enzyme activity changes. Solvent(guanidine hydrochloride and urea)-induced denaturation is well described by a two-state model involving significant populations of only the folded dimer and unfolded monomer. Neither a folded, active monomeric form nor stable unfolding intermediates were detected. The conformational stability, $\Delta G_u(H_2O)$, of the native dimer was estimated to be about 25.3 ± 2 kcal/mol at 20°C and pH6.5.

Glutathione S-transferases (EC 2.5.1.18) are a family of multifunctional proteins which play a crucial role in protecting cells against the harmful effects of cytotoxic and genotoxic chemicals [1]. The mammalian cytosolic enzymes, which exist as multiple homodimeric and heterodimeric forms, are represented by three species-independent classes, namely α, μ and π [2]. Class π isozymes are of considerable interest because of their potential as early markers for cancer and their association with the development of antineoplastic drug resistance (for a review see ref 3). The class π isozyme from porcine lung is a homodimer (M_r ~48000) with no disulfide bridges [4], and our 2.3Å-resolution X-ray structure shows that each polypeptide (207 amino acid residues) is folded into two dissimilar structural domains [5]. Residues 1-74 form domain I which contains a central four-stranded β-sheet flanked on one side by two α-helices and on the other side by an irregular helix structure. The G-site for

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glutathione is situated in this domain. Domain II, residues 81-207, contains five α-helices. In this paper we describe the fluorescence properties of the porcine isozyme and, through equilibrium denaturation experiments, examine structural behaviour during dissociation and unfolding, the thermodynamic stability of the protein, and the existence of stable intermediate states.

EXPERIMENTAL METHODS

Class π glutathione S-transferase was isolated from porcine lung and purified as described by Dirr et al. [4]. Protein concentration was determined by the Bradford method [6] using bovine serum albumin as standard. All protein concentrations reported are for the dimer unless otherwise specified. Glutathione S-transferase was dissolved in 20mM Mes-NaOH, 100mM NaCl, 1mM EDTA, 0.02% NaN_3, pH6.5. Guanidine hydrochloride and N-acetyl-L-tryptophanamide were obtained from Merck and urea (Aristar) and acrylamide were from BDH.

Denaturation experiments were performed at 20°C in 20mM Mes-NaOH, 100mM NaCl, 1mM EDTA, 5mM dithiotreitol, 0.02% NaN_3, pH6.5. Glutathione S-transferase was denatured in various concentrations of guanidine hydrochloride (0-6M) and urea (0-7.75M), and the solutions were allowed to stand for at least 1h for guanidine hydrochloride and 24h for urea, times that were determined to be sufficient for equilibrium to be attained. The unfolding process was monitored by steady-state fluorescence, second-derivative spectroscopy, and by measuring the enzyme's activity. Equilibrium measurements were made after all changes in spectroscopic properties and enzyme activity were complete.

Steady-state fluorescence spectra were recorded over 300-400nm with a Perkin-Elmer MPF 3-L spectrofluorimeter operating in the ratio mode. Tryptophan fluorescence was induced by excitation at 295nm. Quantum yields were determined according to Parker and Rees [7], assuming a quantum yield of 0.13 for N-acetyl-L-tryptophanamide [8]. Fluorescence quenching titrations with acrylamide were performed as described elsewhere [9,10], and a correction factor was applied for the attenuation of excitation energy by added acrylamide [11], which in these experiments has a molar extinction coefficient of $0.24 M^{-1}.cm^{-1}$ at 295nm.

Ultraviolet-absorption spectra over 320-240nm as well as the second-derivative analyses thereof, were carried out on a Varian DMS 200 UV/VIS spectrophotometer. The number of tyrosine residues in glutathione S-transferase which are exposed to solvent was estimated from the second-derivative spectroscopic data as described by Ragone et al. [12].

The enzyme activity of glutathione S-transferase was measured spectrophotometrically at 340nm and at 20°C in 0.1M potassium phosphate, 0.02% NaN_3, pH6.5, containing 1mM 1-chloro-2,4-dinitrobenzene, 3% ethanol, and 1mM reduced glutathione [13]. To prevent the reactivation of unfolded enzyme from occurring during the assay, trypsin was added to the standard assay mixture at a concentration of 62μg/ml. This had no effect on the activity of the native enzyme. Reactivation studies were performed by preincubating the unfolded enzyme at various times in assay buffer without trypsin before measuring activity. Residual concentrations of denaturant (less than 20mM) did not interfere with the assay.

Experimental data were compared by normalizing each unfolding transition curve to the fraction of protein present in the unfolded conformation, f_u [14]:

$$f_u = (Y_f - Y_{obs})/(Y_f - Y_u) \quad (1)$$

where Y_{obs} is the observed ratio of tryptophan fluorescence intensity at 348nm and 335nm, the maximum wavelength of tryptophan emission, the degree of tyrosine exposure to solvent, or the enzymatic activity at a given denaturant concentration; Y_f and Y_u are those obtained with folded and unfolded protein, respectively. Estimates of Y_f and Y_u in the transition region were obtained by linear extrapolation from the pre- and post-transition base lines, respectively [14].

Size-exclusion chromatography was performed at 20°C in a 8mm x 40mm Sephadex G-75 superfine gel filtration column. Glutathione S-transferase samples (0.4 ml containing 0.16-0.24 protein) were applied onto the column and subjected to isocratic elution at a flow rate of 11ml/h with 20mM Mes-NaOH, 100mM NaCl, 1mM EDTA, 0.02% NaN_3, pH6.5, with or without guanidine hydrochloride.

Solvent-accessible surfaces of the amino acids in glutathione S-transferase were calculated from the X-ray coordinates for our molecular model [5], by using the DSSP programme of Kabsch and Sander [15].

RESULTS AND DISCUSSION

Fluorescence properties of class π glutathione S-transferase: Class π glutathione S-transferase from porcine lung contains two tryptophan residues (Trp-28 and Trp-38) and ten tyrosine residues per subunit [4]. In its native state, the enzyme exhibits an emission spectrum with a maximum at 335nm, a spectral band width of 53nm, and a quantum yield of 0.021. These features are characteristic of tryptophan residues partially buried in the protein matrix [16]. Quenching studies with acrylamide support this, and indicate that approximately 60% of the tryptophan residues are solvent-accessible. The Stern-Volmer plot, with an effective quenching constant of $1.1M^{-1}$, is illustrated in Fig.1 (curve a). The absence of any curvature in this plot for the native protein suggests two classes of tryptophan residues differing slightly in solvent accessibility [10]. Accessible surface areas of 42-57Å² for Trp-28 and 39Å² for Trp-38, computed from our X-ray data of glutathione S-transferase complexed with glutathione sulfonate [5], would seem to support this. Trp-38 is involved in sequestering the inhibitor at the active site, however, and this is likely to bring about a change in the accessibility of the residue. When glutathione S-transferase is denatured in guanidine hydrochloride and urea, its emission maximum is significantly red-shifted to 348nm, which is similar to that of N-acetyl-L-tryptophanamide in water (351nm).

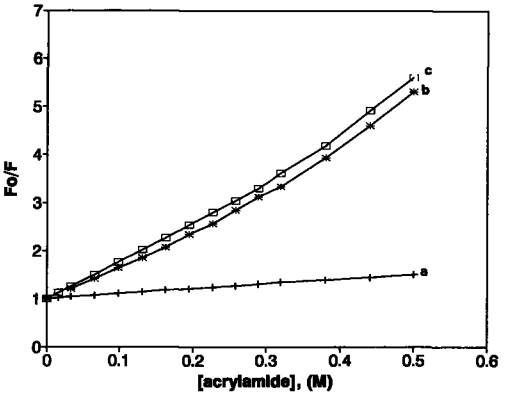

FIGURE 1. Stern-Volmer plots for the quenching of the tryptophan fluorescence of class π glutathione S-transferase in the absence (+), and presence of 2M guanidine hydrochloride (*) and 6M urea (□). F_o and F are the fluorescence intensities in the absence and presence of acrylamide, respectively.

This shift was accompanied by a broadening in the spectral band width to 58nm together with a 4.3-fold increase in the quantum yield to 0.091. Therefore, both tryptophan residues become fully exposed to solvent upon unfolding, which is substantiated by the finding that all (100%) of the tryptophan fluorescence becomes available to quenching by acrylamide under denaturing conditions. The Stern-Volmer plots for unfolded glutathione S-transferase with the typical upward curvature observed for denatured proteins and free tryptophan [9,10], are illustrated in Fig. 1 (curves b and c). Collisional (dynamic) quenching constants obtained from these plots were $5.3M^{-1}$ and $5.9M^{-1}$ for guanidine hydrochloride and urea, respectively, with a static quenching constant of $0.8M^{-1}$.

Two-state unfolding of glutathione S-transferase: The denaturation of glutathione S-transferase induced by guanidine hydrochloride and urea, was followed under equilibrium conditions by using the fluorescence properties (emission wavelength and intensity) of Trp-28 and Trp-38 located in domain I, the solvent-exposure of the ten tyrosine residues distributed throughout the protein molecule (three in domain I, one in the connector region and six in domain II), and the catalytic activity. Figures 2A and 2B illustrate the conformational transition of the enzyme from the native to the unfolded state. The unfolding reaction is highly cooperative, and the individual curves obtained with the different probes are coincident within experimental error and all have midpoints near 1.4M for guanidine hydrochloride and 4.4M for

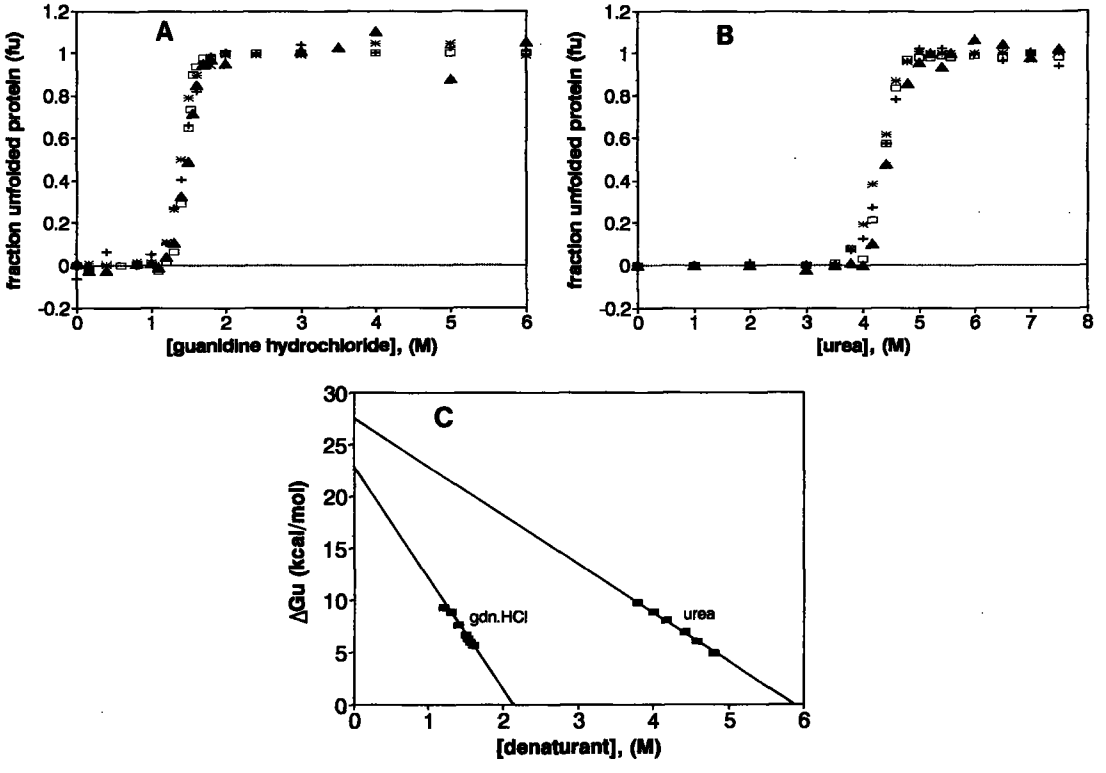

FIGURE 2. Guanidine hydrochloride(A) and urea(B)-induced unfolding transitions of class π glutathione S-transferase in 20mM Mes-NaOH, 100mM NaCl, 1mM EDTA, 5mM dithiotreitol, pH6.5, at 20°C. The protein concentration was 4.5μM. Fractions of protein in the unfolded state, f_u, were determined by the ratio of fluorescence intensity at 348nm and 335nm (+), the maximum emission wavelength (*), the extent of tyrosine exposure (▲), and catalytic activity (□). C; Free energy of unfolding, G_u, of glutathione S-transferase as a function of guanidine hydrochloride and urea concentration.

urea. Although stable intermediates of unfolding appear not to be highly populated at equilibrium, as suggested by the monophasic character of the transition, it was possible that the dimer dissociates before the transition region and that only the equilibrium between folded monomer and unfolded monomer was observed. This possibility is ruled out by size-exclusion chromatography experiments (Fig.3), which show that catalytic activity is associated only with the native dimer and that variations in activity, therefore, directly measure the disappearance(formation) of this species. Inactivation is also shown to be reversible since the unfolded glutathione S-transferase can be reactivated by dilution of the denaturant (Fig.3, curve c).

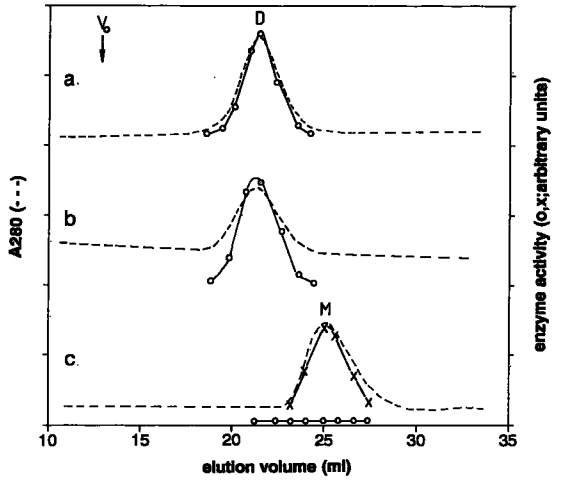

FIGURE 3. Size-exclusion chromatography in Sephadex G-75 superfine of glutathione S-transferase in the absence (a), and presence of 1.2M (b) and 2M (c) guanidine hydrochloride at pH6.5 and 20°C. Protein was determined at 280nm (---). Enzyme activity was measured with (o) and, for reactivation measurements, without (x) trypsin in the standard assay buffer. D is the native dimer, and M the unfolded monomer. V_o indicates the void volume of the column.

Thus, the equilibrium transition between folded(F) and unfolded(U) glutathione S-transferase can be described, to a first approximation, by a two-state mechanism [17]:

$$F \underset{}{\overset{K_u}{\rightleftharpoons}} 2U$$

The equilibrium constant, K_u, and the free energy change of unfolding, ΔG_u, were calculated for the denaturant concentrations within the transition region according to the relationship:

$$K_u = [U]^2/[F] = 2P_t[f_u^2/(1-f_u)] = \exp(-\Delta G_u/RT)$$

with P_t being the total protein concentration and f_u the fraction of unfolded protein. To estimate the conformational stability of glutathione S-transferase in the absence of denaturant, $\Delta G_u(H_2O)$, it was assumed that ΔG_u is linearly dependent upon denaturant concentration [14]:

$$\Delta G_u = \Delta G_u(H_2O) - m[\text{denaturant}]$$

where m reflects the responsiveness of the enzyme to solvent-induced unfolding. Figure 2c shows unfolding free energies, ΔG_u, as a function of denaturant concentration, from which $\Delta G_u(H_2O)$ values of about 23.3kcal/mol (m = 11.1kcal/mol/M) for guanidine hydrochloride and 27.2kcal/mol (m = 4.6kcal/mol/M) for urea could be estimated.

All cytosolic glutathione S-transferases exist only as dimers [1]. While the crystal structure of the porcine class π isozyme shows two discrete domains [5], the dissociation and unfolding reactions of the protein are closely coupled, resembling a concerted two-state process with only the native dimer and unfolded monomers present at substantial levels at equilibrium. The present findings suggest that the soluble glutathione S-transferases need to dimerize in order to maintain a stable and functional folded structure.

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