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Effect of glutathione, glutathione sulphonate and S-hexylglutathione on the conformational stability of class pi glutathione S-transferase

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Abstract The glutathione S-transferases (GST) are a supergene family of phase II detoxification enzymes which catalyse the S-conjugation between glutathione and an electrophilic substrate. The active site can be divided into two adjacent functional regions, a highly specific G-site for binding the physiological substrate glutathione and a nonspecific H-site for binding nonpolar electrophilic substrates. Equilibrium and kinetic unfolding experiments employing tryptophan fluorescence and enzyme activity measurements were preformed to study the effect of ligand binding to the G-site on the unfolding and stability of the porcine class pi glutathione S-transferase against urea. The presence of glutathione caused a shift in the equilibrium-unfolding curves towards lower urea concentrations and enhanced the first-order rate constant for unfolding suggesting a destabilisation of the pGSTP1-1 structure against urea. The presence of either glutathione sulphonate or S-hexylglutathione, however, produced the opposite effect in that their binding to the G-site appeared to exert a stabilising effect against urea. The binding of these glutathione analogues also reduced significantly the degree of cooperativity of unfolding indicating a possible change in the protein's unfolding pathway.

Key words: Conformational stability; Unfolding; Denaturation; Glutathione S-transferase; Ligand binding; Glutathione

1. Introduction

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a family of intracellular proteins that participate in the phase II metabolism of electrophiles and in ligand transport [1,2]. GSTs realize their catalytic function by catalysing the nucleophilic addition of the thiol group of glutathione to an electrophilic centre in a variety of nonpolar potentially toxic compounds, thus, facilitating their excretion from the cell. Cytosolic GSTs are dimers (M_r ~ 50 000) and can be grouped into five distinct gene classes, alpha, mu, pi, theta and sigma, according to their structural and catalytic properties [3–5]. The first three-dimensional structure solved for the family of cytosolic GSTs was that for the porcine class pi enzyme (pGSTP1-1) [6,7], and has turned out to be the structural archetype for the family [8]. pGSTP1-1 is homodimeric with two structural domains per subunit and a catalytically independent active site on each subunit. The active site consists of two adjacent regions: the polar G-site which is highly specific for the tripeptide glutathione, and a hydrophobic H-site which can bind a range of structurally diverse nonpolar electrophiles. The active site cleft is located primarily on domain I but a fully functional active site also requires structural elements from domain II of the neighbouring subunit [6,7,9]. The recognition and binding of glutathione at the G-site is highly specific involving good surface complementarity and an extensive network of polar interactions between moieties of the extended tripeptide and the protein (Fig. 1). Numerous site-directed mutagenesis studies have confirmed most of the polar interactions implicated by the various crystal structures for class pi and other gene class enzymes [9–15]. The general conformation of bound glutathione as well as the strategic interactions between it and the protein are similar to those observed at the G-sites of other gene classes. Data from chemical modification and structural studies with class pi enzymes suggest a small glutathione-induced conformational change near the G-site (see [8,16] and references therein). The conformational flexibility of this region is, however, restricted by a conserved hydrophobic lock-and-key interaction located at the subunit interface of class pi/alpha/mu/*S. japonicum* enzymes [6,7,17–20]. Crystallographic data for class alpha [18] and *Schistosoma japonicum* enzymes [19,20] reveal that no significant conformational changes occur upon glutathione binding. pGSTP1-1 displays a two-state unfolding/refolding process which is highly populated with folded dimer and unfolded monomer at equilibrium [21,22]. Stable intermediates are not detected. Therefore, changes in the solvent-accessible surface area appears to be the major structural determinant for its cooperativity (i.e. m-value) of unfolding/refolding (see [23]). Oxidation but not alkylation of the highly reactive and conserved Cys45/47 in class pi glutathione S-transferases is markedly reduces enzyme stability [32]. To investigate further the conformational dynamics of the cytosolic glutathione S-transferases, we performed equilibrium and kinetics unfolding experiments to study the effect of ligand binding to the G-site on the unfolding and stability of pGSTP1-1.

2. Materials and methods

2.1. Materials

pGSTP1-1 was purified from porcine lung [16]. Urea (Aristar) was purchased from BDH. 1,4-Dithiothreitol and reduced glutathione were purchased from Boehringer Mannheim, glutathione sulphonate was obtained from Sigma, and 1-chloro-2,4-dinitrobenzene was from Merck. S-hexylglutathione was prepared according to the method of Vince et al. [24] and washed with ethanol. All other chemicals were of analytical grade quality.

2.2. Enzyme activity measurements

Catalytic activity and reactivation of pGSTP1-1 were measured as described previously [22].

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Abbreviations: GST, glutathione S-transferase; pGST P1-1, porcine glutathione S-transferase class pi with two type-1 subunits; GSH, reduced glutathione; S-hexGSH, S-hexylglutathione; G-site, glutathione-binding site; H-site, hydrophobic binding site for electrophiles

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2.3. Fluorescence methods

All fluorescence measurements were performed at room temperature using a Hitachi model 850 Fluorescence Spectrophotometer. The excitation bandwidth was set to 5 nm and the emission bandwidth to 10 nm. Samples were irradiated at 295 nm to selectively excite tryptophan residues and the emission monitored at 335 nm and 350 nm.

2.4. Unfolding/refolding studies

Equilibrium unfolding and reversibility studies as well as the analysis of the unfolding data were performed as described elsewhere [22]. pGSTP1-1 (1 μM) was equilibrated in the absence or presence of ligand (glutathione, glutathione sulphonate or S-hexylglutathione) in 0–8 M urea in 20 mM sodium-phosphate buffer, pH 6.5, containing 0.1 M NaCl, 1 mM EDTA, 0.02% NaN_3 at 23°C. All tryptophan fluorescence and enzyme activity measurements were performed after equilibrium was attained.

Unfolding kinetic studies were performed at 18°C by mixing 200 μl pGSTP1-1 with or without ligand in phosphate buffer with 800 μl of 10 M urea to yield a desired final concentration of ligand and 1 μM pGSTP1-1. The appearance of the unfolded enzyme form was monitored with time by measuring the increase in fluorescence intensity at 350 nm. Since dissociation and unfolding of oligomeric proteins are unimolecular processes, first-order kinetics data were obtained from the unfolding progress curves by plotting log $F_{max}/(F_{max}-F_{obs})$ against time; where F_{obs} represents the fluorescence intensity at a particular time and F_{max} the maximal fluorescence signal reached. Values for the first-order rate constants (in arbitrary fluorescence units per second) were obtained from the slope of the linear plots. All data were averaged over three experiments.

3. Results

3.1. Fluorescence properties of pGSTP1-1 and reversibility of unfolding

The binding of glutathione or its S-analogues quenches about 20% of the protein's tryptophan fluorescence without shifting the maximum emission wavelength indicating that the polarity of the tryptophan microenvironment does not change. The quenching effect is most likely a consequence of the direct interaction between bound glutathione and the indole fluorophore of Trp[38] located at the G-site [7]. Unfolding of pGSTP1-1 in the absence or presence of ligand results in a red shift in the emission maximum from 335 nm to 350 nm accompanied by a 3-fold increase in the fluorescence intensity. Refolded pGSTP1-1 (following a 10-fold dilution in non-denaturing buffer) shows a native-like tryptophan fluorescence spectrum in the presence of ligands and at least 85% of functional pGSTP1-1 is recovered.

3.2. Equilibrium studies

Monophasic unfolding transition curves for pGSTP1-1 in the presence of glutathione, glutathione sulphonate or S-

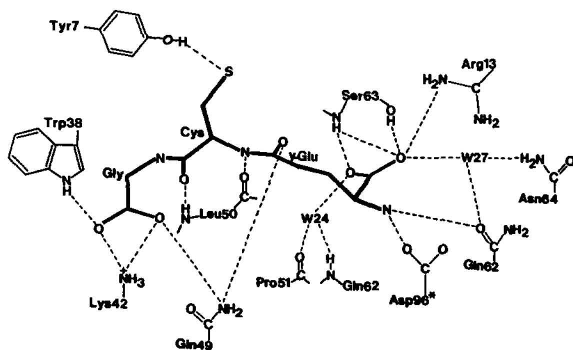

Fig. 1. Binding of glutathione at the G-site of pGSTP1-1. The hatched lines indicate hydrogen bonding interactions [7,8,31].

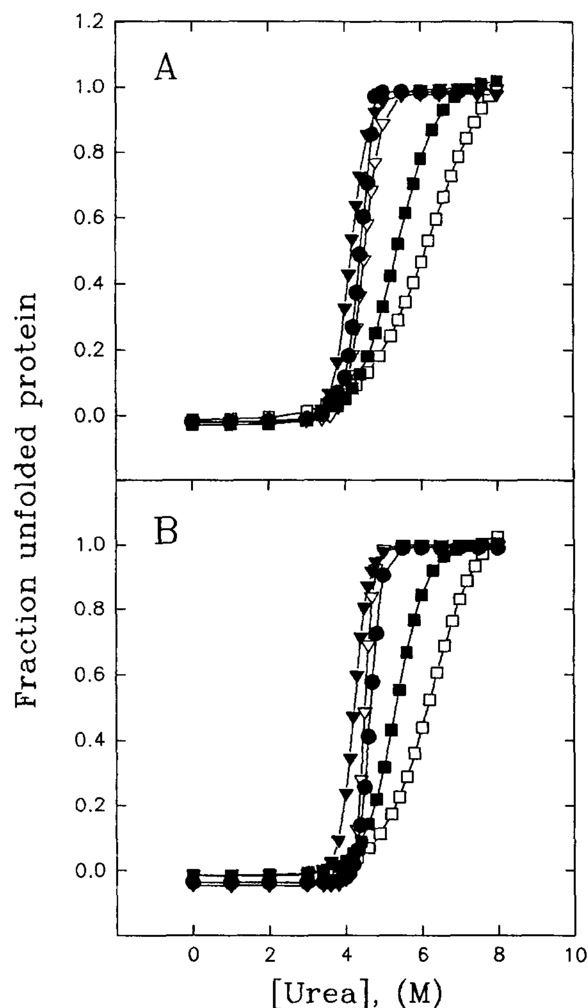

Fig. 2. Influence of glutathione, glutathione sulphonate and S-hexylglutathione on the equilibrium unfolding of pGSTP1-1. Fraction of unfolded protein determined from (A) fluorescence data and (B) enzyme activity data. (●) without ligands; (▽) with 1 mM GSH; (▼) with 5 mM GSH; (■) with 0.5 mM S-hexGSH; (□) with 0.5 mM glutathione sulphonate.

hexylglutathione are shown in Fig. 2. The data obtained from fluorescence (Fig. 2A) and activity measurements (Fig. 2B) are essentially coincident. At a saturating concentration of 5 mM glutathione (K_d of 120 μM [25]), the transition midpoint of pGSTP1-1 is shifted from 4.6 M to 4.1 M urea, whereas saturating concentrations of S-hexylglutathione (K_d of 2 μM [16]) and glutathione sulphonate (K_d of 4 μM [16]) shift the transition midpoint to higher urea concentrations of 5.4 M and 6 M, respectively. The slope of the unfolding transition is not changed dramatically in the presence of glutathione. The dependence of a protein's free energy of unfolding upon urea concentration (which is also known as the m-value [23,26]) can be determined from the data in the unfolding transition (see [22]). The m-values for pGSTP1-1 without and with 1 mM and 5 mM glutathione are 4.5, 4.4 and 3.8 kcal/mol per M urea, respectively. However, the enzyme's m-value is lowered significantly from 4.5 kcal/mol per M urea to 1.1 and 1.4 kcal/mol per M urea in the presence of glutathione sulphonate and S-hexylglutathione, respectively. Assuming a two-state equilibrium unfolding/refolding process in the absence [21,22] and presence of glutathione, conformational sta-

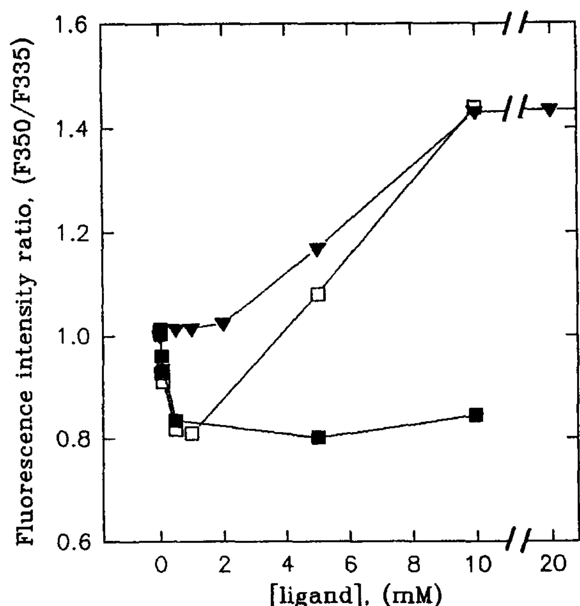

Fig. 3. Dependence of fluorescence intensity ratio of pGSTP1-1 in 4.4 M urea upon the concentration of (▼) glutathione, (■) S-hexylglutathione, and (□) glutathione sulphonate. F350 and F335 are the fluorescence intensities at 350 nm (unfolded protein) and 335 nm (folded protein), respectively.

bilities, $\Delta G(H_2O)$, of 21 kcal/mol, 20 kcal/mol and 16 kcal/mol were determined from the unfolding data in Fig. 2 for pGSTP1-1 without and with 1 mM and 5 mM glutathione, respectively. Free energy difference values for pGSTP1-1 in the presence of the glutathione analogues were not determined in light of the large lowering of the m-value suggesting that a two-state model is no longer valid [26,27]. Changes in a protein's m-value arise from changes in its cooperativity of unfolding reflecting a protein's changed responsiveness to denaturant.

The dependence of the ratio of unfolded to folded pGSTP1-1 in 4.4 M urea (i.e. urea concentration near midpoint of pGSTP1-1 without ligand) upon the concentration of glutathione and its analogues, is shown in Fig. 3. Fluorescence ratio values (intensity at 350 nm for unfolded protein to intensity at 335 nm for folded protein) of about 0.8 and 1.43 represent fully folded and unfolded pGSTP1-1, respectively. Increasing the concentration of reduced glutathione to 3 mM causes a gradual increase in unfolded pGSTP1-1 followed by a greater increase in the fraction of unfolded protein at higher concentrations. pGSTP1-1 in 4.4 M urea becomes fully unfolded at glutathione concentrations of 10 mM or greater. The glutathione analogues exerted the opposite effect of glutathione in that both glutathione sulphonate and S-hexylglutathione up to a concentration of about 1 mM increased markedly the fraction of folded protein (i.e. pGSTP1-1 in 4.4 M urea is fully folded at 1 mM ligand). At concentrations greater than 1 mM, S-hexylglutathione does not exert any further effect on the protein whereas glutathione sulphonate begins to increase the fraction of unfolded protein to fully unfolded at 10 mM.

3.3. Kinetic studies

Linear plots of log $F_{max}/(F_{max}-F_{obs})$ against time were obtained for the fluorescence unfolding progress curves indicating first-order kinetics (data not shown). The dependence of the unfolding first-order rate constant for pGSTP1-1 in 8 M urea upon the concentration of glutathione, glutathione sulphonate and S-hexylglutathione is shown in Fig. 4. The kinetics data correlates well with the equilibrium data described above in that the presence of glutathione has a destabilising effect on folded pGSTP1-1 and increases the rate of unfolding (Fig. 4A) whereas the two glutathione analogues stabilise folded pGSTP1-1 and decrease the rates of unfolding (Fig. 4B). Kinetics data for glutathione sulphonate beyond 0.1 mM are not included because of the lack of linearity of the log $F_{max}/(F_{max}-F_{obs})$ against time plots indicating a deviation from first-order unfolding kinetics.

4. Discussion

Glutathione S-transferases bind the physiological tripeptide thiol glutathione (present up to 10 mM in cells) and its S-

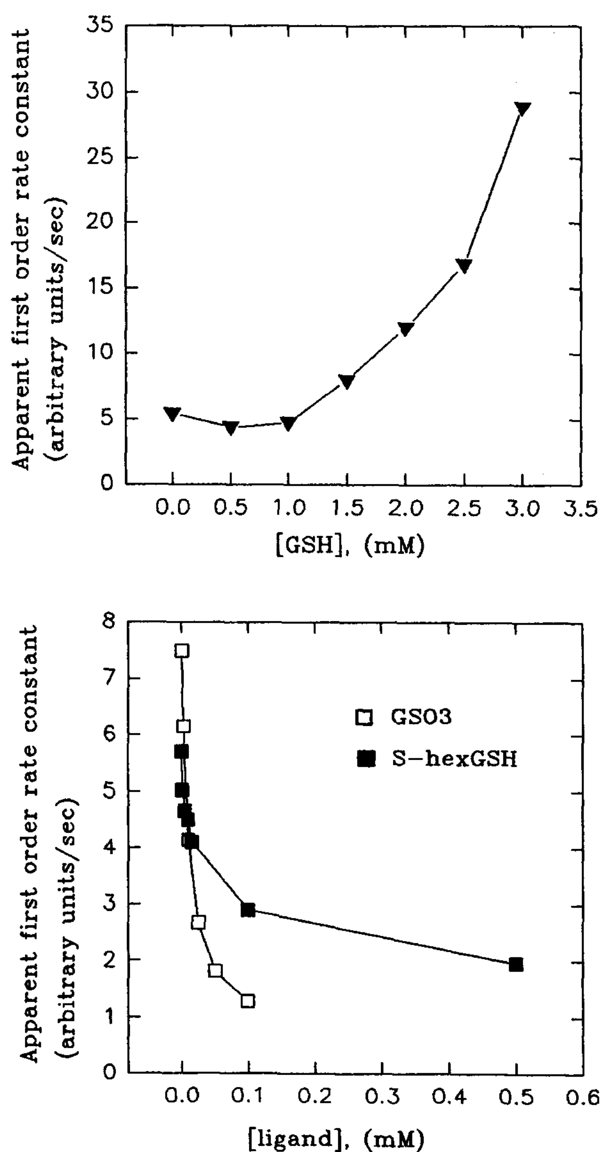

Fig. 4. Dependence of the first-order unfolding rate constant of pGSTP1-1 in 8 M urea upon the concentration of (▼) glutathione, (■) S-hexylglutathione, and (□) glutathione sulphonate.

analogues in a highly specific manner at the polar G-site (for reviews, see [8,31]). The effects of G-site ligand binding on the conformational dynamics of these enzymes were investigated by equilibrium unfolding and first-order unfolding kinetics studies with pGSTP1-1. The data presented in this study indicate a glutathione-induced destabilisation and a glutathione analogue-stabilisation of the pGSTP1-1 conformation against urea. Reasons for the destabilising effect of glutathione sulphonate observed at high concentrations are not known at present but could be due to it stabilising unfolded states through nonspecific interactions via its negatively charged sulphonate. The magnitude of m-values have been shown for a number of proteins to correlate well with protein size [23], and changes in m-values indicate changes in the cooperativity of unfolding/refolding [26]. According to the m-value data reported in this study, the binding of glutathione appears not to affect significantly the way the protein responds to urea-induced unfolding, whereas its sulphonate and S-hexyl analogues seem to alter significantly the protein's unfolding pathway. Uncomplexed pGSTP1-1 exists in either a completely folded or unfolded state at equilibrium [21,22] and its experimentally determined m-value (4.5 kcal/mol per M) compares very well with the expected value of 4.51 kcal/mol per M calculated according to [23]. This indicates that the amount of pGSTP1-1 surface area exposed to solvent upon unfolding is the major structural determinant for its m-value. It is possible that, during unfolding in the presence of the glutathione analogues, certain intermediate conformational states of pGSTP1-1 become stabilised thus giving rise to a multi-state unfolding process and, therefore, a lowered m-value [23,26,27].

The structural basis for the effects observed may not just be steric but may also involve the activation of the thiol group of reduced glutathione at the G-site. Glutathione and its S-analogues are sequestered at the G-site via a conserved hydrogen-bonding network (see Fig. 1). The hydrogen bonding between the γ-glutamyl moiety of glutathione on one subunit and the side chain of Asp*96* from the neighbouring subunit (a topologically conserved feature at the subunit interface of class alpha/mu/pi/sigma/Sj structures) could have a stabilising effect at the dimer interface. For S-hexylglutathione (a product analogue; $K_d = 2$ μM), nonspecific apolar contacts with the hexyl moiety at the adjacent H-site provide additional binding energy resulting in tighter binding. The stabilised anionic side chain of glutathione sulphonate (which mimics the thiolate form of glutathione; $K_d = 4$ μM) interacts more tightly with Tyr^7 than does the thiol moiety of reduced glutathione ($K_d = 200$ μM) (see also [28,29]). The interaction with Tyr^7 is not essential for the binding of glutathione but it is essential for catalysis [4,9–12]. Its hydroxyl group appears to act as a hydrogen bond donor to promote glutathione thiolate formation by decreasing the pK_a of the tripeptide's thiol group [7]. Therefore, a possible explanation for the observed destabilising effect glutathione exerts on the conformation of pGSTP1-1 when compared with glutathione sulphonate, is that some binding energy is lost to stabilisation against urea as a result of it being required for the activation of the thiol group of glutathione.

Ligand-induced stabilisation/destabilisation effects could impact significantly on protein turnover in vivo since unfolded proteins are highly susceptible to proteolytic degradation [30]. The effects observed here may have an important role in determining the life times of glutathione S-transferases in vivo.

References

[1] Armstrong, R.N. (1994) Adv. Enzymol. Relat. Areas Mol. Biol.69, 1–44.
[2] Listowsky, I. (1993) in: Hepatic Transport and Bile Secretion: Physiology and Pathophysiology (Tavoloni, N. and Berk, P.D., Eds.), pp. 397–405, Raven Press, New York.
[3] Mannervik,B., Alin, P., Guthenberg, C., Jensson, H., Tahir, M. K., Warholm, M. and Jornvall, H. (1985) Proc. Natl Acad. Sci. USA 82, 7202–7206.
[4] Meyer, D.J., Coles, B., Pemble, S.E., Gilmore, K.S., Fraser, G. M. and Ketterer, B. (1991) Biochem. J. 274, 409–414.
[5] Ji, X., vonRosenvinge, E.C., Johnson, W.W., Tomarev, S. I., Piatigorsky, J., Armstrong, R.N. and Gilliland, G.L. (1995) Biochemistry, 34, 5317–5328.
[6] Reinemer, P., Dirr, H.W., Ladenstein, R., Schäffer, J., Gallay, O. and Huber, R. (1991) EMBO J. 10, 1997–2005.
[7] Dirr, H., Reinemer, P. and Huber, R (1994a) J. Mol. Biol. 243, 72–92.
[8] Dirr, H., Reinemer, P and Huber, R. (1994b) Eur. J. Biochem. 220, 645–661.
[9] Manoharan, T.H., Gulick, A.M., Reinemer, P., Dirr, H.W., Huber, R. and Fahl, W.E. (1992) J. Mol. Biol. 226, 319–322.
[10] Wang, R.W., Newton, D.J., Huskey, S.W., McKeever, B.M., Pickett, C.B. and Lu, A.Y.H. (1992) J. Biol. Chem. 267, 19866–19871.
[11] Liu, S., Zhang, P., Ji, X., Johnson, W.W., Gilliland, G.L. and Armstrong, R.N. (1992) J. Biol. Chem. 267, 4296–4299.
[12] Kong, K., Nishida, M., Inoue, H. and Takahashi, K. (1992) Biochem. Biophys. Res. Commun. 182, 1122–1129.
[13] Xia, C., Meyer, D.J., Chen, H., Reinemer, P., Huber, R. and Ketterer, B. (1993) Biochem. J. 293, 357–362.
[14] Stenberg, G., Board, P.G. and Mannervik, B.(1991) FEBS Lett. 293, 153–155.
[15] Kong, K.-H., Inoue, H. and Takahashi, K. (1993) Protein Engineering 6, 93–99.
[16] Dirr, H.W., Mann, K., Huber, R., Ladenstein, R. and Reinemer, P. (1991) Eur. J. Biochem. 196, 693–698.
[17] Ji, X., Zhang, P., Armstrong, R.N. and Gilliland, G.L. (1992) Biochemistry 31, 10169–10184.
[18] Sinning, I., Kleywegt, G.J., Cowan, S.W., Reinemer, P., Dirr, H.W., Huber, R., Gilliland, G.L., Armstrong, R.N., Ji, X., Board, P.G., Olin, B., Mannervik, B. and Jones, T.A. (1993) J. Mol. Biol. 32, 192–212.
[19] McTigue, M.A., Williams, D.R. and Tainer, J.A. (1995) J. Mol. Biol. 246, 21–27.
[20] Lim, K., Ho, J.X., Keeling, K., Gilliland, G.L., Ji, X., Rüker, F. and Carter, D.C. (1994) Protein Sci. 3, 2233–2244.
[21] Dirr, H. W. and Reinemer, P. (1991) Biochem. Biophys. Res. Commun. 180, 294–300.
[22] Erhardt, J. and Dirr, H. (1995) Eur. J. Biochem. 230, 614–620.
[23] Myers, J.K., Pace, C.N. and Scholtz, J.M. (1995) Protein Sci. 4, 2138–2148.
[24] Vince, R., Daluge, S. and Wadd, W.B. (1971) J. Med. Chem. 14, 402–404.
[25] Bico, P, Erhardt, J., Kaplan, W. and Dirr, H. (1995) Biochim. Biophys. Acta, 1247, 225–230.
[26] Carra, J.H. and Privalov, P.L. (1995) Biochemistry 34, 2034–2041.
[27] Pace, C.N. (1986) Methods Enzymol. 131, 266–280.
[28] Graminski, G.F., Zhang, P., Sesay, M.A., Ammon, H.L. and Armstrong, R.N. (1989) Biochemistry 28, 6252–6258.
[29] Adang, A.E.P., Brussee, J., Gen, A. and Mulder, G.J. (1990) Biochem J., 269, 47–54.
[30] Pace, C.N. (1990) Trends Biochem. Sci. 15, 14–17.
[31] Reinemer, P., Dirr, H. and Huber, R. (1995) Methods Enzymol. 251, 243–254.
[32] Sluis-Cremer, N. and Dirr, H. (1995) FEBS Lett. 371, 94–98.