Effects of C-Terminal Deletions on the Conformational State and Denaturation of Phosphoglycerate Kinase†

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ABSTRACT: Phosphoglycerate kinase (PGK) contains two domains of approximately equal size, both of the α/β type. An α-helix consisting of the middle section of the 415-amino acid polypeptide chain, and the N- and C-termini reside in the interdomain hinge region [Watson, H. C., et al. (1982) *EMBO J. 1,* 1635−1640]. The C-terminal end is an integral part of the N-terminal domain. The consequences of the deletion of fifteen and three C-terminal amino acids on the conformational state and on the guanidine hydrochloride-induced and thermal unfolding of PGK were investigated by using near- and far-UV CD, tryptophan fluorescence, 1-anilinonaphthalene-8-sulfonic acid binding, accessibility to chemical modification, and differential scanning calorimetry. The results of these studies indicate that the conformations of both domains and of the interdomain region were altered by these deletions. In the absence of the 15-amino acid C-terminal peptide [Δ(401−415)], the N-terminal domain exhibits several characteristics of a molten globule state, whereas the C-terminal domain retains native-like, although distinctly different, tertiary structure. Deletion of three C-terminal amino acids [Δ(413−415)] also globally affects PGK conformation, although to a much lesser extent. Both C-terminal deletions resulted in a significant decrease in protein stability, as demonstrated by their increased susceptibility to guanidine-induced and thermal denaturation. These results suggest that the formation of a native tertiary fold of PGK requires the presence of a complete polypeptide chain.

PGK[1] structure is composed of two domains with extensive domain−domain interactions. Each domain contains approximately 200 amino acids, mostly corresponding to the N- and C-terminal halves of the amino acid sequence. The interdomain region includes N- and C-termini and a helix formed by the amino acids located in the middle of the amino acid sequence (Figure 1). Thus, the interdomain region in this protein contains segments of the polypeptide chain that are very distant in the unfolded state but become close in the folded conformation. The C-terminal end of the polypeptide chain constitutes an integral part of the N-terminal domain. This arrangement of the N- and C-termini suggests their important role in the folding of PGK domains and their pairing during the folding process. Genetically engineered individual domains of yeast PGK have been isolated by Yon and co-workers (Minard et al., 1989), who demonstrated that, despite their native-like structure (Fairbrother et al., 1989; Missiakas et al., 1990), they failed to reassociate. The role of these interactions at various stages of the folding process is unknown and is under investigation in this laboratory (Szpikowska et al., 1994; Sherman et al., 1995; J. M. Beechem, M. A. Sherman, and M. T. Mas, in preparation; M. P. Lillo, J. M. Beechem, B. K. Szpikowska, and M. T. Mas, in preparation).

It has been shown previously (Mas & Resplandor, 1988) that deletion of the C-terminal peptide (residues 401−415) from phosphoglycerate kinase results in a dramatic decrease in activity and loss of sulfate-dependent activation of this enzyme. Changes in the far-UV CD spectra and in the sedimentation constant, as well as an increased susceptibility to thermal inactivation, implied an important role of the carboxy-terminal region of the enzyme in the structural integrity and thermal stability (Mas & Resplandor, 1988). In this paper, we have further investigated the role of the carboxy-terminal region in the structure, function, and stability of PGK by characterizing the effects of the C-terminal deletions on the conformation and stability of PGK. In addition to the Δ(401−415)PGK constructed previously, two new mutants with shorter C-terminal deletions, Δ(406−415)PGK and Δ(413−415)PGK, were constructed by using site-directed mutagenesis. Unfortunately, aggregation of the Δ(406−415)PGK mutant, lacking 10 C-terminal amino acids, precluded its characterization. The Δ(413−415)PGK mutant, which lacks three C-terminal amino acids, has been purified to homogeneity and its enzymic properties have been characterized. This paper describes the characterization of the conformational state and the chemical and thermal denaturation properties of the Δ(401−415)PGK and Δ(413−415)PGK deletion mutants, using fluorescence, circular

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[1] Abbreviations: PGK, 3-phosphoglycerate kinase; ATP, adenosine 5′-triphosphate; 3-PG, 3-phosphoglycerate; Tris, tris(hydroxymethyl)-aminomethane; CD, circular dichroism; DSC, differential scanning calorimetry; ANS, 1-anilinonaphthalene-8-sulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); WT, wild type; Gdn-HCl, guanidine hydrochloride.

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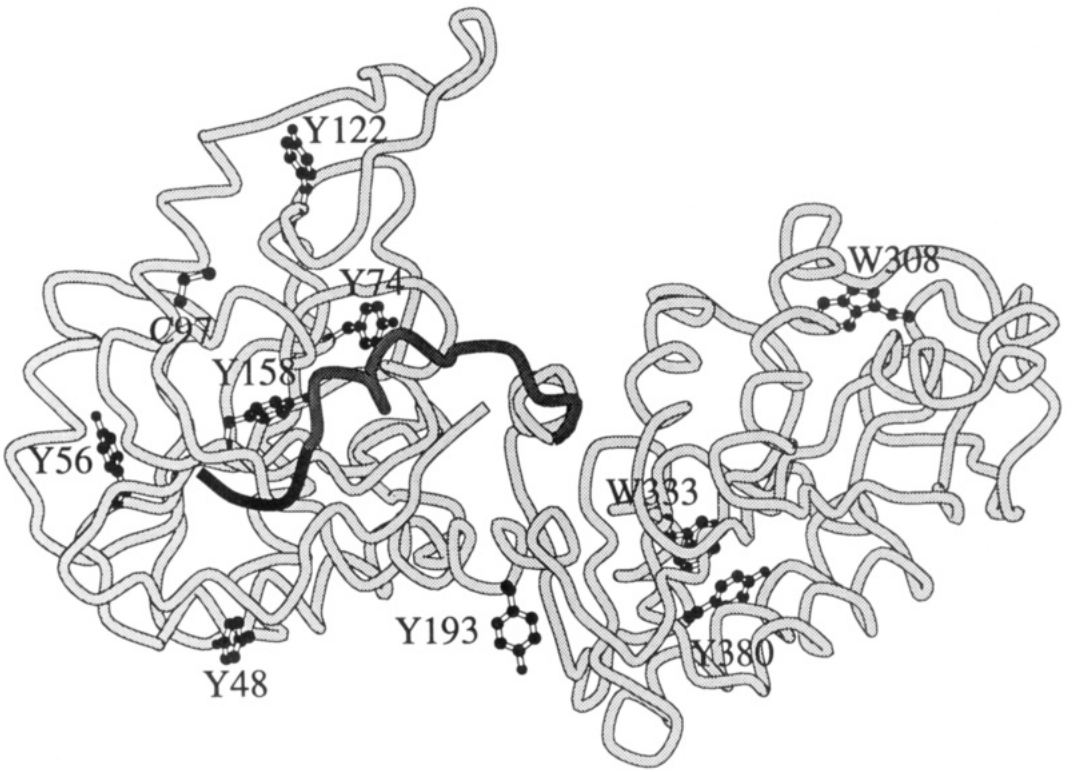

FIGURE 1: Ribbon drawing of PGK structure (Watson et al., 1982) made using MOLSCRIPT (Kraulis, 1991). Segments of the polypeptide chain shown in different shades of gray correspond as follows: dark, residues 413−415; intermediate, residues 401−412; light, residues 1−400. Locations of seven tyrosines, two tryptophans, and the single cysteine-97 are also shown.

dichroism, chemical modification, ANS binding, and differential scanning calorimetry.

MATERIALS AND METHODS

Materials. ANS (ultrapure) was purchased from Molecular Probes, Inc. (Eugene, OR). DTNB was from Sigma (St. Louis, MO). The sources of all other reagents were reported previously (Mas et al., 1987, 1988; Szpikowska et al., 1994).

Site-Directed Mutagenesis and Protein Purification. Oligonucleotide-directed mutagenesis and protein purification were carried out according to previously published procedures (Mas et al., 1986, 1987, 1988).

Determination of Enzyme Activity. The formation of 1,3-diphosphoglycerate was measured spectrophotometrically at 25 °C as previously described (Mas et al., 1986) in the presence or absence of 50 mM sodium sulfate.

CD Spectra. CD spectra were recorded at 25 °C using a Jasco-600 spectropolarimeter (Jasco) and quartz cuvettes of 1 mm and 1 cm path lengths for far-UV and near-UV CD, respectively. For the far-UV CD spectra four scans from 190 to 250 nm and for near-UV CD ten scans from 250 to 310 nm were recorded for each sample at a scan speed of 20 nm/min and a bandwidth of 1 nm. The scans for each sample were then averaged and corrected by subtracting a buffer baseline.

Fluorescence Spectra. Steady-state fluorescence measurements were performed at 25 °C using a Fluorolog-2 photon counting spectrofluorometer (Spex Industries, Edison, NJ). Spectral measurements were performed as previously described (Szpikowska et al., 1994).

DSC Measurements. DSC measurements were performed on a Microcal MC-2 calorimeter (Northampton, MA), interfaced with an IBM computer. The experimental details were identical to those described previously for PGK studies (Bailey et al., 1990).

DTNB Reaction Time Course. The reactivity of the thiol group of cysteine-97 in WT and the truncated versions of PGK was measured using DTNB. Protein solutions (0.7 mg/mL) were prepared in 20 mM sodium phosphate buffer (pH 7.5). The reaction was initiated by the addition of 100 μL of 10 mM DTNB stock solution in the same buffer to 900 μL of protein solution. The kinetics was followed by recording the variation in absorbance at 412 nm every 0.1 min for 10 min at 30 °C. The data were corrected for the contribution of the same concentration of DTNB in buffer alone and analyzed using the ENZFITTER program (a nonlinear regression data analysis program for IBM PC by Robin J. Leatherbarrow, purchased from Biosoft, Cambridge, UK). DTNB kinetics data were fitted to a pseudo-first-order equation, and the data and the calculated fits were plotted using the SIGMAPLOT 5.0 program from Jandel Scientific (San Rafael, CA).

ANS Binding Experiments. A stock solution of ANS was prepared in 20 mM sodium phosphate (pH 7.5), and its concentration was determined at 350 nm by using an extinction coefficient of 4950 $M^{-1}cm^{-1}$, according to the procedure described by Weber and Young (1964). Diluted solutions of ANS and protein (0.1 mg/mL) were mixed 1:1 (300 μL of each) in a 1-cm quartz cuvette. Fluorescence emission spectra of ANS were recorded at an excitation wavelength of 365 nm from 400 to 650 nm (Wiksell & Larsson-Raznikiewicz, 1982).

Equilibrium Unfolding Experiments. Equilibrium unfolding experiments were performed as previously described (Sherman et al., 1995). Data analysis was performed by

Table 1: Kinetic Parameters for WT PGK and Δ(413−415) PGK[a]

enzyme	$K_m^{3\text{-PG}}$ (mM)	K_m^{ATP} (mM)	V_{max}[b] (units mg^{-1})[c]
wild-type PGK	0.50	0.30	786
Δ(413−415)PGK	0.52	0.28	680
Δ(401−415) PGK	3.85	0.23	1.7

[a] The kinetics measurements were performed in 20 mM triethanolamine-acetate buffer (pH 7.5) containing 50 mM Na_2SO_4, as described in Materials and Methods. [b] The V_{max} values are the maximal specific activities observed at saturating substrate concentrations. [c] One unit is defined as the number of micromoles of substrate converted to product per minute per milliliter.

nonlinear least-squares fits of raw data to a two- or three-state unfolding model as previously described (Santoro & Bolen, 1988; Jackson et al., 1993; Eftink, 1994; Sherman et al., 1995). Standard errors of C_m determinations were obtained by using equation 6 in Jackson et al. (1993).

RESULTS AND DISCUSSION

Comparison of the Enzymatic Properties of Δ(413−415)-PGK and WT PGK. We have shown previously (Mas & Resplandor, 1988) that the deletion of 15 amino acids from the C-terminus of PGK [Δ(401−415)] resulted in a dramatic loss of activity and about 8-fold increase in the K_m for 3-PG. The K_m value for ATP was essentially unchanged in comparison with that for WT PGK. The maximum velocity for this mutant measured in the presence of 50 mM sodium sulfate was equal to approximately 0.2% of the V_{max} for the WT enzyme (Mas & Resplandor, 1988). In this study, the steady-state kinetics measurements for the Δ(413−415)PGK mutant were carried out under analogous conditions, as described in the Materials and Methods. The Michaelis constants (K_m) for ATP and 3-PG were determined at 25 °C in the presence of 20 mM triethanolamine−acetate buffer (pH 7.5) containing 50 mM sodium sulfate. As shown in Table 1, the K_m values for both substrates are virtually identical for the mutant and for WT PGK. The V_{max} = 680 units/mg for Δ(413−415)PGK is approximately 13% lower than V_{max} = 786 units/mg for WT PGK under these conditions.

The maximum velocities were also measured in the absence of sulfate. In sharp contrast to Δ(401−415)PGK, which exhibited only about 1% of the activity of WT PGK, the Δ(413−415)PGK exhibits a specific activity essentially identical to the activity of WT PGK under these conditions (465 and 457 units/mg, respectively).

Activation by Sulfate. The effect of sulfate on the maximum velocities was explored further by measuring the specific activities of Δ(413−415)PGK and WT PGK as a function of sulfate concentration, under conditions described in previous studies of Δ(401−415)PGK (Mas & Resplandor, 1988). It was shown previously that, in the presence of low substrate concentrations, the WT enzyme is activated at low sulfate concentrations and inhibited at higher concentrations. The concentration of sulfate at which maximum activation occurs and the extent of activation depend on the relative concentrations of both substrates in the assay (Larsson-Raznikiewicz & Jansson, 1973; Scopes, 1978; Mas et al., 1987). As shown previously for the Δ(401−415)PGK mutant, the deletion of a 15-amino acid, C-terminal peptide resulted in a complete loss of activation of the enzyme by

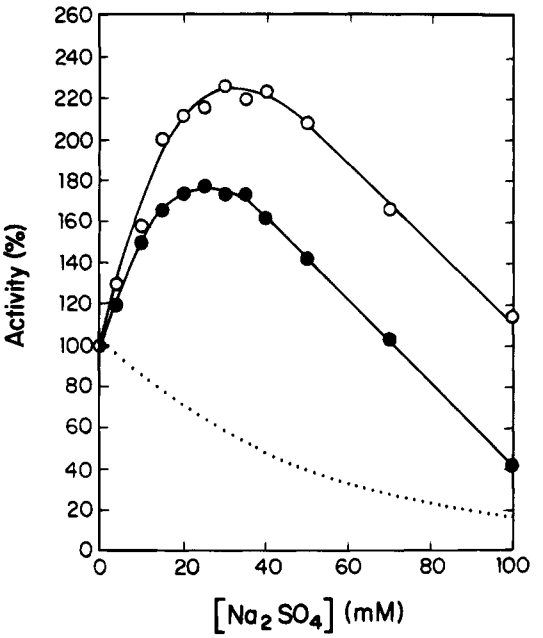

FIGURE 2: Effect of sulfate on the specific activities of wild-type PGK and of the deletion mutants. Effect of sulfate on enzymatic activity (v_{obsd}) was measured in 50 mM Tris-HCl buffer (pH 7.8) in the presence of 1 mM ATP and 2 mM 3-PG. Activities are expressed as a percentage of the activity of each enzyme in the absence of sulfate. Each point represents the average of at least two determinations: (○) WT PGK; (●) Δ(413−415)PGK; (•••) Δ(401−415)PGK [from Mas and Resplandor (1988)].

sulfate ions (Mas & Resplandor, 1988). Figure 2 shows a comparison of the activities as a function of sulfate concentration for WT PGK, Δ(401−415)PGK, and Δ(413−415)-PGK. In contrast to Δ(401−415)PGK, which exhibits progressive inhibition as the concentration of sulfate increases, the Δ(413−415)PGK exhibits activation, although not as great as WT PGK. The maximum activation of about 76% is observed at approximately 25 mM sulfate for the Δ(413−415)PGK mutant, compared to the maximum activation of 125% observed at 30 mM sulfate for WT PGK. A decrease or elimination of sulfate-dependent activation has been observed previously for several PGK mutants containing point mutations in the hinge region, including those situated outside of the anion binding site (Mas et al., 1987, 1988; Bailey et al., 1990; B. K. Szpikowska and M. T. Mas, unpublished results). These mutations were postulated to affect conformational changes associated with the activation mechanism. Similar results were also observed as a result of perturbing the anion binding site located in the interdomain cleft on the inner surface of the N-terminal domain (a "basic patch region"), as confirmed by site-directed mutagenesis (Sherman et al., 1990, 1992). Given the hinge location of the C-terminal end and its association with the N-terminal domain (Figure 1), the decreased activation by sulfate in the Δ(413−415)PGK mutant and its loss in the Δ(401−415)-PGK mutant are likely to result from the perturbation of one or both of these regions in the truncated proteins.

CD Spectra. As previously reported, the CD spectrum of the Δ(401−415)PGK mutant in the far-UV range (Figure 3A, spectrum 1) revealed significant changes in secondary structure as a result of deleting 15 carboxy-terminal amino acids (Mas & Resplandor, 1988). In contrast, the far-UV CD spectrum of Δ(413−415)PGK (Figure 3A, spectrum 2) was almost identical to the spectrum of WT PGK (Figure

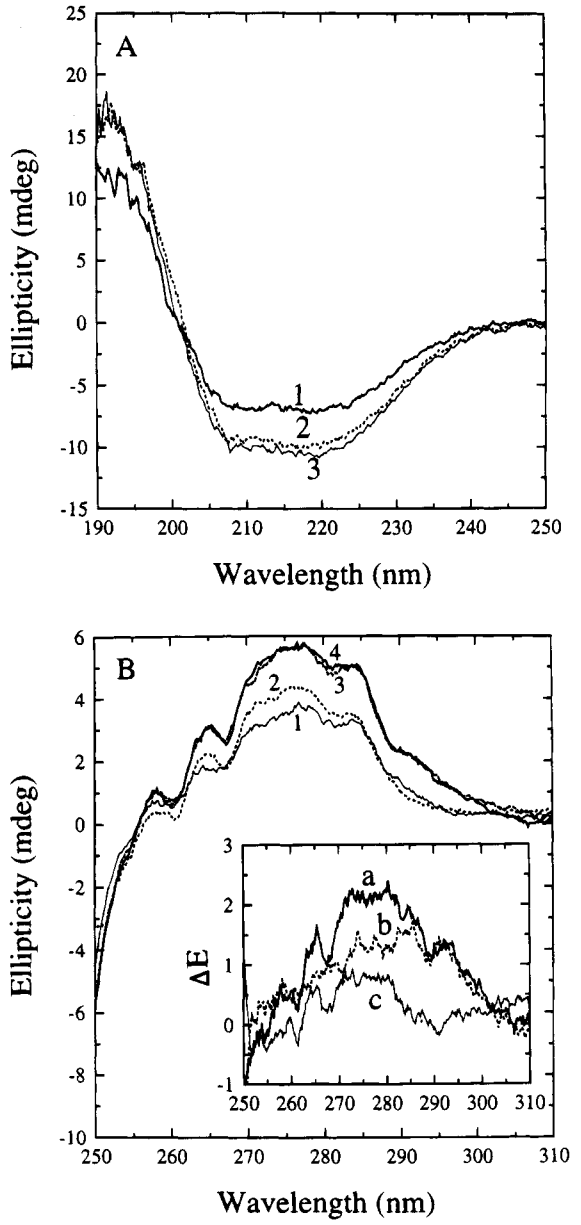

FIGURE 3: Circular dichroism spectra. (A) Far-UV CD: (1) Δ(401−415)PGK; (2) Δ(413−415)PGK; (3) wild-type PGK. (B) Near-UV CD: (1) Δ(401−415)PGK; (2) tryptophan-less PGK; (3) Δ(413−415)PGK; (4) wild-type PGK. Inset: The difference spectra obtained by subtracting (a) WT PGK − Δ(401−415), (b) Δ(413−415)PGK − W− PGK, and (c) W− PGK − Δ(401−415)PGK.

3A, spectrum 3), indicating that no significant changes in secondary structure occurred in this mutant.

CD spectra in the near UV (Figure 3B) also show close similarity between the near-UV CD spectra for Δ(413−415)-PGK and WT PGK (Figure 3B, spectra 3 and 4, respectively). It has been shown previously, by comparing the spectra of WT PGK and the tryptophan-less mutant (W− PGK), that in PGK the positive CD signal in the aromatic region is contributed predominantly by tyrosines (Sherman et al., 1995). Substitution of both C-terminal tryptophans with phenylalanines in the W− mutant resulted in a 22% decrease in ellipticity at 278 nm (Sherman et al., 1995). The difference spectrum b (Figure 3B, inset), obtained by subtracting the spectrum of the tryptophan-less PGK (Figure 3B, spectrum 2) from that of the Δ(401−413)PGK mutant (Figure 3B, spectrum 3; note that this spectrum is essentially identical to WT spectrum 4 shown in Figure 3B), represents the contribution of tryptophans in PGK to the near-UV CD spectrum above 270 nm.

A significant decrease in the ellipticity (34% decrease at 278 nm) was observed in the spectrum of Δ(401−415) PGK, when compared to that of WT. The difference spectrum (spectrum a in the inset to Figure 3B) obtained by subtracting the spectrum of Δ(401−415)PGK from that of WT PGK illustrates changes in the aromatic region of the spectra that resulted from the deletion of the 15 C-terminal amino acids (note that the aromatic amino acid content in both proteins is the same). The decrease in the CD signal observed for Δ(401−415)PGK encompasses the entire aromatic range. In order to estimate the extent of spectral changes contributed by changes in the tryptophan environment, the spectrum of Δ(401−415)PGK (spectrum 1, Figure 3B) was compared to the spectrum of a PGK mutant in which both tryptophans had been replaced with phenylalanines (spectrum 2, Figure 3B). Difference spectrum c in the inset to Figure 3B was obtained by subtracting spectrum 1 from 2. The lack of a positive signal above 300 nm, which in WT PGK can be ascribed to tryptophans (Sherman et al., 1995), suggests the disruption of tertiary interactions involving the C-terminal tryptophans, which is spectrally equivalent to the removal of both tryptophans in the tryptophan-less mutant. The 34% change in the near-UV spectrum in the 270−300-nm region, where tyrosine and tryptophan residues absorb, is greater than the 22% decrease in the spectrum of the W−mutant (Sherman et al., 1995) and suggests that the environment of some of the tyrosines has been perturbed as a result of the deletion of the C-terminal peptide. Of the seven tyrosine residues, five are situated in the N-domain, one in the hinge, and one in C-domain adjacent to the hinge region. Only three of these tyrosines (48 and 122 in the N-domain and 193 in the hinge) are significantly exposed to solvent and relatively independent from the adjacent secondary structure elements in the native structure; others, including Tyr-74 which, although solvent-exposed, has van der Waals contacts with the N-terminus and with Phe-185 in the hinge and thus would be expected to be sensitive to unfolding of these regions, are tightly packed against neighboring secondary structure elements (Figure 1). It can therefore be inferred that some of the tyrosines in the N-domain of the truncated protein have retained their native-like tertiary contacts and that the hydrophobic core of the N-domain must have a relatively compact structure. A small decrease in ellipticity in this region suggests changes in the tertiary environment of some tyrosine residues, presumably those located in the shell of the N-domain and in the hinge.

Fluorescence Spectra. The fluorescence emission spectrum of yeast PGK at an excitation wavelength of 280 nm is dominated by tyrosine emission and has an emission maximum at 311 nm (Szpikowska et al., 1994). Both C-terminal tryptophans have been shown to be significantly quenched in the native protein (Nojima et al., 1976; Szpikowska et al., 1994). At an excitation wavelength of 295 nm, at which tryptophans are selectively excited, the fluorescence emission maximum occurs at 337 nm. In WT PGK, a significant enhancement in tryptophan fluorescence is observed upon unfolding, concomitant with the red shift of its maximum to 355 nm (Szpikowska et al., 1994).

As shown in Figure 4A, the tryptophan emission spectra for the truncated proteins in the absence of denaturant have

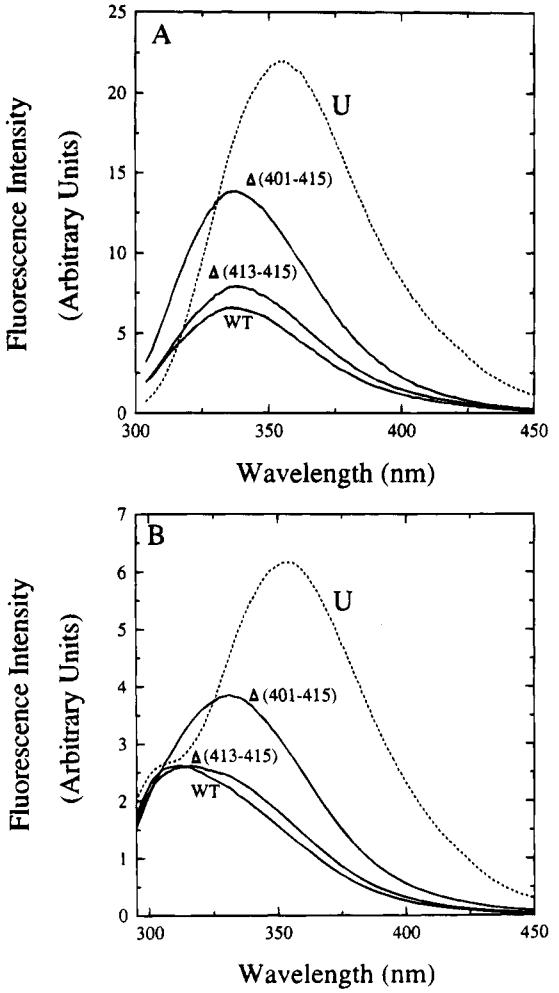

FIGURE 4: Fluorescence emission spectra of wild-type PGK (WT), Δ(401−415)PGK, and Δ(413−415)PGK in 20 mM phosphate buffer (pH 7.5), in the absence and presence of 4 M guanidine hydrochloride: (A) excitation at 295 nm; (B) excitation at 280 nm. Spectra of the folded proteins (0.1 mg/mL) were normalized to the spectrum of unfolded proteins in the presence of 4 M guanidine hydrochloride (U), identical for all three proteins.

approximately the same λ_{max} (337 nm) as WT PGK, whereas their intensities are increased compared to the spectrum of native WT PGK. This enhancement is particularly dramatic for Δ(401−415)PGK, although it is smaller than that in the completely unfolded proteins (Figure 4A). Likewise, the emission spectrum at 280-nm excitation (Figure 4B) has much higher intensity than that of the native WT PGK, with an emission maximum (331 nm) intermediate between those of folded and unfolded PGK (Szpikowska et al., 1994). This result also suggests partial unfolding of the truncated Δ(401−415)PGK. The enhancement of tryptophan fluorescence is likely to be due to an increased distance between tryptophans and quenching groups in the C-domain, suggesting "melting" of this domain in the absence of the C-terminal peptide. The tyrosine fluorescence in PGK is much less sensitive to changes in tertiary or secondary structure [see Figure 3B in Szpikowska et al. (1994)] and therefore is a much less sensitive measure of such changes than the aromatic region of the CD spectrum (Figure 3B and the preceding discussion).

Reactivity of the Single Cysteine Residue Situated in the N-Terminal Domain. DTNB has been used previously to probe the reactivity of the single cysteine-97 in yeast PGK. This residue exhibits very low solvent accessibility in the

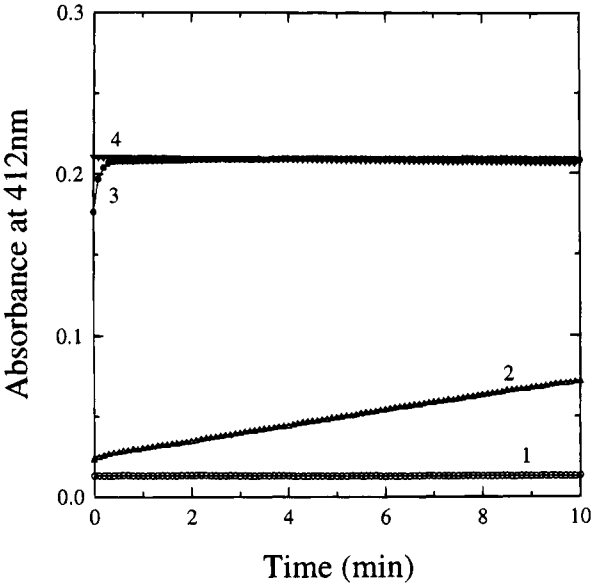

FIGURE 5: Time course of DTNB reaction with the thiol group of single cysteine-97 in 20 mM phosphate buffer (pH 7.5) in the absence of denaturant for (1) WT PGK, (2) Δ(413−415)PGK, (3) Δ(401−415)PGK, and (4) WT PGK in the presence of 1 M guanidine hydrochloride.

structure of yeast PGK by interaction with the adjacent amino acid residues. Previous studies showed that this residue reacts very slowly with DTNB (Markland et al., 1975). The kinetics of the reaction of Cys-97 in the truncated mutants has been compared to that of WT PGK. The time course of these reactions is shown in Figure 5. Under the conditions used in this study, Cys-97 is essentially inaccessible to modification by DTNB in the native enzyme and becomes more accessible in Δ(413−415)PGK [pseudo-first-order rate constant $k = (2.4 \pm 0.3) \times 10^{-4}$ s^{-1}]. In contrast, the same residue in Δ(401−415) PGK reacts very rapidly [$k = (1.60 \pm 0.04) \times 10^{-1}$ s^{-1}], although more slowly than when the protein is completely unfolded, in which case the reaction is instantaneous (occurs within manual mixing time) (Figure 5). These results suggest that the C-terminal deletions affect the conformation of the N-terminal domain, which leads to a greater exposure of Cys-97. This is probably due to at least local unfolding of the structure around Cys-97, which involves contacts with residues of helices III and IV and the loop connecting helix III and β-strand C [nomenclature used by Watson et al. (1982)], resulting in its greater exposure to solvent and reaction with DTNB, particularly in the absence of the 15 C-terminal amino acids.

ANS Binding. ANS has been shown to bind to hydrophobic regions of proteins that become exposed in a molten globule state (Goto & Fink, 1989; Ptitsyn et al., 1990; Semitsonov et al., 1991). Figure 6 shows fluorescence emission spectra of free ANS and ANS in the presence of WT, Δ(413−415)PGK and Δ(401−415)PGK in 20 mM phosphate buffer (pH 7.5) at λ_{ex} = 365 nm. A small enhancement in ANS fluorescence is observed in the presence of WT PGK. ANS has been shown previously to bind to yeast PGK with a dissociation constant in the millimolar range and to inhibit the activity of the enzyme due to binding at the ATP binding site (Wiksell & Larsson-Raznikiewicz, 1982). More ANS sites were detected when ANS concentrations were further increased. Nonsaturating behavior indicated progressive denaturation at high ANS

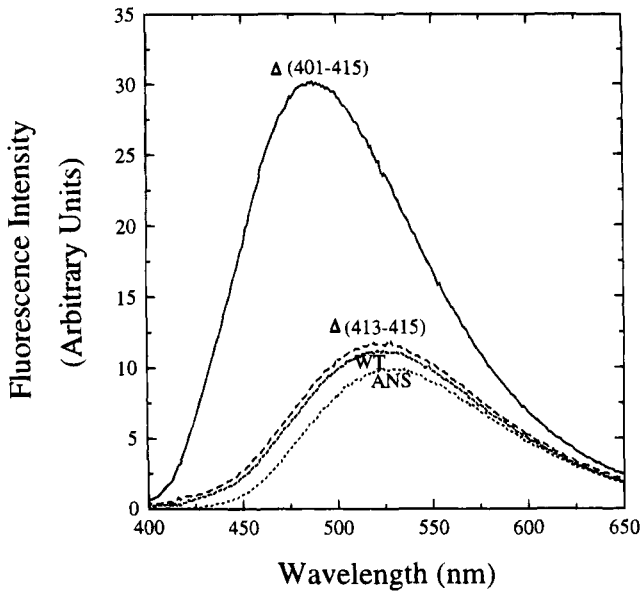

FIGURE 6: Fluorescence spectra of free ANS and ANS in the presence of WT PGK, Δ(413−415)PGK, and Δ(401−415)PGK at an excitation wavelength of 365 nm. ANS (13.2 μM) and protein (0.05 mg/mL) solutions were prepared in 20 mM phosphate buffer (pH 7.5).

concentrations (Wiksell & Larsson-Raznikiewicz, 1982). At the concentrations used in this study (1 μM protein and 13 μM ANS), binding to the ATP site is negligible (less than 1% PGK−ANS complex). A similar spectral change was observed for Δ(413−415)PGK (Figure 6). In contrast, a dramatic increase in ANS fluorescence was observed in the presence of Δ(401−415)PGK. A concomitant 37-nm blue shift in the emission maximum indicates transfer to a more hydrophobic environment, a change that characteristically results from binding of ANS to the hydrophobic surfaces exposed in the molten globule states (Goto & Fink, 1989; Ptitsyn et al., 1990; Semitsonov et al., 1991). In an effort to discern whether ANS binds to both or only one of the domains, the emission spectra of Δ(401−415)PGK in the presence of ANS were also recorded at excitation wavelengths of 295 (selective excitation of tryptophans) and 280 nm (excitation of both tryptophans and tyrosines). Energy transfer from tryptophans to ANS, resulting in an enhancement in ANS fluorescence and a corresponding decrease in tryptophan fluorescence, would be expected (at both excitation wavelengths) if ANS were bound to the C-terminal domain, which contains both tryptophans. An increase in ANS fluorescence, accompanied by a decrease in protein fluorescence, was observed at λ_{ex} = 280 nm but not at 295 nm (data not shown). This result suggests that ANS binds to the N-terminal domain.

Equilibrium Unfolding Transitions Monitored by Far-UV CD and Tryptophan Fluorescence. (i) Δ(413−415)PGK. Equilibrium unfolding transitions for this protein were monitored by CD in the peptide bond absorption region, by shifts in the maximum emission wavelength of tryptophan fluorescence, and by changes in total fluorescence intensity (Figure 7). These transitions were essentially coincident, with C_m = 0.57 ± 0.02 M, suggesting that no significantly populated intermediate is present under equilibrium conditions. A similar cooperative transition was observed under identical conditions for WT PGK, although with a transition midpoint at higher guanidine concentrations, C_m = 0.77 M

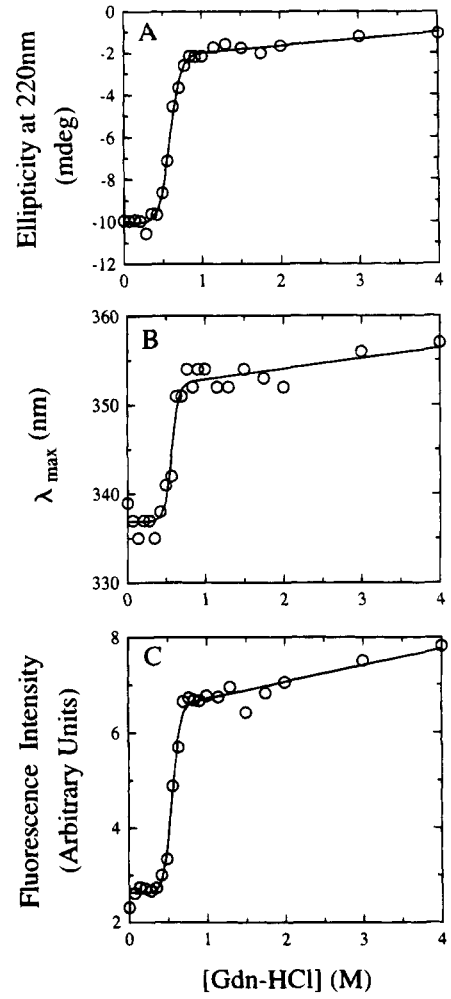

FIGURE 7: Equilibrium unfolding of Δ(413−415)PGK by guanidine hydrochloride monitored by (A) far-UV CD and by (B) tryptophan fluorescence (λ_{ex} = 295 nm) maximum emission wavelength and (C) total fluorescence intensity. Protein samples (0.1 mg/mL) were prepared in 20 mM phosphate buffer (pH 7.5) in the presence of 0−4 M Gdn-HCl as described in Materials and Methods. The solid lines are nonlinear least-squares fits of raw data to a two-state unfolding model, as described in Materials and Methods. Thermodynamic parameters calculated from these data are summarized in Table 2.

(Szpikowska et al., 1994). The difference in the free energy of unfolding, Δ(Δ*G*) = Δ*G*(WT) − Δ*G*(mutant), is ~ 4 kcal/mol. A decrease in stability was also observed in the thermal denaturation profile of Δ(413−415)PGK (described in the following).

(ii) Δ(401−415)PGK. Guanidine-induced denaturation of Δ(401−415)PGK was also monitored by using the same three methods. As shown in Figure 8, the unfolding transitions obtained by different methods differed from one another as well as from the corresponding profiles obtained for Δ(413−415)PGK (Figure 7). First, the transition monitored by ellipticity at 220 nm was clearly biphasic, with C_{m1} = 0.59 ± 0.03 M and C_{m2} = 1.17 ± 0.79 M. Total fluorescence emission intensity changes at λ_{ex} = 295 nm showed a non-cooperative increase in the tryptophan emission from 0 to about 0.8 M Gdn-HCl. A cooperative two-state transition, with C_m = 0.60 ± 0.02 M, was observed when the wavelength of maximum fluorescence emission (λ_{max}) was plotted as a function of guanidine concentration. Comparison of Figure 8B and 8C shows that most of the change in fluorescence intensity occurs in the 0−0.4 M

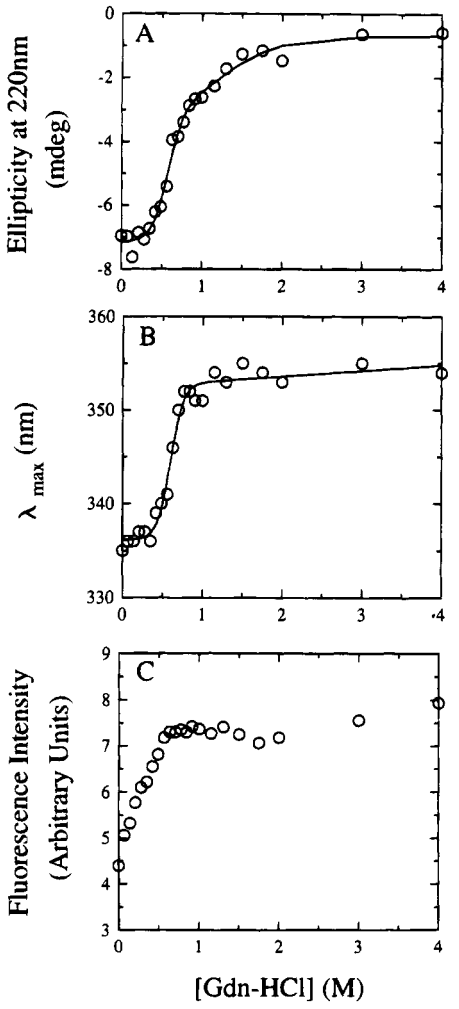

FIGURE 8: Equilibrium unfolding of Δ(401−415)PGK by guanidine hydrochloride monitored by (A) far-UV CD, (B) maximum emission wavelength, and (C) total intensity of tryptophan fluorescence (λ_{ex} = 295 nm). Experimental conditions were as described in the legend to Figure 7. The solid lines are the nonlinear least-squares fits of raw data to a three-state (A) and a two-state (B) unfolding model, as described in Materials and Methods. Thermodynamic parameters calculated from these data are summarized in Table 2.

concentration range, during which λ_{max} remains essentially unchanged. This suggests the presence of an intermediate state(s) in which tryptophan residues become less quenched while the polarity of the environment remains unchanged. This could occur as the result of "melting" of the structure, during which the distance between tryptophans and protein quenching groups would increase, but not enough to allow access of water molecules in the vicinity of the fluorophores. The first transition observed by ellipticity at 220 nm, which coincides with the change monitored by the emission maximum, apparently corresponds to a global change in secondary structure, during which both C-terminal tryptophans become completely solvent exposed. Although the C_m of the first transition for Δ(401−415)PGK coincides with the C_m for the Δ(413−415)PGK, the free energies of unfolding calculated from each transition are very different (Table 2). The decrease in the free energy of unfolding represents the difference in stability of the folded and unfolded states. It is important to note that the "folded" states (conformation in the absence of denaturant) are clearly different for WT, Δ(413−415)PGK, and Δ(401−415)PGK, as demonstrated in this study, and that the unfolded states may differ as well.

In addition to differences detected by circular dichroism, fluorescence, ANS binding, and reactivity of Cys-97, a significantly lower denaturant slope, *m*, in Δ(401−415)PGK (Table 2) may suggest increased exposure of hydrophobic surface area in the "native" state of this protein, compared to the wild type. The denaturant slope, *m*, is proportional to the difference in the solvent-exposed surface areas of the denatured and native states (Schellman, 1978). The nature of the second transition, observed only in the CD-monitored curve, is unclear. It may suggest the presence of a stable intermediate with residual secondary structure that is not observed in WT or Δ(413−415)PGK under the same conditions. Interestingly, a similar intermediate was observed in the unfolding transitions of some site-directed mutants of PGK (Sherman et al., 1995), with single amino acid substitutions within the individual domains and in the hinge region. Therefore, this transition cannot be ascribed to a specific mutation site. Coincidence of the second transition observed in these different mutants suggests a common intermediate whose equilibrium population can be increased as a result of different structural changes.

Thermal Denaturation. Previous experiments showed that the Δ(401−415)PGK mutant exhibited a time-dependent loss of activity when incubated at 55 °C, in contrast to the native enzyme whose activity was unchanged under the same conditions (Mas & Resplandor, 1988). This result suggested that the truncated enzyme was more susceptible to thermal denaturation than WT PGK. Differential scanning calorimetry was used in the present paper to evaluate the thermal stability of the Δ(401−415) and Δ(413−415) deletion mutants.

(i). Figure 9 shows the DSC scans of thermal unfolding of WT PGK (panel A) and the Δ(413−415) mutant (panel B) in the absence of substrates (curve 1) and in the presence of ADP, 3-PG, and Mg^{2+} (curve 2). It was shown previously that, in the absence of substrates, WT PGK exhibits an asymmetric peak that can be resolved into two transitions, A and B (Brandts et al., 1989). It has been shown that the low-temperature transition A (T_m = 52.3 °C) corresponds to the melting N-terminal domain and that the high-temperature transition B (T_m = 54.1 °C) corresponds to the C-terminal domain. The effects of the substrates on these transitions indicate that the primary binding sites for ATP and 3-PG reside in the C-terminal and N-terminal domains, respectively.

In contrast to WT PGK, which in the absence of substrates exhibits one asymmetric peak composed of two transitions (T_{m1} = 52.3 °C and T_{m2} = 54.1 °C), Δ(413−415)PGK exhibits two distinct transitions centered at T_{m1} = 45 °C and T_{m2} = 54.1 °C (Figure 9 and Table 3). Shift of the A transition to a lower temperature may be due to destabilization of the N-terminal domain and/or domain−domain interactions (Brandts et al., 1989). The reduced cooperative behavior observed for the Δ(413−415) mutant (Figure 9B, curve 1) is very similar to that seen for other mutants of PGK containing various point mutations in the interdomain hinge region, for example, H388A, H388L, E190Q, E190D (Brandts et al., 1989), A183P (Bailey et al., 1990), and F194L (J. F. Brandts and L.-N. Lin, unpublished). The thermal denaturation profiles for all of these mutants exhibit two DSC peaks: a low-temperature peak at T_m = 45 °C, corresponding

Table 2: Free Energy Changes, Denaturant Slopes, and Transition Midpoints for Guanidine Hydrochloride-Induced Unfolding Transitions Calculated from Data Shown in Figures 7 and 8[a]

mutant	$\Delta G°_{N\rightarrow U}$ (kcal/mol)	$m_{N\rightarrow U}$ (kcal/mol·M)	$\Delta G°_{N\rightarrow I}$ (kcal/mol)	$m_{N\rightarrow I}$ (kcal/mol·M)	$\Delta G°_{I\rightarrow U}$ (kcal/mol)	$m_{I\rightarrow U}$ (kcal/mol·M)	C_{m1} (M)	C_{m2} (M)
				Fluorescence				
WT[b]	10.90 ± 1.51	13.9 ± 1.90					0.78 ± 0.01	
Δ(413−415)[c]	7.63 ± 2.27	13.4 ± 4.00					0.57 ± 0.02	
Δ(413−415)[b]	6.73 ± 0.93	12.2 ± 1.68					0.55 ± 0.01	
Δ(401−415)[c]	4.46 ± 0.76	7.5 ± 1.26					0.60 ± 0.02	
				Circular Dichroism				
WT	9.34 ± 0.85	12.1 ± 1.08					0.77 ± 0.01	
Δ(413−415)	5.33 ± 0.43	9.0 ± 0.70					0.59 ± 0.01	
Δ(401−415)[d]			3.48 ± 0.21	5.9 ± 1.4	1.78 ± 2.56	1.5 ± 1.2	0.59 ± 0.03	1.17 ± 0.79

[a] Calculated as described in the Materials and Methods. Values reported as ±one standard deviation. [b] Calculated from total intensity data. [c] Calculated from λ_{max} data. [d] Fractional change at 220 nm attributed to the N→I transition fixed at 0.55 during curve fitting.

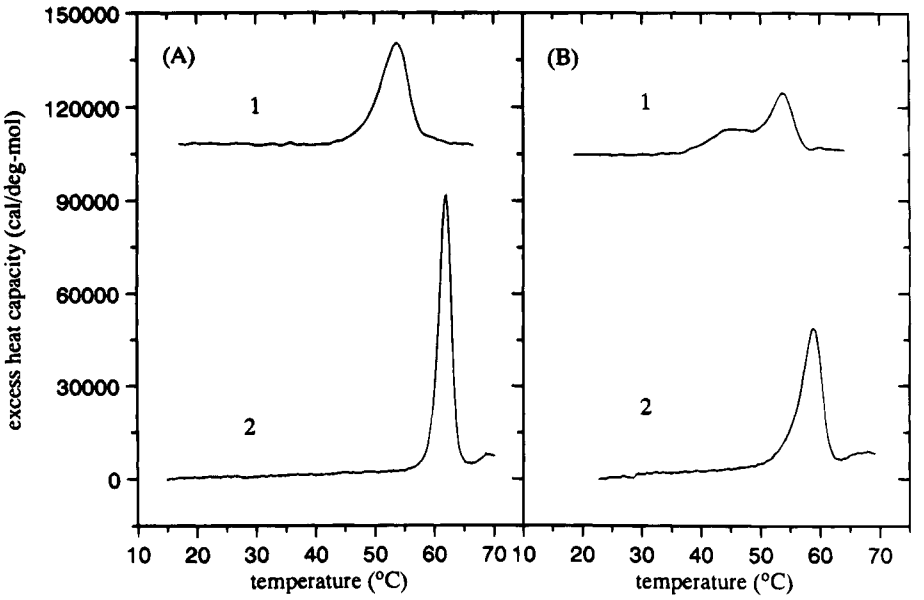

FIGURE 9: Comparison of DSC data for the thermal denaturation of (A) wild-type PGK and (B) Δ(413−415)PGK. Samples were prepared in 0.02 M triethanolamine−acetate buffer (pH 7.5) in the absence of substrates (1) and in the presence of 5.4 mM ADP, 5.4 mM 3-PG, and 6.5 mM Mg^{2+} (2). Concentrations of the wild type and Δ(413−415)PGK were 0.03 mM. Scanning rate was 60 °C/h. Transition temperatures and enthalpies calculated by fitting the experimental curves to one or two independent two-state transitions are shown in Table 3.

Table 3: DSC Parameters for Thermal Unfolding of WT PGK and Δ(413−415)PGK[a]

enzyme	additions	T_{m1} (°C)	ΔH_1 (kcal/mol)	T_{m2} (°C)	ΔH_2 (kcal/mol)	SD
WT PGK	none	52.3	89.9	54.1	141.4	286
	ADP + 3-PG + Mg^{2+}			62.0	278.7	1499
Δ(413−415)PGK	none	45.0	79.4	54.1	122.8	362
	ADP + 3-PG + Mg^{2+}	56.9	78.7	58.8	180.0	609

[a] Protein solutions (1.3−1.6 mg/mL) were in 20 mM triethanolamine−acetate buffer (pH 7.5). Concentrations of ADP, 3-PG, and Mg^{2+} were 5.4, 5.4, and 6.5 mM, respectively. DSC parameters were calculated by fitting the data shown in Figure 9 to one and two independent two-state transition models.

to the N-terminal domain, and the main peak centered near T_m = 54 °C, corresponding to the C-terminal domain (Brandts et al., 1989; Bailey et al., 1990). In light of the diverse nature of the amino acid substitutions in these mutants and their location in the amino acid sequence, the only common feature is their location in the interdomain region. The similarity of their heat denaturation profiles therefore may be related to the perturbation of domain−domain interactions, leading to a similarly reduced cooperativity. The change in ΔG_{AB} of about 2 kcal/mol was estimated from the change in T_m for the PGK mutant containing an A183P substitution in the hinge (Bailey et al., 1990). Although in the case of Δ(413−415)PGK, A183P, and F194L mutants, both CD spectra and kinetic parameters were unchanged (keeping in mind that changed properties indicate structural changes, while lack of change does not exclude perturbations not affecting kinetics or CD), structural information at atomic resolution is necessary to allow any quantitative consideration.

In the presence of ADP, 3-PG, and Mg^{2+}, WT PGK exhibits a single symmetric peak at higher temperature (T_m = 62 °C). Under the same conditions the Δ(413−415)PGK mutant exhibits an asymmetric peak (Figure 9, curve 2). A good fit can be obtained by assuming two independent two-state transitions with T_{m1} = 56.9 °C and T_{m2} = 58.8 °C, suggesting that domain−domain interactions are also weaker in the absence of three C-terminal amino acids than in WT PGK.

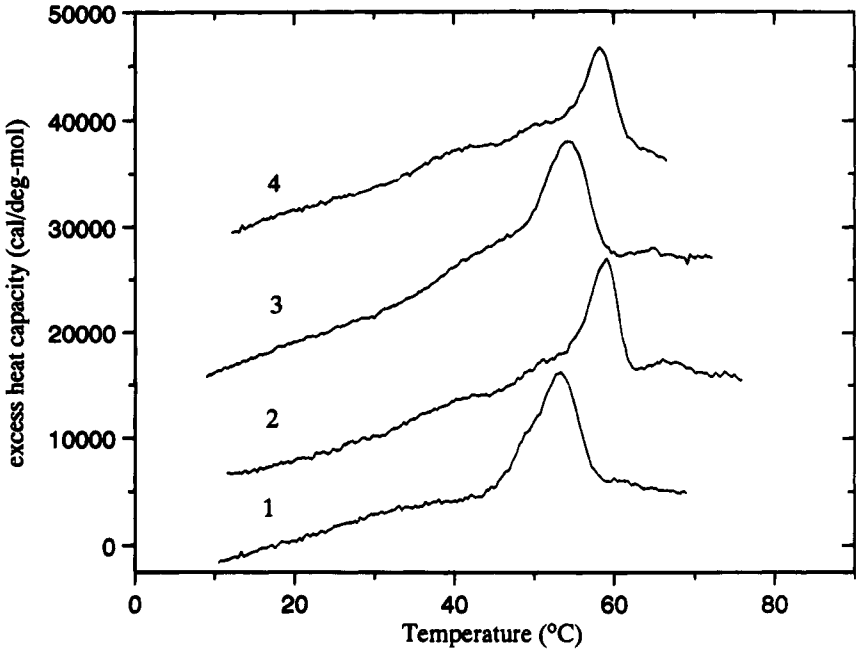

FIGURE 10: DSC scans for thermal unfolding of Δ(401−415)PGK in the absence and presence of substrates: (1) no additions; (2) 0.107 M Mg-ATP; (3) 0.18 M 3-PG; (4) 5.4 mM ADP, 5.4 mM 3-PG, and 6.5 mM Mg^{2+}. All samples were in 0.02 M triethanolamine−acetate buffer (pH 7.5). Protein concentrations were 0.04, 0.026, 0.032, and 0.038 mM, respectively. Scanning rate was 60 °C/h.

(ii). Figure 10 shows DSC scans for the Δ(401−415)-PGK mutant in the absence and presence of added substrates. In the presence of buffer alone (curve 1), a major transition centered near 54 °C is observed, which occurs at the same T_m as the B transition observed in the DSC scan of WT PGK. A small broad A transition centered near 35 °C is barely detectable in curve 1. The presence of a weak transition may indicate that the N-terminal domain is largely unfolded or is in a molten globule state. A low or not detectable heat absorption peak has been reported for other proteins in this state (Kuwajima, 1989; Yutani et al., 1992), although it has been argued that ΔC_p and ΔH cannot be zero and that an apparent lack of heat absorption observed in some cases may be the net result of a specific balance of positive and negative contributions (Haynie & Freire, 1993; Griko et al., 1994). It is also possible that as the T_m decreases, the peak becomes very broad and therefore undetectable (Haynie & Freire, 1993). The lack of detectable heat absorption therefore cannot be used as evidence for a molten globule state or for enthalpic equivalence of the unfolded and molten globule states (Haynie & Freire, 1993; Griko et al., 1994).

Addition of 3-PG results in a slight shift of the major transition by about 1 °C and a shift of a broad minor transition to higher temperature (near 43 °C; Figure 10, curve 3), suggesting partial refolding of the N-terminal domain. The addition of Mg-ATP alone (Figure 10, curve 2) or of ADP + 3-PG + Mg^{2+} (Figure 10, curve 4) produces a very similar effect, i.e., transition B shifts higher ($T_m \sim 59$ °C) and two additional minor transitions appear at lower temperatures, approximately 40 and 45 °C, again suggesting some refolding of the N-domain and the possible presence of intermediates with different degrees of structure. The DSC curves for this mutant could not be fitted satisfactorily to simple models; therefore, no quantitative data can be presented for this mutant.

SUMMARY AND CONCLUSIONS

The results of our studies demonstrate that short carboxy-terminal deletions affect the conformation of the entire molecule, with the N-domain and hinge perturbed more than the C-domain. Deletion of only three C-terminal amino acids resulted in a slight melting of the structure, reflected in altered enzymatic properties, thiol reactivity, and tryptophan fluorescence but not the near-UV CD spectrum. These results indicate that the C-terminus is important for attaining a native tertiary fold.

Conformational changes caused by the deletion of 15 C-terminal residues were much more dramatic.

(i) Effects on the N-Domain. The N-terminal domain in Δ(401−415)PGK exhibited several characteristics of a molten globule state: (1) A relatively small decrease in the far-UV CD signal indicates that *most of the secondary structure* has remained *intact.* (2) The *relative compactness* of the N-terminal domain (compact hydrophobic core) is suggested by small changes in the near-UV CD range of the tyrosine spectrum, indicating unchanged asymmetry of the environment of some of the tyrosine residues, presumably those buried in a relatively hydrophobic environment. A small decrease (10%) in the sedimentation constant (Mas & Resplandor, 1988) also indicates the compactness of Δ(401−415)PGK. (3) A decreased tyrosine contribution to the near-UV CD spectrum and an increased susceptibility of Cys-97, which in the native state is protected by tight packing interactions, to chemical modification both indicate the *disruption of tertiary interactions.* (4) Increased *ANS binding* and (5) a *barely detectable heat absorption peak* during thermal denaturation are also indicative of a molten globule state. The dramatic effect of C-terminal deletion on the conformational state of the N-terminal domain is not unexpected, because the C-terminal segment of the polypeptide chain is an integral part of the N-terminal domain.

(ii) Effects on the Interdomain Region. Perturbation of the interdomain region, which in PGK is essential for enzyme

activity and activation by sulfate (both require an intact interdomain cleft), can be postulated from the dramatic effect on both of the above characteristics and from the decreased binding affinity of 3-PG. Perturbation of domain−domain interactions may also be inferred from the effect on protein stability and the effect of substrates on thermal transitions in DSC experiments.

(iii) Effects on the C-Domain. Whether the structure of the C-terminal domain can also be classified as a molten globule state is less clear on the basis of the available information. It appears that the C-terminal domain in Δ-(401−415)PGK has some native-like features, as suggested by the unchanged K_m for ATP and by the native-like C-domain thermal transition and its response to ATP binding. At the same time, certain features characteristic of a molten globule state are also present. The lack of spectral features ascribed to tryptophan in the near-UV CD and increased tryptophan fluorescence (without a change in λ_{max}) both indicate the disruption of tertiary interactions, which can be attributed to melting of the C-domain. Melting can be described as a slight loosening of the structure, which would result in decreased quenching by internal protein groups, and changes in asymmetry but not in the polarity or solvent accessibility of the tryptophan environment. Since the disruption of tertiary interactions and compactness are only two of the criteria used to define the molten globule state that can be demonstrated directly for the C-domain, it might be more appropriate to describe the conformational state of this domain in the truncated protein [Δ(401−415)PGK] as a *native-like molten globule* (with a native-like tertiary fold) and the N-domain as a *disordered molten globule*, according to terminology proposed by Ptitsyn (1992). It is conceivable that the molten globule-like state detected in Δ(401−415)-PGK corresponds to an intermediate in the PGK folding pathway.

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