An engineered amino-terminal domain of yeast phosphoglycerate kinase with native-like structure

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Abstract

Previous studies have suggested that the carboxy-terminal peptide (residues 401–415) and interdomain helix (residues 185–199) of yeast phosphoglycerate kinase, a two-domain enzyme, play a role in the folding and stability of the amino-terminal domain (residues 1–184). A deletion mutant has been created in which the carboxy-terminal peptide is attached to the amino-terminal domain (residues 1–184) plus interdomain helix (residues 185–199) through a flexible peptide linker, thus eliminating the carboxy-terminal domain entirely. CD, fluorescence, gel filtration, and NMR experiments indicated that, unlike versions described previously, this isolated N-domain is soluble, monomeric, compactly folded, native-like in structure, and capable of binding the substrate 3-phosphoglycerate with high affinity in a saturable manner. The midpoint of the guanidine-induced unfolding transition was the same as that of the native two-domain protein ($C_m \sim 0.8$ M). The free energy change associated with guanidine-induced unfolding was one-third that of the native enzyme, in agreement with previous studies that evaluated the intrinsic stability of the N-domain and the contribution of domain–domain interactions to the stability of PGK. These observations suggest that the C-terminal peptide and interdomain helix are sufficient for maintaining a native-like fold of the N-domain in the absence of the C-domain.

Keywords: autonomously folding domains; isolated domains; phosphoglycerate kinase; protein engineering; protein folding

As early as 1973, it was proposed that protein domains represent stable folding units (Wetlaufer, 1973). Since then, a number of isolated domains have been designed, expressed, and characterized (Minard et al., 1989; Herold et al., 1991; Gilkes et al., 1993; Berger et al., 1994; Bessalle et al., 1994; Campbell & Downing, 1994; Constantine et al., 1994; Jecht et al., 1994; Li et al., 1994; Munro et al., 1994; Alexandrescu et al., 1995; Bertsch & Soll, 1995; Gale & Schimmel, 1995; Ghosh et al., 1995; Lappalainen et al., 1995; Lemmon et al., 1995; Nock et al., 1995; Parry et al., 1995; Riechmann & Davies, 1995; Shabb et al., 1995; Weiss et al., 1995; Klemm & Pabo, 1996). Many of these isolated domains adopt native-like folds capable of binding ligands and substrates. Others adopt a quasi-native fold of limited stability. The two domains of phosphoglycerate kinase are an example of the latter.

The crystal structure of yeast PGK (Watson et al., 1982; McPhillips et al., 1996) reveals that the amino-terminal domain (residues

1–184) is connected to the carboxy-terminal domain (residues 200–415) by a long α -helix (residues 185–199) (Fig. 1). When expressed individually (1–184, 185–415), both domains failed to adopt fully native-like structures (Fairbrother et al., 1989b; Minard et al., 1989; Missiakas et al., 1990). However, the isolated C-domain retained its ability to bind ATP, although with 30-fold lower affinity. In contrast, the N-domain lost completely its ability to bind 3-phosphoglycerate and other anions. Fairbrother and his colleagues (1989b) attributed this anomaly to the fact that the N-domain is discontinuous: the C-terminal peptide (residues 401–415) folds back onto the N-terminal domain and is thus an integral part of it, making numerous contacts with residues that contribute side chains to the “basic patch,” a cluster of positively charged residues that participates in anion and 3-PG binding (Fairbrother et al., 1989c, 1990; Sherman et al., 1990; Harlos et al., 1992; May et al., 1996; McPhillips et al., 1996).

The essential role of the C-terminal peptide in maintaining the stability of the N-domain was confirmed recently through a series of deletion mutants in which amino acids were deleted from the end of the complete enzyme (Mas & Resplandor, 1988; Mas et al., 1995). In the absence of the 15-amino acid tail ($\Delta 401$ –415), only 0.2% of the enzyme’s activity remains: the N-terminal domain becomes highly flexible, exhibiting properties of a molten globule, and the K_m for 3-PG increases eightfold. In contrast, the C-terminal

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Abbreviations: PGK, 3-phosphoglycerate kinase; 3-PG, 3-phosphoglycerate; ANS, 1-anilinonaphthalene-8-sulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); WT, wild-type; Gdn-HCl, guanidine hydrochloride.

Fig. 1. Structure of yeast phosphoglycerate kinase drawn using MOLSCRIPT (Kraulis, 1991). Coordinates are from Brookhaven file 1QPG (McPhillips et al., 1996). Features include the C-terminal peptide (residues 401–415, black), the interdomain helix (residues 185–199, shaded), cysteine-97 (black spheres), and basic patch histidines (His 62, His 167, His 170). A black dot indicates the location of residue 185, the site chosen by Minard et al. (1989) for engineering two isolated domains.

domain remains largely intact, the K_m for ATP changing little. NMR studies of a closely related mutant ($\Delta 404$ –415) revealed that removal of the last 11 amino acids significantly increases the flexibility of both domains and alters the tertiary environment of the adjacent basic patch region (Ritco-Vonsovici et al., 1995).

Given the essential role of the C-terminal "tail" in maintaining the integrity and stability of the N-terminal domain, an engineered version of this domain that incorporates the tail was designed (Fig. 2), thus making a discontinuous domain continuous (a similar approach was employed recently to create an autonomously folding coenzyme binding domain from D-glycerate 3-phosphate de-

hydrogenase; Jecht et al., 1994). The new construct ($\Delta 198$ –400) consists of amino acids 1–197 followed by C-terminal residues 401–415. A two-amino acid linker (Gly-Gly) was used to terminate the interdomain helix and create the sharp turn required for chain reversal. This paper describes the construction of this domain and the characterization of its stability and structural integrity. Because many multidomain proteins have "crossover linkages" (C-terminal segments that interact with N-terminal domains; Thornton & Sibanda, 1983), this methodology should prove useful in designing other isolated domains capable of folding autonomously.

Results

Design of the $\Delta 198$ –400 domain

Examination of the refined crystal structure of yeast PGK (McPhillips et al., 1996) revealed that five hydrophobic residues (Leu 405, Pro 406, Val 408, Phe 410, and Leu 411) belonging to the C-terminal tail (residues 401–415) pack against the N-domain (residues 1–185). Suitable fusion sites were therefore sought between residues 401 and 404. Of the four, residue 401 is positioned most favorably, being 12.8 Å from residue 197 of the interdomain helix (C_α – C_α distance). Molecular modeling suggested that a two-residue linker would span the distance, once some minor adjustments were made to the backbone torsion angles of residues 401 and 402.

Great care was taken in selecting amino acids for the linker. Terminating the interdomain helix a full turn ahead of its natural terminus meant that the first residue of the linker must also function as a helix breaker. Glycine was therefore selected as the first linker residue because about one third of all helices end with this amino acid (Schellman, 1980). Coordinates for the C-cap were borrowed from triosephosphate isomerase (residues 120–121 of Brookhaven file 1TIM), which includes a classic example of a helix terminated by glycine (Richardson & Richardson, 1989). Glycine was also chosen as the second linker residue because it is the only amino acid capable of accommodating the sharp chain reversal required for appending the natural C-terminus. The crude model was then refined using molecular dynamics and minimization.

Fig. 2. Stereo view of the modeled structure of $\Delta 198$ –400 PGK. Features include the glycine–glycine linker (white segment preceding C-terminal peptide), bound 3-PG (white spheres), and Tyr 193 (labeled with an asterisk). See Figure 1 legend for additional features.

Purification, identification, and preliminary characterization

The $\Delta 198$ –400 gene product was overexpressed in a yeast cell line that lacks endogenous PGK (Lam & Marmur, 1977). SDS-PAGE of the purified protein revealed a single band with no detectable proteolytic degradation products. Yields were comparable to those obtained for other PGK mutants (5 mg/L of culture). N-terminal sequencing confirmed that the N-terminus is blocked (in WT PGK, the N-terminal serine is N-acetylated).

Analysis by mass spectrometry yielded a molecular weight of 23,673 g/mol. This compares favorably with the calculated molecular weight of 23,667 g/mol. An apparent molecular weight was obtained using HPLC size-exclusion chromatography (Superose-12 column). In this procedure, a standard curve is generated based on the elution volumes of proteins of known molecular weight and Stokes radius. The elution volume of the $\Delta 198$ –400 domain corresponds to an apparent molecular weight of 23,793 g/mol and apparent Stokes radius of 22.93 Å. The latter compares favorably with the theoretical Stokes radius (22.91 Å) of a fully folded protein of molecular weight 23,673 g/mol (Uversky, 1993, Equation 1). No evidence of dimerization was apparent.

Reactivity of Cys 97

The reactivity of single Cys 97 is a good indicator of the compactness and structural integrity of PGK. This residue is essentially inaccessible to modification by DTNB in the native enzyme, but becomes more accessible when as few as three residues are deleted from the C-terminal tail ($k = 2.4 \times 10^{-4} \text{ s}^{-1}$) and highly accessible when the C-tail is deleted completely ($k = 1.6 \times 10^{-1} \text{ s}^{-1}$), even though Cys 97 is located 21 Å from the tail (Mas et al., 1995). The reactivity of Cys 97 in the $\Delta 198$ –400 domain ($k = 2.4 \times 10^{-3} \text{ s}^{-1}$) was greater than that of the $\Delta 413$ –415 mutant, but far less than that of the $\Delta 401$ –415 mutant. In contrast, Cys 97 in the previously reported N-domain ($\Delta 185$ –415) was found to be highly reactive ($k = 55 \text{ M}^{-1} \text{ s}^{-1}$; Minard et al., 1989).

CD spectra

CD spectra of the $\Delta 198$ –400 domain in the near- and far-UV range are shown in Figure 3. For comparative purposes, spectra of a PGK mutant in which both native tryptophans (W308 and W333, C-domain) have been replaced with phenylalanines (W–, Szpikowska et al., 1994) are superimposed. The W– mutant has seven tyrosines, six of which are also present in the isolated N-domain. The presence of a significant signal above 275 nm in the near-UV CD spectrum suggests that the tertiary environments of the six tyrosines in $\Delta 198$ –400 PGK are largely intact. The observed 27% decrease in signal at 278 nm relative to the W– mutant can be attributed to the absence of one tyrosine and/or perturbation of the environment of Tyr 193, an interdomain helix residue in the native enzyme (in the molecular model of $\Delta 198$ –400 PGK, this residue is almost fully exposed to solvent).

Surprisingly, the mean residue ellipticity of the $\Delta 198$ –400 domain at 220 nm is only half of that observed for the W– mutant, even though the predicted helix content of each protein is nearly the same (36% versus 40%). This observation is difficult to interpret given the strong signal in the near-UV range. One possible explanation is that the N-domain contributes less to θ_{220} than the C-domain. This explanation is consistent with CD-monitored guanidine-induced unfolding studies of single-tryptophan PGK mutants, all of which exhibit biphasic transitions (Sherman et al.,

Fig. 3. CD spectra in the (A) far-UV and (B) near-UV range. In each case, $\Delta 198$ –400 PGK is compared to W– PGK, a mutant in which both native tryptophans (308 and 333) have been replaced with phenylalanines. Because of these mutations, nearly all of the strongly absorbing aromatic residues (six of seven tyrosines) now reside in the N-terminal domain.

1995). The first unfolding transition, which reflects unfolding of the C-terminal domain, results in a 68% decrease in the CD signal at 220 nm, even though other parameters indicate that the N-terminal domain is still folded (Beechem et al., 1995; Sherman et al., 1995). It is also possible that not all of the signal at 220 nm derives from helix peptide bonds. Recent studies of BPTI and helical peptides have indicated that aromatic side chains often contribute significantly to this region of the far-UV CD spectrum (Manning & Woody, 1989; Chakrabarty et al., 1993). One can therefore speculate that Tyr 380, the only aromatic residue that is present in the W– mutant but absent in the $\Delta 198$ –400 domain, contributes to the observed difference, or perhaps even Tyr 193, whose environment is altered upon deletion of the C-domain. It has been established previously that the two tryptophans of WT PGK are responsible for 14% of the CD signal at 220 nm (Szpikowska et al., 1994).

Fluorescence spectra

Fluorescence emission spectra (not shown) were gathered at an excitation wavelength of 280 nm. Because no tryptophans are present, the spectra exhibit the expected maximum at 304 nm. A 10% increase in total intensity accompanied by a slight blue shift in the wavelength of maximum intensity (303 nm) is observed upon unfolding of $\Delta 198$ –400 PGK in 4 M Gdn-HCl. In contrast, unfolding of the W- mutant results in a 23% decrease in total intensity (Fig. 4). Again, the differences may be attributed to the absence of Tyr 380 in the $\Delta 198$ –400 domain or the altered environment of Tyr 193, whose exposure to solvent increases threefold upon removal of the C-domain according to a molecular model of this domain (Fig. 2).

Fig. 4. Equilibrium unfolding of $\Delta 198$ –400 PGK by Gdn-HCl as monitored by: (A) the intensity of tyrosine fluorescence measured at 304 nm (the wavelength of maximum emission), and (B) the far-UV CD signal at 220 nm. The fluorescence-monitored unfolding profile of W- PGK, a mutant that lacks both native tryptophans, is included for comparative purposes (closed symbols). In each case, the protein concentration was 2.24 μ M in 20 mM sodium phosphate, pH 7.5. Solid lines represent the nonlinear least-squares fits of raw data in the 0–4 M Gdn-HCl range to a two-state unfolding model. The pre- and posttransition baselines (dotted lines) were added to the figure using the slope and intercept values obtained from the nonlinear least-squares fit results.

Equilibrium unfolding transitions monitored by changes in far-UV CD signal and tyrosine fluorescence

Guanidine-induced equilibrium unfolding transitions were monitored by CD in the peptide bond absorption region (220 nm) and by changes in fluorescence intensity at the wavelength of maximum tyrosine fluorescence (304 nm), as shown in Figure 4. In each case, the transitions are monophasic and highly cooperative, suggesting that no intermediate is significantly populated at equilibrium. As shown in Table 1, the transition midpoints coincide, (~ 0.79 M Gdn-HCl), as do the calculated free energy changes ($\Delta G_0 \sim 3.25$ kcal/mol) and denaturant slopes ($m \sim 4.1$ kcal/mol·M).

The observed transition midpoint is nearly the same as that of the native enzyme (0.77 M Gdn-HCl, Szpikowska et al., 1994). Interestingly, it also coincides with the second transition midpoint of the W- mutant unfolding profile, a transition that has been attributed to unfolding of the N-domain (Sherman et al., 1995). In contrast, the free energy change of the $\Delta 198$ –400 domain is only one-third of that reported for the native enzyme. A similar value was reported for the $\Delta 185$ –415 isolated N-domain (Missiakas et al., 1990). Because domain–domain interactions contribute significantly to the free energy of folding of PGK (Brandts et al., 1989), it is not surprising that the sum of the isolated domain values does not equal the native enzyme value. The denaturant slope m , which is proportional to the difference in the solvent-exposed surface areas of the denatured and native states (Schellman, 1978), is also only one-third that of the native enzyme. However, it should be noted that deletion of the C-domain exposes a portion of the N-domain that is buried in the native enzyme, thus increasing the domain's surface area in the folded state. Therefore, a smaller net change in the solvent-accessible surface area of the isolated domain upon unfolding would be expected.

Also noteworthy is the twofold increase in the slope of the pre-transition baseline in the $\Delta 198$ –400 fluorescence profile relative to the W- profile. This is most likely due to an increase in the exposure of one or more tyrosines to solvent. According to our molecular model, the most likely candidate is Tyr 193 (Fig. 2), located near the domain–tail fusion site: its solvent-exposed surface increases threefold upon removal of the C-domain.

Binding of ANS

ANS is known to bind to hydrophobic regions of proteins that become exposed when a protein unfolds partially or enters the molten globule state (Semisotnov et al., 1991). When $\Delta 198$ –400 (2.24 μ M) was added to 13 μ M ANS, a 58% increase in total fluorescence intensity occurred, accompanied by an 18-nm blue shift in the intensity of maximum fluorescence (Fig. 5). This value is only slightly larger than that observed for the fully folded native enzyme (34%, 8-nm blue shift), and far less than that observed for the $\Delta 401$ –415 mutant (232%, 37-nm blue shift), whose N-domain is in the molten globule state (Mas et al., 1995). The moderate increase observed for $\Delta 198$ –400 PGK may represent binding of ANS to adjacent hydrophobic residues (Leu 2, Leu 191, Phe 194, Phe 163, Leu 405) that are exposed partially as a result of deleting the C-domain.

The binding of ANS was also studied as a function of denaturant concentration. These data (Fig. 6) indicate that the small amount of ANS that binds in the absence of denaturant is released in a cooperative manner as the protein unfolds. Stabilization of the protein due to bound ANS may explain the slightly higher unfolding

Table 1. Free energy changes, denaturant slopes, and transition midpoints for Gdn-HCl-induced unfolding of $\Delta 198-400$ PGK as monitored by fluorescence and CD spectroscopy^a

Technique	ΔG° (kcal/mol)		m (kcal/mol·M)		C_m (M)	
	$\Delta 198-400$	WT PGK ^b	$\Delta 198-400$	WT PGK	$\Delta 198-400$	WT PGK
Fluorescence	3.3 ± 0.5	10.9 ± 1.5	4.2 ± 0.6	13.9 ± 1.9	0.78 ± 0.03	0.78 ± 0.01
CD	3.2 ± 0.7	9.3 ± 0.8	4.0 ± 0.7	12.1 ± 1.1	0.80 ± 0.05	0.77 ± 0.01

^aValues reported are ± 1 standard deviation.^bAs reported by Mas et al. (1995).

transition midpoint calculated from these data ($C_m = 0.91 \pm 0.02$ M Gdn-HCl). No evidence for a molten globule steady-state unfolding intermediate is apparent in the ANS profile.

Structural integrity as assessed by NMR

A 1D proton NMR spectrum of the $\Delta 198-400$ domain in D₂O is shown in Figure 7. The extent of the chemical shifts' dispersion is indicative of a native-like protein. Many of the resonances assigned previously to histidine C2-H protons in the native enzyme (Fairbrother et al., 1989c) are present in the spectrum of the $\Delta 198-400$ domain, although some are shifted slightly (Table 2). Additional spectra acquired after storing the sample for 2–4 weeks at 4 °C were virtually identical, suggesting that it is stable. Also, no precipitation of the NMR sample at relatively high protein concentration was observed during this time, unlike the $\Delta 185-415$ N-domain reported previously (W.J. Fairbrother, pers. comm.).

Stability of the structure was also assessed by following the time course of amide exchange after rapidly exchanging the solvent from H₂O to D₂O. NMR spectra gathered after incubating the sample at 27 °C for 48 h (not shown) indicated that a significant number of amide resonances remain (7.9–9.6 ppm). These data suggest that the hydrogen bonded secondary and tertiary structures

of $\Delta 198-400$ PGK are more stable than those of the $\Delta 185-415$ isolated N-domain, in which all amide protons exchanged for deuterons with 48 h, even at 4 °C (Fairbrother et al., 1989b).

Substrate binding assessed by NMR titrations

The binding of 3-PG to the native enzyme causes NMR peaks assigned to various N-domain histidine resonances to shift (Wilson et al., 1988; Fairbrother et al., 1989c). Three of these histidines (His 167, His 170, His 62, Fig. 2) belong to a cluster of positively charged residues referred to as the "basic patch," a region located on the cleft side of the N-domain adjacent to the C-terminus (Watson et al., 1982; McPhillips et al., 1996). These resonances failed to shift when 3-PG was added to the $\Delta 185-415$ N-domain fragment (Fairbrother et al., 1989b). Titration experiments were therefore performed with the $\Delta 198-400$ domain to see if the "basic patch" was intact and capable of binding substrate.

Four of the well-resolved histidine C2-H proton peaks in the aromatic region were observed to shift in a saturable fashion upon addition of 3-PG (Fig. 8; Table 2), thus confirming the structural

Fig. 5. Fluorescence spectra of free ANS and ANS in the presence of WT PGK and the $\Delta 198-400$ N-domain. ANS (13.2 μ M) and protein (2.24 μ M) were prepared in 20 mM sodium phosphate buffer, pH 7.5. The excitation wavelength was 390 nm.

Fig. 6. Equilibrium unfolding of $\Delta 198-400$ PGK by Gdn-HCl in the presence of ANS (13.2 μ M). The fluorescence of bound ANS (wavelength of maximum emission = 482 nm) is plotted as a function of denaturant. The excitation wavelength was 390 nm. Fluorescence due to bound ANS was obtained by subtracting the spectrum of free ANS in denaturant from the spectrum of ANS plus protein in denaturant. Protein concentration was 2.24 μ M in 20 mM sodium phosphate, pH 7.5. Solid lines represent the nonlinear least-squares fits of raw data to a two-state unfolding model.

Fig. 7. 500 MHz ^1H -NMR spectrum of the $\Delta 198$ –400 N-domain in D_2O , 100 mM d_3 -acetate, pH 7.14, 27°C. Protein concentration was 0.7 mM. Inset shows detail of aromatic region. Several well-resolved histidine C2-H protons are labeled: (1) His 123; (2a, 2b) His 149 and His 53, unresolved; (3) His 62; (4) His 167.

integrity of the 3-PG binding site in this domain. These shifts are qualitatively the same as those observed when the native enzyme is titrated with 3-PG, but reduced in magnitude substantially (15–20% of the native enzyme values), much like shifts observed for many basic patch mutants (Fairbrother et al., 1989c; Sherman et al., 1992). The titration-induced shifts associated with resonances assigned tentatively to basic patch histidines 62 and 167 were used to calculate a binding constant for 3-PG. When the data are fit to an equation describing a single binding site (Live & Chan, 1976), a K_d value of 0.39 mM is obtained in both cases (Fig. 9). Although 40-fold greater than the value obtained for the native enzyme (0.01 mM), this value is comparable to values obtained for many basic patch mutants, described previously, containing single amino acid substitutions (Fairbrother et al., 1989a, 1989c; Sherman et al., 1992), all of which were active enzymati-

Table 2. Chemical shifts of C2-H histidine resonances in the 500 MHz ^1H -NMR spectrum of the $\Delta 198$ –400 N-domain construct and effects of 3-PG binding

Resonance	WT PGK chemical shift (ppm) ^a	$\Delta 198$ –400 PGK chemical shift (ppm)	3-PG induced change in chemical shift (ppm)	
			WT ^a	$\Delta 198$ –400
1 (His 123)	8.87	8.88	–0.05 ^b	–0.06
2a (His 149) ^c	8.16	8.04	+0.06	–0.07
2b (His 53)	8.10	8.04	+0.01	–0.09
3 (His 62)	7.95	7.98	+0.72	+0.17
4 (His 167)	7.81	7.71	+0.26	+0.04

^aAs reported by Fairbrother et al. (1989c).

^b(–) upfield shift; (+) downfield shift.

^cPeaks 2a and 2b resolve only upon addition of 3-PG.

Fig. 8. 3-PG-induced perturbations of the aromatic region of the 500-MHz ^1H -NMR spectrum of $\Delta 198$ –400 PGK. **A:** No 3-PG added. **B:** Fivefold molar excess of 3-PG added.

cally. For $\Delta 198$ –400 PGK, changes in the microenvironment of the basic patch region due to lack of the C-domain may explain the decrease in binding affinity.

Discussion

Previous attempts to isolate and characterize the amino-terminal domain of PGK have met with limited success. A fragment (residues 1–173) obtained by cyanogen bromide cleavage (Adams et al., 1985) was reported to refold into a native-like structure but to dimerize, thus precluding further characterization. An engineered version of the complete domain (residues 1–184) was characterized as having a “quasi-native” structure (Minard et al., 1989). Although the isolated domain ($\Delta 185$ –415) unfolded in a cooperative, reversible manner with a transition midpoint in Gdn-HCl equal to that of the complete enzyme (Missiakos et al., 1990), its structure was clearly not native-like based on its inability to bind 3-PG and on the high reactivity of Cys 97, normally inaccessible to DTNB. Subsequent NMR studies confirmed this interpretation (Fairbrother et al., 1989b). The authors concluded that interactions between the two domains, particularly those involving the C-terminal end and the N-domain, are essential for maintaining the integrity of the active site. PGK mutants in which portions of the C-terminal tail (but not the C-domain) have been deleted also suggest that the C-tail makes a significant contribution to the stability of the N-domain in the intact enzyme (Mas & Resplandor, 1988; Mas et al., 1995; Ritco-Vonsovici et al., 1995). We therefore designed

Fig. 9. Changes in chemical shifts of peaks 3 (●, His 62) and 4 (○, His 167) plotted as a function of 3-PG:Δ198-400 PGK molar ratio. Continuous lines represent theoretical binding curves corresponding to a dissociation constant (K_d) of 0.39 mM.

an isolated N-domain, referred to as Δ198-400 PGK, in which the C-terminal tail (residues 401-415) of the complete enzyme is connected to the N-domain using a linker that consists of a portion of the natural interdomain helix followed by two glycine residues.

A number of observations suggest that a soluble, monomeric, compactly folded protein with native-like structure was produced: (1) The Δ198-400 domain showed no tendency to aggregate *in vivo* or *in vitro*. (2) Gel filtration chromatography revealed a single species that eluted at the molecular weight corresponding to a monomer of 23,793 g/mol. (3) NMR peak widths were narrow, suggesting that aggregation did not occur even at a protein concentration of 0.7 mM. (4) The reactivity of Cys 97 toward chemical modification by DTNB was significantly less than that observed for Δ401-415 PGK, characterized previously as having a molten globule-like N-domain (Mas et al., 1995), and far less than that observed for the Δ185-415 isolated domain (Minard et al., 1989). (5) The Stokes radius of Δ198-400 PGK determined by size-exclusion chromatography (22.93 Å) compared favorably with the theoretical value of a fully folded protein of this molecular weight. (6) The amide exchange rate was far less than that observed for the Δ185-415 N-domain, in which all amide hydrogens exchanged for deuterons within 48 h at 4°C. For Δ198-400 PGK, resonances beyond 8.6 ppm were still visible in the NMR spectrum after one week at 27°C. (7) The signal at 278 nm in the near-UV CD spectrum of Δ198-400 (six tyrosines, no tryptophans) was strong, being only 30% less than the signal measured for a full-enzyme mutant containing seven tyrosines and no tryptophans (W-; Sherman et al., 1995). (8) The midpoints of the guanidine-induced unfolding transitions of Δ198-400 PGK monitored by CD and fluorescence spectroscopy coincided, and corresponded to that of the native enzyme (~0.79 M Gdn-HCl). (9) The binding of ANS to Δ198-400 PGK, a fluorescent dye used routinely to test for the presence of hydrophobic patches that are exposed in the molten globule state, was minimal. (10) The chemical-shift dispersion seen in the 1D proton NMR spectrum of Δ198-400 PGK was

extensive, indicating that most protons experience a unique environment. (11) The binding of 3-PG to Δ198-400 PGK, as monitored by shifts in NMR peaks assigned to basic patch histidines, was saturable, with a K_d comparable to values observed for many fully active PGK mutants. As stated by Herold et al. (1991), ligand binding is perhaps the most rigorous proof of native-like structure. In summary, these data indicate that the structure of the Δ198-400 domain closely resembles that of the N-domain in the native enzyme and suggest that the presence of the engineered "tail" promotes a more native-like fold.

These data also underscore the importance of long-range interactions in protein folding. In the linear sequence of the complete enzyme, 200 residues separate the N-domain from the C-terminal tail. One might argue that the native-like behavior of this domain derives not only from the presence of the C-terminal peptide, but also from the presence of a portion of the interdomain helix (residues 185-197), a segment of the chain not present in the Δ185-415 isolated domain. This, however, does not appear to be the case because the C-terminal deletion mutants described previously, Δ401-415 (Mas & Resplandor, 1988; Mas et al., 1995) and Δ404-415 (Ritco-Vonsovici et al., 1995), retain this helix, but still lack a native-like N-domain.

Although our results indicate that the C-terminal peptide plays a major role in maintaining the structural integrity of the N-domain, it does not fully restore the protein's affinity for 3-PG, suggesting that an intact basic patch may require the presence of adjacent elements, such as helix 14 (residues 394-401). Helix 14 precedes the C-terminal tail in the linear sequence; in the three-dimensional structure, it occupies the interdomain cleft (McPhillips et al., 1996).

The role of interdomain contacts in maintaining the thermodynamic stability of the individual domains within a multi-domain protein has been examined by a number of investigators (Hu & Sturtevant, 1987; Brandts et al., 1989; Freire et al., 1992). These studies were prompted by the observation that the unfolding behavior of multi-domain proteins is often highly cooperative (i.e., the domains unfold simultaneously), even though the intrinsic stabilities of each domain differ. In order to accommodate this phenomenon, Brandts and his colleagues proposed a thermodynamic model illustrating how the energy associated with domain-domain pairing in a two-domain protein (ΔG_{AB}) can mask differences in the intrinsic stabilities of the individual domains when large enough, thus resulting in cooperative unfolding behavior. For PGK, ΔG_{AB} amounts to several kilocalories per mole (Brandts et al., 1989).

This may explain why the free energy change associated with unfolding of the Δ198-400 isolated domain in Gdn-HCl ($\Delta G^\circ = 3.25$ kcal/mol) is only one-third of that released when the full enzyme unfolds ($\Delta G^\circ \sim 10$ kcal/mol, Mas et al., 1995) rather than the expected value of one-half (assuming that each domain contributes equally to the free energy of unfolding). A differential scanning calorimetry study of six mutant forms of PGK (Brandts et al., 1989) showed that destabilizing amino acid replacements in the interdomain region lowered the heat-induced unfolding transition midpoint of the N-domain without affecting the C-domain midpoint, thus illustrating the importance of interdomain interaction energy (ΔG_{AB}) in maintaining cooperative unfolding behavior. In addition to the native state, A-B, of a two-domain protein, in which ΔG_{AB} reflects the strength of the domain pairing interaction, Brandts et al. defined a reference state A*-B*, in which both domains are still folded but not interacting ($\Delta G_{AB} = 0$). The authors then proposed that it may be possible to isolate the reference state by introducing mutations that critically destabilize the

AB interface, or by isolating individual domains. Our previous study (Brandts et al., 1989) is an example of the former case, whereas the current study is an example of the latter.

Materials and methods

Reagents

3-PG, ANS, and all buffer components were obtained from Sigma Chemicals (St. Louis, Missouri). Gdn-HCl (sequanal grade) was from Pierce (Rockford, Illinois). Thermostable polymerase was purchased from Perkin-Elmer (Norwalk, Connecticut), and restriction enzymes were from New England Biolabs (Cambridge, Massachusetts). Deuterated solvents were from Aldrich (Milwaukee, Wisconsin).

Gene constructs

Nucleotides coding for amino acids 198–400 were replaced with TGG-TGG (Gly-Gly) using splice overlap extension PCR (Higuchi et al., 1988). Template DNA consisted of the WT yeast PGK gene cloned into shuttle vector yEP9T (Chen et al., 1984). The sequences of the four oligonucleotide primers were as follows:

- Primer 1: 5' -GAT-GCC-TTC-GGT-ACC-GCT-CAC-AGA-3' (Kpn I site)
 Primer 2: 5' -TCC-TTA-CCT-TCA-CCA-CCA-GCC-TTA-CCG-AAG-TAC-TTC-3' (Gly-Gly)
 Primer 3: 5' -CGG-TAA-GGC-TGG-TGG-TGA-AGG-TAA-GAA-ATT-GCC-AG-3' (Gly-Gly)
 Primer 4: 5' -GCA-TAT-AGC-GCT-AGC-AGC-ACG-CCA-3' (Nhe I site).

Primer 1, a forward primer, includes the codons of amino acids 161–168. Primer 2, a reverse primer, includes codons for amino acids 192–197 and 401–404, separated by a Gly-Gly linker. Primer 3, a forward primer, includes the codons of amino acids 195–197 and 401–407, separated by a Gly-Gly linker. Primer 4, a reverse primer, incorporates the *Nhe* I restriction site located 485 bases downstream of the PGK stop codon.

PCR reactions 1a and 1b (100 μ L each) included 5 ng of template and 100 pmol each primer (reaction 1a: primers 1 and 2; reaction 1b: primers 3 and 4). After a 2-min incubation of 94 °C, 35 cycles of amplification were performed (1 min, 94 °C; 1 min, 55 °C; 30 s, 72 °C), followed by a 7-min incubation at 72 °C. Primers for reaction 1a amplify a 129-base pair fragment; primers for reaction 1b amplify a 570-base pair fragment. Products of reactions 1a and 1b, purified by agarose gel electrophoresis and anion exchange column chromatography (Qiagen, Inc. Chatsworth, California), were then mixed, heated to 95 °C for 1 min, and annealed at 37 °C for 20 min. Primers 1 and 4 were then added (100 pmol each) and the above amplification cycle repeated (PCR reaction 2). The resulting product, a single 659-base pair fragment, was then digested with *Kpn* I and *Nhe* I and subcloned into the similarly digested WT PGK-yEP9T vector. Double-stranded dideoxy sequencing (Chen & Seeburg, 1985) using Sequenase version 2.0 (U.S. Biochemicals) was used to confirm the presence of the desired junctions and the absence of any secondary mutations in the PGK gene.

Expression and purification

The Δ 198–400 domain was overexpressed in *Saccharomyces cerevisiae* strain XSB44–35D, as described previously (Sherman

et al., 1992). This strain lacks endogenous PGK (Lam & Marmur, 1977). The overexpressed protein was purified by procedures used routinely for purification of the complete enzyme (Mas et al., 1986, 1988). The only modification involved extending the salt gradient used for eluting the Blue Sepharose dye-affinity column (the Δ 198–400 domain elutes between 0.45 M and 0.60 M KCl). A single band appeared on Coomassie-stained SDS-PAGE gels. Fifty milligrams were obtained routinely from 10 L of cells grown to an optical density of 2.0 (600 nm) in media containing glycerol/ethanol as a carbon source. Protein concentration was determined using a molar extinction coefficient of 8,940 $M^{-1} cm^{-1}$, a value calculated using the equation given by Pace et al. (1995). Our experience with other single-tryptophan and no-tryptophan PGK mutants indicates that coefficients predicted using this equation compare favorably with coefficients determined experimentally using the method of Gill and von Hippel (1989) (for the W-mutant, calculated value = 10,430 $M^{-1} cm^{-1}$, measured value = 10,728 $M^{-1} cm^{-1}$).

Mass spectrometry

Electrospray mass spectrometry was performed after desalting on a Vydac 3- μ m C₁₈ reverse-phase column using a linear acetonitrile/water gradient containing 0.1% trifluoroacetic acid. Spectra were gathered in positive ion mode using a TSQ-700 triple quadrupole instrument (Finnigan-MAT, San Jose, California).

Amino-terminal sequencing

The sequence of the amino terminus was checked by automated Edman degradation on a G1005A protein sequencing system manufactured by Hewlett-Packard. The sample consisted of 100 pmol of protein.

Gel filtration

Apparent molecular weights and Stokes radii were measured using a BioCADTM/SPRINTTM HPLC system (PerSeptive Biosystems, Framingham, Massachusetts) equipped with a Superose 12 column (Pharmacia Biotech, Piscataway, New Jersey). The column was equilibrated with 20 mM sodium phosphate, pH 7.5, 0.2 M NaCl at room temperature. Blue-dextran and acetone were used to determine the void and bed volumes, respectively. The system was calibrated with molecular weight standards ranging from 12 to 200 kDa (Sigma, St. Louis, Missouri). Injected samples (60 μ g) were detected at 226 nm.

Reaction of thiol groups with DTNB

The reactivity of cysteine-97 was measured using 5,5'-dithiobis(2-nitrobenzoic acid) as described previously (Mas et al., 1995).

Binding of ANS

The binding of 1-anilinonaphthalene-8-sulfonic acid was studied as described previously (Mas et al., 1995). ANS binding was also studied as a function of Gdn-HCl. Protein samples (2.5 μ M) were unfolded overnight at 25 °C, then ANS was added to a final concentration of 13 μ M. Fluorescence emission spectra (excitation = 390 nm) were recorded between 410 and 650 nm after a 5-min incubation. Samples lacking protein and/or ANS were measured in parallel.

Fluorescence spectra

Steady-state fluorescence measurements were performed at 25 °C using a Fluorolog-2 photon-counting spectrofluorometer (Spex Industries, Edison, New Jersey) as described previously (Sherman et al., 1995).

CD measurements

CD measurements in both the near- and far-UV range were conducted using a Jasco-600 spectropolarimeter as described previously (Sherman et al., 1995). Protein concentrations were 22.4 μ M and 2.24 μ M, respectively, in 20 mM phosphate buffer, pH 7.5. In each case, 10 scans were averaged.

Equilibrium unfolding experiments

Unfolding transitions were monitored at 25 °C using far-UV CD (ellipticity at 220 nm) and steady-state fluorescence techniques (excitation 280 nm, emission 290–450 nm), as described previously (Sherman et al., 1995). In each case, the protein concentration was 2.24 μ M in 20 mM sodium phosphate, pH 7.5. Guanidine hydrochloride (sequanal grade, Pierce) was used as a denaturant (0–4 M). Data analysis was performed by nonlinear least-squares fits of raw data to a two-state unfolding model (Santoro & Bolen, 1988; Jackson et al., 1993; Eftink, 1994) using commercially available software (SigmaPlot for Windows, Jandel, San Rafael, California).

NMR measurements

One-dimensional proton NMR spectra were obtained at 27 °C using a Varian Unity Plus 500 NMR spectrometer operating at 499.82 MHz proton frequency. Protein samples (550 μ L, 0.7 mM) were prepared in 100 mM sodium d_3 -acetate, 0.02% NaN_3 , in D_2O , as described previously (Fairbrother et al., 1989c). The pH, measured with an Ingold combination electrode, was adjusted to 7.14 with 0.4% (w/v) NaOD or DCl. All values quoted are uncorrected pH-meter readings. Proton chemical shifts were referenced to acetone at 2.21 ppm. The spectra were zero-filled from 4K to 16K real data points. A 90-degree sine-bell weighting function was applied to the free-induction decay prior to Fourier transformation.

3-PG binding

The binding of 3-PG to the Δ 198–400 domain was investigated by titrating a protein sample (0.7 mM, 550 μ L) with 1–4 μ L aliquots of 60 mM 3-PG prepared in D_2O /acetate buffer, pH 7.14, as described previously (Fairbrother et al., 1989c). The pH of the protein sample was remeasured after the titration was complete to ensure that the observed spectral changes were not due to changes in pH. Dissociation constants (K_d) were determined from the change in chemical shifts of resonances assigned to the basic patch histidines (Wilson et al., 1988; Fairbrother et al., 1989c).

Amide exchange time course

Rapid solvent exchange (H_2O to D_2O) was achieved using pre-packed Sephadex G-25 spin columns (5 Prime $\rightarrow$ 3 Prime, Inc., Boulder, Colorado) equilibrated with 100 mM d_3 -acetate, pH 7.14 in D_2O . One-dimensional NMR spectra (27 °C) were gathered at regular intervals over a one-week period starting 5 min after solvent exchange.

Molecular modeling

Modeling of the Δ 198–400 domain was accomplished on a 250-MHz Silicon Graphics Indigo2 High Impact (R4400) processor running the InsightII/Discover software package marketed by Biosym/MSI, Inc. (San Diego, California). The consistent valence forcefield (Dauber-Osguthorpe et al., 1988) was used for all simulations. The conformational space accessible to the Gly-Gly linker was explored using multiple short molecular dynamics runs (1 ps, 400 K) followed by conjugate gradient minimizations to a maximum derivative of 0.1 kcal/mol $\cdot$ Å.

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