Role of the C-Terminal Helix in the Folding and Stability of Yeast Phosphoglycerate Kinase†

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ABSTRACT: In order to determine the role of the C-terminal helix in the folding and stability of yeast phosphoglycerate kinase, a mutant deleted of the 12 C-terminal residues (PGK Δ404−415) was constructed. This mutant folds in a conformation very similar to that of the wild-type protein, but exhibits a very low activity (0.1% of that of the wild-type enzyme). The main structural effect of the deletion of the C-terminal helix is an increase in flexibility of the whole protein and a decrease in stability by about 5 kcal/mol. The structural properties of the truncated protein are very similar, at least qualitatively, to those in the isolated domains. The accessibility of the thiol group of Cys 97 is identical to that in the isolated N-domain. The large solvent effect on the tryptophan fluorescence in the native protein at very low concentration of denaturant reveals an increase of flexibility of the C-domain, similar to that observed on the isolated C-domain. NMR measurements show that the pH dependence of His C2H and C4H chemical shifts in the truncated protein perfectly matches those of the isolated domains. The addition of the missing peptide provokes a 40-fold increase in enzyme activity at saturation. A dissociation constant of 80 μM was determined. This peptide, which displays a random structure in solution, folds in a helical structure in the region 405−410 as assessed by TRNOESY. All these results show that the C-terminal part of yeast phosphoglycerate kinase is not necessary for most of the initial folding steps but acts to lock the C-domain on the N-domain, thus ensuring the expression of full enzyme activity. Without this sequence, the protein has the sum of the properties of the two isolated domains.

Phosphoglycerate kinase is a typical α/β monomeric protein. The three-dimensional structures of horse muscle (Banks *et al.*, 1979), yeast (Watson *et al.*, 1982), pig muscle (Harlos *et al.*, 1992), and *Bacillus stearothermophilus* (Davies *et al.*, 1991) phosphoglycerate kinases are very similar. The polypeptide chain is folded into two continuous structural domains of approximately the same size, referred to as the N- and C-domains. The folding properties have been intensively studied by several different groups (Betton *et al.*, 1984, 1985; Ballery *et al.*, 1990, 1993; Vas *et al.*, 1990; Semisotnov *et al.*, 1991; Varley & Pain, 1991; Nojima & Noda, 1979; Gast *et al.*, 1993) as a model to investigate the role of domains in the folding process. In the native enzyme, domain interactions involve helix V that links the two domains and C-terminal helices XIII and XIV interacting with the N-domain (Figure 1).

A detailed study of the folding process of the yeast enzyme has shown that the region located around residue 183 in the N-domain, which is in contact with helix XIV, becomes buried during the last phase of the folding process (Ballery *et al.*, 1993). This suggests that the contact between the N-domain and the C-terminal sequence (residues 404−415, including helix XIV) takes place only at a late stage of the folding process. Therefore, it is expected that the deletion of this C-terminal sequence might prevent the structural arrangement and block the protein conformation in a widely but incompletely folded state.

FIGURE 1: Three-dimensional structure of yeast PGK (Watson *et al.*, 1982). The deleted sequence is indicated in grey. Residues C97, W308, and W333 are shown.

Previous functional studies have shown that the enzyme devoid of the last 15 amino acids, corresponding to helix XIV and the preceding loop, exhibits approximately 1% of the activity of native PGK[1] and loses the ability to undergo sulfate-induced activation (Mas & Resplandor, 1988).

The isolated domains have been also produced by recombinant DNA methods and are able to refold independently

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in vivo as well as *in vitro* (Minard *et al.*, 1989a; Fairbrother *et al.*, 1989a; Missiakas *et al.*, 1990) to a structure very close to their native structure. However, local differences related to the absence of interdomain interactions have been observed by NMR, such as a perturbation of a cluster of basic residues in the isolated N-domain. Moreover, the chemical reactivity of the unique cysteine and the increased H/D exchange rate have suggested that the N-domain is more flexible when isolated than in the whole enzyme (Fairbrother *et al.*, 1989a).

The two isolated domains are unable to reassociate and do not regenerate an active nicked enzyme. This suggests that the interdomain interactions are, in this case, not strong enough to produce a fully complemented enzyme, although the isolated domains are folded and stable.

Conversely, several other pairs of contiguous PGK fragments obtained by chemical cleavage have been studied. Some of these fragments have a low degree of structure but are able to complement the complementary fragment to generate an active enzyme (Pecorari *et al.*, 1993). Altogether, these data indicate that the ability of protein fragments to reassociate functionally is not related to their ability to refold autonomously. Therefore, this property should not be used, without other information, as a criterion to define domain or structural unit.

The observation that the C- and N-terminal extremities of polypeptide sequences in proteins are often close one to another and involved in the domain interface has been pointed out by Thornton and Sibanda (1983). The structural arrangement of the C-terminal sequence observed in PGK could reflect a general constraint of folding and stability of many other proteins.

In order to obtain further information about the role of the C-terminal residues 404−415 in the folding and stability of PGK, we studied a PGK truncated by insertion of a stop codon at position 404; the 12 residues removed include helix XIV (PGK Δ404−415). Structural and functional properties of the mutant as well as its stability were characterized. The interactions of the complementary peptide (404−415) with the truncated protein were also investigated.

MATERIALS AND METHODS

Site-Directed Mutagenesis. The codon coding for Glu 404 was mutated in stop codon TAA by using a method adapted from Zoller and Smith (1982) as described previously (Minard *et al.*, 1989b). The coding sequence of the mutated gene was completely sequenced in order to check the absence of other mutations. It was then inserted in a pYE vector and overexpressed in *pgk Saccharomyces cerevisiae* strain BC3 (Piper & Curran, 1990), according to the previously published procedures (Minard *et al.*, 1989a).

Protein Purification. Protein purification was performed according to Minard *et al.* (1989a). The initial steps of purification were the same as for the wild-type enzyme, the only difference being the replacement of affinity chromatography on blue-Sepharose CL-6B by ion-exchange chromatography on a S-Sepharose Fast-flow column (6 × 2.5 cm) equilibrated in a 20 mM Bis-Tris buffer, pH 6.35, containing 1 mM EDTA, 1 mM 2-mercaptoethanol, and 500 μM PMSF. Elution was performed by a linear gradient of NaCl (0−500 mM; 10 cm/min) in the same buffer. Typically, 110 mg of PGK Δ404−415 was obtained from 30 g of fresh cells. The purity was determined by SDS/PAGE.

Peptide 404−415. HPLC-purified peptide (>95%) prepared by chemical synthesis was purchased from Neosystem (Strasbourg, France).

Enzyme Activity Measurements. Enzyme activity was measured by a coupled assay adaptated from Bücher (1955) as described by Betton *et al.* (1985). Enzyme activity unit (IU) was expressed in micromoles of 3-phosphoglycerate produced per minute. The protein concentration was determined by the absorbance at 278 nm using the same extinction coefficient as for the wild-type protein ($\epsilon = 0.49$ mL mg^{-1} cm^{-1}; Watson *et al.*, 1982) since only one aromatic residue (Phe 410) is included in the 12 missing residues.

Molecular Weight Determination. Analytical gel filtration was performed by HPLC on a Superose 12 column equilibrated in a 50 mM phosphate buffer, pH 7.5, containing 200 mM NaCl. The reference proteins used were transferrin (81 000), ovalbumin (43 000), β-lactalbumin (35 000), carbonic anhydrase (29 000), and α-lactalbumin (14 200). Typically, 100 μg (50 μL) of protein sample was injected on the column at each run.

Electrospray mass spectrometry was performed on a V.G. TRIO 2000 spectrometer. Protein samples (400 μg) were desalted by reverse-phase HPLC on an Aquapore BU300 column, by using a linear gradient of CH_3CH-H_2O containing 0.1% trifluoroethanol. Measurements were performed after injection of 10 μL of samples 10 μM, the solvent being 50% CH_3CN−49% H_2O−1% HCOOH at a temperature of 70 °C.

Reactivity of the Thiol Groups. The reactivity of the thiol groups toward 5,5′-dithiobis(2-nitrobenzoate) (NbS_2) was measured at 20 °C in a 20 mM Tris-HCl buffer, pH 7. The reaction was started by the addition of a small volume of 5 mM NbS_2 (150−300 μM final concentration) to 1 mL of 7 μM PGK solution. The reaction was followed by monitoring the absorbance of the NbS^- at 412 nm ($\epsilon = 14\,150$ M^{-1} cm^{-1}; Riddles *et al.*, 1979) with a Cary spectrophotometer equipped with a thermostated cell holder and connected to a microcomputer for data acquisition. The apparent first-order rate constants were calculated from the whole data set by using a nonlinear regression (Marquardt algorithm; Press *et al.*, 1986). Second-order rate constants were obtained by linear regression of the dependence of the apparent first-order rate constant upon NbS_2 final concentration.

Unfolding−Refolding Studies. All the unfolding−refolding experiments were carried out in a 20 mM Tris-HCl buffer, pH 7.5, containing 500 μM EDTA and 1 mM 2-mercaptoethanol at 22 °C. The final concentration of protein during incubation in Gdn-HCl was 2 μM except for enzymatic activity assays where the protein concentration during incubation in Gdn-HCl was 5 μM. Activity or spectroscopic measurements were performed after 12 h incubation. For the denaturation experiments, the protein solutions were incubated in increasing concentrations of

[1] Abbreviations: Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; Gdn-HCl, guanidine hydrochloride; NbS_2, 5,5′-dithiobis(2-nitrobenzoate); NOESY, nuclear Overhauser effect spectroscopy; PAGE, polyacrylamide gel electrophoresis; PGK, phosphoglycerate kinase (EC 2.7.2.3); PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; TRNOESY, transferred nuclear Overhauser effect spectroscopy; UV, ultraviolet.

Gdn−HCl, while for renaturation experiments, a stock protein solution (about 100 μM) was incubated 7 h in buffer containing 4 M Gdn-HCl before dilution to the desired final concentration of denaturant.

Ultrapure Gdn-HCl was obtained from Pierce; the denaturant concentrations were chekced by refractometry, using the relationship provided by Nozaki (1970).

The fluorescence emission spectrum of each sample was recorded from 280 to 400 nm on a SPEX fluorimeter equipped with a thermostated cell holder and connected to a microcomputer for data acquisition. Excitation was at 293 nm. Transition curves were constructed by plotting either the shift in maximum wavelength or the variation of fluorescence intensity versus the denaturant concentration.

When the transition was assessed by circular dichroism, the CD spectrum was recorded from 260 to 210 nm for each sample, on a Mark V dichrograph (Jobin-Yvon) equipped with a thermostated cell holder and connected to a microcomputer for data acquisition. Transition curves were constructed by plotting the variation in ellipticity at 220 nm as a function of the denaturant concentration.

The enzyme activity was also used as a signal. The transition curves were constructed by plotting the variation of residual activity of each sample as a function of denaturant concentration. Control experiments were performed to show that, during the time required to perform activity measurements, the renaturation of PGK was negligible.

The experimental data were analyzed by using the model of linear dependency of ΔG upon denaturant concentration x (Pace, 1986):

$$\Delta G_x = \Delta G_0 - mx \tag{1}$$

In order to take into account of the solvent effect on signals, the following equation was used:

$$y_x = y_n + s_n x + \left\{\frac{e(\Delta G_0 - mx)/RT}{1 + e(\Delta G_0 - mx)/RT}\right\}[A + (s_d - s_n)x] \tag{2}$$

where y_x is the experimental signal in the presence of x molar Gdn-HCl, y_n is the signal of the native form, s_n and s_d are the solvent effects on the native and denatured protein signal, respectively, and A is the amplitude of the transition. Experimental data were fitted according to eq 2 by using a simplex procedure based on the Nelder and Mead algorithm (Press *et al.*, 1986).

Fluorescence Quenching. Fluorescence quenching experiments were carried out by the addition of a 4 M acrylamide solution to 4 μM protein solutions previously incubated overnight in Gdn-HCl. The recorded fluorescence intensity at 350 nm after excitation at 293 nm was adjusted for dilution and acrylamide absorbance using a molar extinction coefficient for acrylamide of 0.236 M^{-1} cm^{-1} (Eftink & Ghiron 1976). Quenching data were analyzed according to Stern−Volmer plot (Lakowicz, 1983).

^{1}H-NMR Spectra and pH Titration. The proteins (wild-type PGK and PGK Δ404−415) were dissolved in 100 mM sodium acetate-d_3/D_2O buffer, pH 7.00, and were desalted on a Sephadex G25 column equilibrated with the same buffer. The protein solutions were concentrated using Amicon Centricon 30 microconcentrators. The final concentration of each sample was determined using UV absorbance and was about 1 mM.

NMR spectra were recorded on a Bruker AM400 spectrometer using a spectral width of 5000 Hz. The spectra were collected with 8192 data points as sums of 64 or 128 transients. Presaturation of the residual solvent protons was performed during the 1.4 s relaxation delay prior to each acquisition. The FIDs were zero-filled to 16 384 points before Fourier transformation. Spectra were recorded at 297 K.

pH adjustments of the protein samples were made with either NaOD or DCl (0.01 N). The pH values quoted are uncorrected meter readings on a Knick 646 pH meter, using an Ingold combination electrode inserted directly into a 5 mm NMR tube. The chemical shifts were determined using acetone as an internal standard (δ = 2.214 ppm).

RESULTS

Characterization of Truncated PGK. In gel filtration, a single symmetric peak was observed with an elution volume corresponding to a monomer of about 44 500, indicating that the protein does not aggregate and possesses a compact globular structure. More accurate data were obtained by electrospray mass spectrometry. The molecular weights of the wild-type and truncated PGK were 44 662 and 43 360, respectively. These data are in good agreement with the theoretical molecular weights expected from the amino acid composition of each molecule (44 640 and 43 350, respectively). The specific activity of the truncated PGK was 1.8 IU/mg, which represents only 0.1% of wild-type specific activity (2100 IU/mg). This result is consistent with previous studies that have shown that the C-terminal part of the protein contributes significantly to the expression of the catalytic activity (Mas & Resplandor, 1988).

The far-UV CD spectrum of the truncated protein is identical, within experimental errors, to that of the wild-type protein (data not shown). Deconvolution using Chang's method (Yang *et al.*, 1986) revealed about the same content of α helices as expected from the crystallographic data (31% of α helices instead of 33% expected from X-ray data of wild-type protein).

The near-UV CD spectra in the region 340−250 nm are presented in Figure 2. The spectrum of the truncated protein shows a slightly weaker intensity, indicating a partial loss of asymmetry in the environment of aromatic residues. However, the polarity of the environment of the tryptophan residues is not significantly modified, as indicated by the maximum wavelength emission fluorescence which is shifted by only 1 nm (334 nm for the truncated protein instead of 333 nm for the wild-type protein; data not shown).

Accessibility of Cys 97 Residue. The unique thiol group, Cys-97, located in the N-domain, is buried in wild-type PGK, and the rate constant of the reaction with NbS_2 is independent of reagent concentration, the first-order rate constant being $k = 6 \times 10^{-5}$ s^{-1}. In the truncated protein, the thiol group is more reactive and the labeling occurs with a second-order rate constant of k = 63 M^{-1} s^{-1}, indicating that this side chain is more accessible in the truncated protein than in the wild-type protein. A similar value (55 M^{-1} s^{-1}) has been reported for the isolated N-domain (Minard *et al.*, 1989a). The reactivity of the thiol group is at least increased 5-fold when the protein is denatured in 1 M Gdn-HCl (k = 393

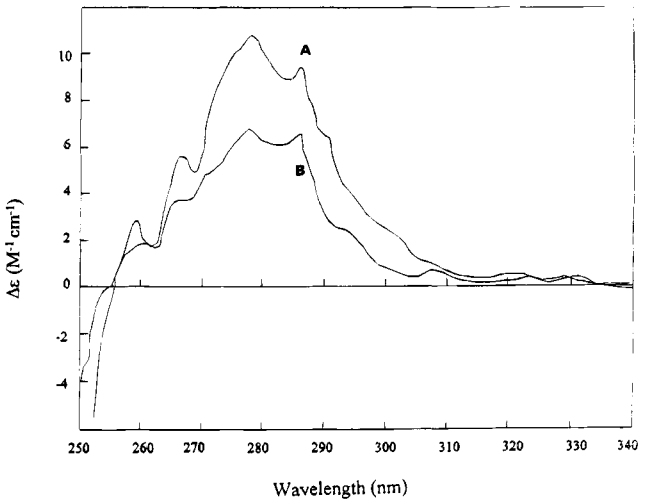

FIGURE 2: Near-UV CD spectra of the wild-type protein (A) and mutant Δ404−415 (B). Spectra were recorded at 22 °C in a 10 mM phosphate buffer, pH 7.5, containing 1 mM EDTA and 500 μM DTT. Protein concentration was 20 μM in a 10 mm path cell.

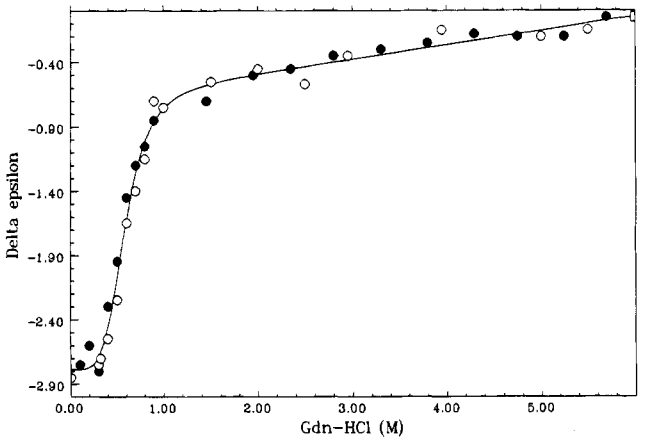

FIGURE 3: Unfolding−refolding transition as assessed by circular dichroism at 220 nm: (●) unfolding process; (○) refolding process. Experimental data were fitted according to eq 2.

M^{-1} s^{-1}). Thus, the Cys 97 residue is more accessible in PGK Δ404−415 than in the native wild-type protein but significantly less than in the fully denatured protein.

Unfolding−Refolding Transition of PGK Δ404−415 As Assessed by Circular Dichroism. The transition of PGK Δ404−415 was followed by measuring the variation in ellipticity at 220 nm, which reflects the decrease in ordered structures for increasing concentrations of Gdn-HCl. The transition curve is shown in Figure 3. This curve is symmetrical and the transition reversible as reported for wild-type yeast PGK (Ballery *et al.*, 1990). Quantitative analysis of this curve (Table 1) shows that the C_m value (0.54 M) and cooperativity of the truncated protein were significantly lower than for wild-type PGK. This reflects the lower stability of the mutant: the $\Delta G°$ value obtained by curve fitting using eq 2 is only 2.7 kcal/mol, compared to 7.8 kcal/mol for the wild-type and 4.0 and 4.5 kcal/mol for the N- and C-domains, respectively (Missiakas *et al.*, 1990).

Unfolding−Refolding Transition of PGK Δ404−415 As Assessed by Tryptophan Fluorescence. The two tryptophan residues of PGK are localized in the C-domain. Thus, the effect of Gdn-HCl on the fluorescence properties of PGK reflects the conformational events that occur in the C-domain.

The analysis of fluorescence emission spectra at different denaturant concentrations shows the existence of two different events during the denaturation: a shift in the maximum emission fluorescence wavelength (from 334 nm for the native form to 352 nm for the fully denatured protein) and a change in the fluorescence intensity which occurs in three steps: (i) an increase in fluorescence intensity without change in the position of the maximum emission fluorescence wavelength, for concentrations of denaturant lower than 0.5 M Gdn-HCl; (ii) a decrease in fluorescence intensity accompanied by the shift in the maximum emission fluorescence wavelength; and (iii) a new increase in fluorescence intensity for concentrations of denaturant higher than 2.0 M. Due to the complexity of the phenomenon, two kinds of transition curves were drawn: one reflecting the shift in the maximum fluorescence emission wavelength, and the second reflecting the change in fluorescence intensity versus denaturant concentration.

The transition curve obtained from the shift in the maximum fluorescence emission wavelength is reported in Figure 4. The transition was found to be completely reversible and symmetrical, with a C_m value of 0.59 ± 0.01 M, the corresponding $\Delta G°$ value being equal to 2.8 kcal/mol, comparable to that obtained by CD measurements.

The transition curve obtained from the change in fluorescence intensity at 335 nm is reported in Figure 5. This transition is reversible but asymmetrical. It differs from the transition observed for wild-type PGK, for which the asymmetrical transition can be deconvoluted in two resolved transitions of opposite amplitude. However, this transition is qualitatively comparable to that observed for the isolated C-domain (Missiakas *et al.*, 1990).

Analysis of Low Gdn-HCl Concentration Effects. In order to further analyze the denaturant effect on the protein for Gdn-HCl concentrations below 0.6 M, two kinds of experiments were performed: on the one hand, the unfolding transition was followed by variation of enzyme activity, and on the other hand, tryptophan accessibility at different concentrations of Gdn-HCl was studied by fluorescence quenching studies.

The transition curve displayed in Figure 6 is highly cooperative, with a midpoint transition of 0.59 M. Furthermore, it clearly appears that the activity of the truncated protein is not modified by the denaturant up to 0.5 M Gdn-HCl.

Tryptophan fluorescence quenching studies were performed by using acrylamide as quencher. The relative tryptophan susceptibility to quenching was determined for the truncated protein in different denaturant concentrations: 0, 0.25, 0.5, 0.6, and 2 M Gdn-HCl. All fluorescence measurements were carried out at 350 nm. Stern−Volmer plots are shown in Figure 7. In Figure 7A, the relative tryptophan accessibilities of truncated and wild-type PGK are compared. These data indicate that the Stern−Volmer constant is slightly higher for the truncated protein than for the wild-type protein. This is consistent with the fact that the maximum fluorescence emission wavelength of native PGK Δ404−415 is red shifted by only 1 nm and with data obtained from near-UV CD measurements indicating a slight loss of asymmetry around tryptophan residues. All these data indicate that the environment of the tryptophans in truncated and wild-type PGK is not significantly different.

Table 1: Unfolding−Refolding Parameters of Wild-Type Yeast Phosphoglycerate Kinase, Mutant Δ404−415, and the Isolated Domains

	signal	wild-type[a]	mutant Δ404−415	isolated C-domain[a]	isolated N-domain[a]
	CD	0.80 ± 0.02	0.54 ± 0.01	0.63 ± 0.02	0.80 ± 0.02
C_m (M)	$\Delta\lambda_{max}$	0.73 ± 0.02	0.59 ± 0.01	0.73 ± 0.02	
	F_{Int}[b]	0.73 ± 0.02	0.58 ± 0.07[c]	0.57 ± 0.08	
Gdn-HCl	activity	0.70 ± 0.02	0.59 ± 0.01	na[d]	na
	CD	12.8 ± 0.1	4.4 ± 0.4	7.8 ± 0.1	6.5 ± 0.1
n	$\Delta\lambda_{max}$	9.0 ± 0.1	5.1 ± 0.5	6.8 ± 0.1	
	F_{Int}[b]	13.0 ± 0.1	3.2 ± 0.8[c]	4.5 ± 0.1	
$\Delta G°$ (kcal/mol)	CD	7.8 ± 0.5	2.7 ± 0.4	4.5 ± 0.5	4.0 ± 0.5
	$\Delta\lambda_{max}$		2.8 ± 0.3		
m (kcal/mol^2)	CD	9.7 ± 1.3	4.6 ± 0.6	7.6 ± 1.5	5.1 ± 1.1
	$\Delta\lambda_{max}$		4.8 ± 0.5		

[a] From Missiakas *et al.*, 1990. [b] Data for the first transition assessed by variation in fluorescence intensity. [c] From Figure 5 for mutant Δ404−415. [d] nd: not available.

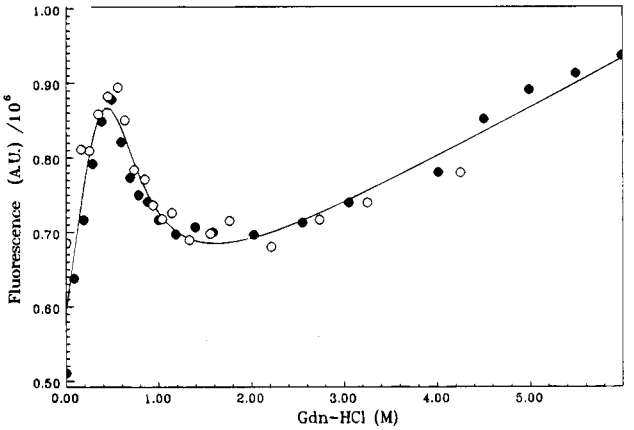

FIGURE 4: Unfolding−refolding transition as assessed by variation of the maximum fluorescence emission wavelength (excitation wavelength: 293 nm). (●) Unfolding process; (○) refolding process.

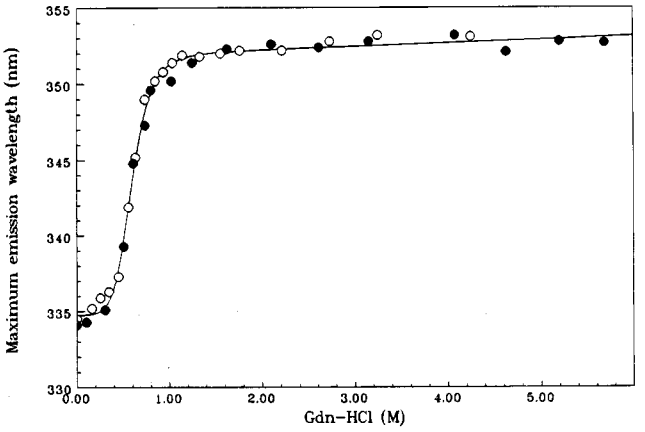

FIGURE 5: Unfolding−refolding transition as assessed by variation of the fluorescence emission at 335 nm (excitation wavelength: 293 nm). (●) Unfolding process; (○) refolding process. Experimental data were fitted according to eq 2.

The Stern−Volmer plots of fluorescence quenching measured in different denaturant concentrations are shown in Figure 7B. Two families of related curves can be distinguished: the first one corresponds to the truncated protein in denaturant concentrations lower than 0.6 M, and the second one corresponds to protein in denaturant concentrations of 0.6 M and higher.

NMR Spectrum of PGK Δ404−415. The 1D NMR spectrum of yeast PGK Δ404−415 was acquired in D_2O in order to simplify the spectrum by removing labile amide

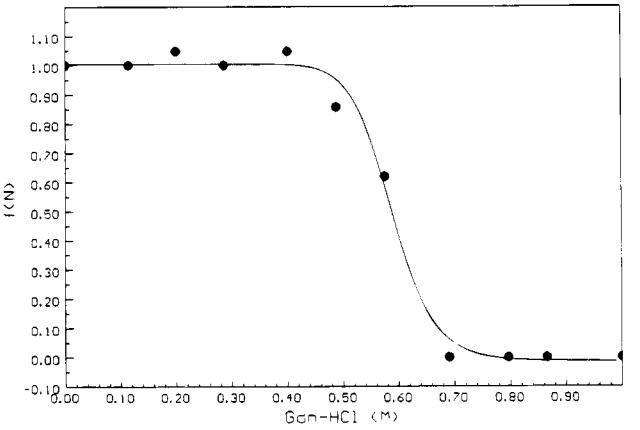

FIGURE 6: Unfolding−refolding transition as assessed by variation of the enzymatic activity (unfolding process).

resonances so that the chemical shifts of the aromatic resonances can be monitored. This spectrum (data not shown) displayed well-spread resonances and was roughly similar to the wild-type PGK spectrum (Fairbrother *et al.*, 1989a). No tentative assignments were made other than for one well-isolated downfield resonance at about 8.8 ppm which we assigned to the C-2 proton of the histidine 123 on the basis of its similar chemical shift in wild-type PGK. This assignment was confirmed by the pH dependence of its chemical shift.

The chemical shifts of the aromatic resonances arising from histidine residues of mutant Δ404−415 were measured as a function of pH over the pH range 5.1−8.8. The titration curves obtained are compared with those of the wild-type protein in Figure 8. The data were fitted to an adapted Hill equation with a nonlinear least-squares fitting procedure (Markley, 1975). The pK_A values of a number of the imidazole ring resonances deviate from those expected for histidines in a "random peptide" (pK_A = 6.6; Markley, 1975). It is notable that all the observable histidine peaks of the mutant shift within the pH range investigated, in contrast to wild-type PGK where some resonances do not shift with pH. Such a behavior indicates that these residues which have an abnormal pK_A in the wild-type PGK recover normal pK_A in PGK Δ404−415 mutant, reflecting either a greater solvent accessibility or a change in local environment.

If we compare the data we obtained to similar experiments made on the two isolated domains, it is interesting to note that the pH titration curves of mutant PGK appear to be simply the combination of the curves established for the

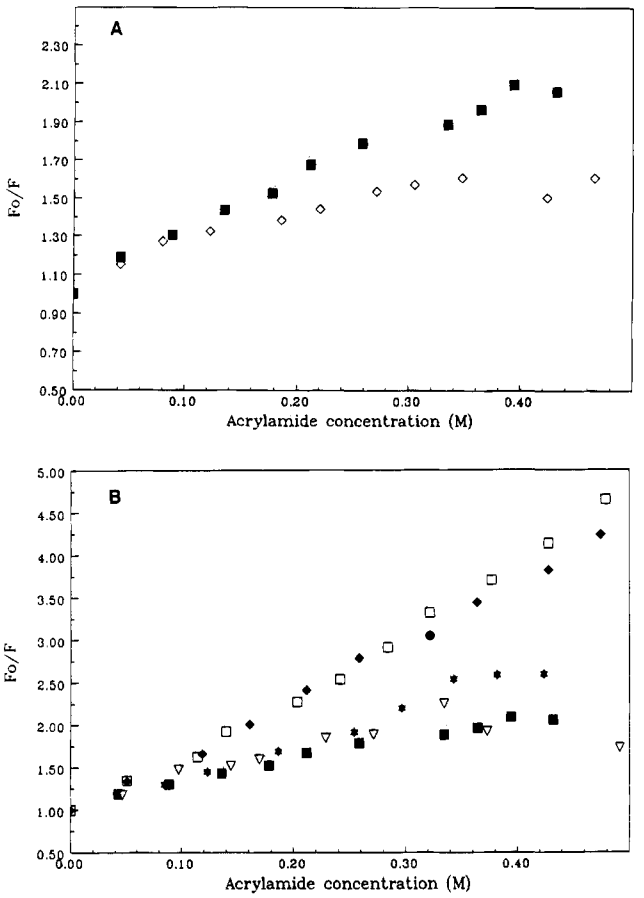

FIGURE 7: Stern–Volmer plot of the steady-state quenching of PGK by acrylamide at 350 nm. (A) Comparison of tryptophan accessibility in wild-type protein (◇) and mutant Δ404–415 (■). (B) Stern–Volmer plot of the steady-state quenching of mutant Δ404–415 in presence of 0 M (■), 0.25 M (▽), 0.5 M (★), 0.6 M (◆), and 2 M (□) Gdn-HCl.

isolated N- and C-domains (see Figures 2 and 4 in Fairbrother *et al.*, 1989b). For the resonances we observed, the pH titration curves match almost perfectly those of the isolated domains. Hence it appears that the structural statements made on these domains can be directly applied to the N- and C-domains of PGK Δ404–415 mutant, i.e., that these domains are destabilized compared to wild-type PGK.

Complementation Experiments. Previous studies (Pecorari *et al.*, 1993) have shown that protein fragments smaller than a domain can associate with complementary fragments to form complexes with enzymatic activity although the fragments are devoid of structure when isolated. This question was also addressed in the present work, and the interactions of the truncated protein with the complementary peptide 404–415 were investigated.

Functional complementation was assessed by studying the dependence of the enzymatic activity upon peptide concentration. Addition of the peptide to PGK Δ404–415 induces an increase in the enzymatic activity during the dead time of manual mixing. This spontaneous complementation depends on the peptide concentration according to a saturation curve (Figure 9) which displays a plateau corresponding to 4.15% of the wild-type specific activity, i.e., a 40-fold increase in specific activity of the truncated protein. A dissociation constant of 80 μM was measured.

Since a functional complementation was observed, the effect of the peptide binding onto the protein structure was studied by transferred NOE NMR experiments. These experiments showed large structural differences between the structures of the free peptide and the peptide when bound to the protein. The peptide in solution displays an NMR spectrum characteristic of a random structure. However, TRNOESY spectra of the peptide in the presence the protein exhibit cross-peaks characteristic of a helical structure in the region 405–410 (see following paper: Andrieux *et al.*, 1995).

DISCUSSION

As an initial step, it was important to check that the truncated protein was homogeneous and corresponded to the gene sequence since truncated proteins are often susceptible to proteolytic attack. Gel chromatography as well as gel electrophoresis both showed that the purified protein corresponded to a monomer of the expected molecular mass. These results indicate that the truncated protein behaves as expected and possesses a globular structure. The exact molecular weight measured by electrospray mass spectrometry corresponded, in the limits of accuracy of the method, to the molecular weight expected from the gene sequence. This protein has a very reduced specific activity (0.1% of the wild-type activity). Previous studies performed on another truncated protein (PGK Δ404–415) also displayed a very low specific activity (1%) although this activity has been measured for the reverse reaction (Mas & Resplandor, 1988). The work presented in this paper brings further details about structural effects of the deletion. PGK Δ404–415 possesses four more amino acids than PGK Δ401–415 and quite the same residual activity. It is likely that the decrease in enzyme activity mainly results from the loss of interactions between the N-domain and the sequence 404–415. The main structural effect of the deletion of the C-terminal helix is an increase in accessibility of different parts of the protein. The high reactivity of residue Cys 97 indicates that this residue is more accessible to the solvent in the truncated protein than in the wild-type protein.

In the wild-type protein, Cys 97 is totally buried. The increase in its accessibility is probably not caused by a drastic change in the protein structure since the far-UV CD spectrum of Δ404–415 mutant is comparable to that of wild type. Moreover, the Δ404–415 mutant displays enzymatic activity. Thus, it is more likely that this higher accessibility of the thiol group reflects an increase in the flexibility of the protein. This hypothesis is supported by the comparison of these data with results previously reported on isolated domains. For example, the reactivity of Cys 97 in PGK Δ404–415 is comparable to that in the N-domain. Furthermore, NMR data concerning histidine residues in truncated protein indicate that the pH titration curve appears to be the simple combination of the curves previously obtained for the isolated N- and C-domains (Fairbrother *et al.*, 1989b). It has been reported that the isolated N-domain is more flexible than the whole enzyme. Indeed, NMR measurements have shown that all amide protons exchange faster than in the whole enzyme (Fairbrother *et al.*, 1989b). A significant degree of flexibility of the structure of the N-domain is also consistent with the reactivity of the thiol group (Minard *et al.*, 1990a) and the susceptibility to proteolysis (Betton *et al.*, 1992).

So, at least for the N-domain of the truncated protein, it seems reasonable to interpret the change in reactivity of the

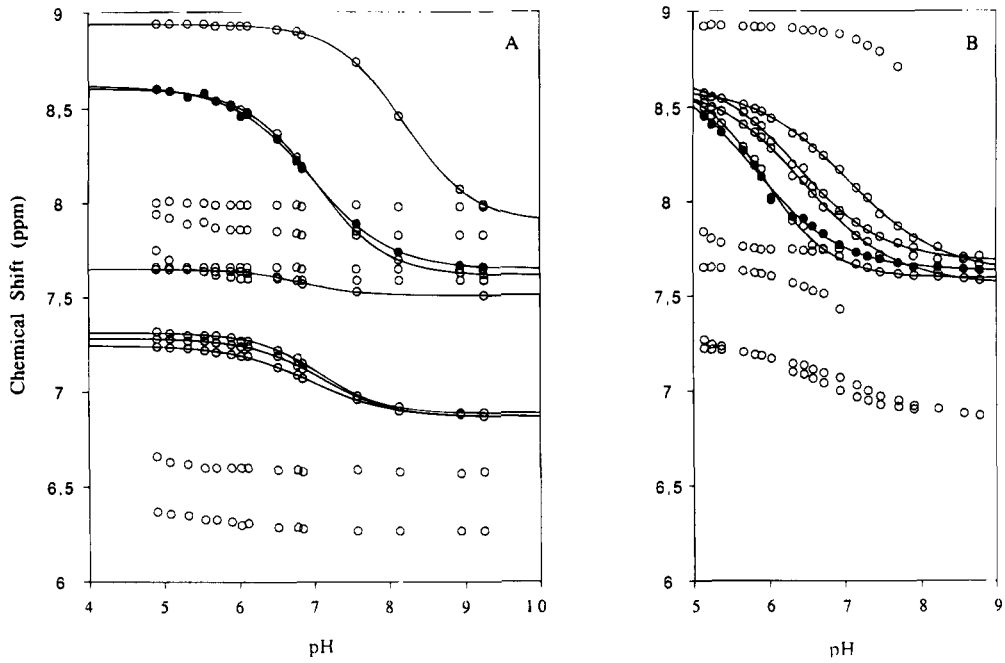

FIGURE 8: pH dependence of chemical shifts for His C2H and C4H resonances of the wild-type PGK (A) and the Δ404−415 PGK (B). A number of fit curves have been drawn and some experimental points shaded for clarity.

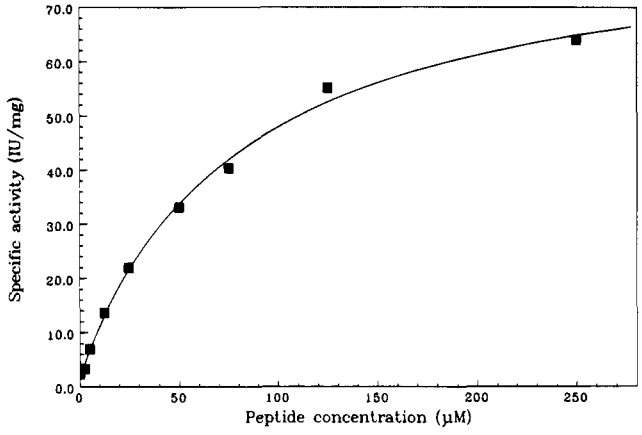

FIGURE 9: Effect of peptide 404−415 on enzymatic activity of mutant Δ404−415 (0.11 μM).

cysteinyl residue by an increase of the flexibility of the protein. The fact that the cysteinyl residue is located on the opposite side of the N-domain relative to the missing helix XIV probably reflects an increase in flexibility propagated throughout the N-domain. The data obtained in this work confirm the hypothesis proposed to explain the increase in flexibility of the isolated N-domain as resulting from the loss of contacts between helix XIV and the N-domain.

Surprisingly, the deletion of helix XIV also has an effect on the C-domain. This domain contains the only two tryptophan residues of the molecule. These two tryptophans are localized far from the hinge region, and one of them, W333, is totally buried, while W308 is localized at the surface of the molecule. These residues are located far away from helix XIV, and their environments are not significantly different from that of the wild-type protein as indicated by the small red shift of the maximum fluorescence emission wavelength of native PGK Δ404−415. However, the tryptophan fluorescence is strongly affected by low denaturant concentrations. Such data are consistent with the hypothesis of an increase in flexibility of the whole protein.

The increase in flexibility upon the deletion of the C-terminal sequence is accompanied by a decrease in stability of the protein as indicated by the unfolding−refolding transition curves. The transition curve followed by CD being symmetrical (Figure 3), it appears that the two domains are destabilized to the same extent by the deletion. This is strengthened by the fact that the same C_m and $\Delta G°$ values were obtained from the transition followed by the shift in maximum fluorescence wavelength (Figure 4). Moreover, the analysis of the fluorescence intensity transition curve gave further information. The complex behavior of the signal upon denaturant concentration is the consequence of two phenomena. In denaturant concentrations lower than 0.5 M Gdn-HCl, the increase in fluorescence intensity is not the consequence of a drastic change in protein structure. Indeed, at these denaturant concentrations, the protein has the same specific activity as in the absence of denaturant (Figure 6) and the tryptophans have the same accessibility as in the native protein (Figure 7). A change in enzymatic activity and an increase in the tryptophan accessibility was only observed for denaturant concentrations higher than 0.5 M Gdn-HCl. Thus, the variation in fluorescence intensity observed between 0.5 and 0.8 M Gdn-HCl corresponds to a complete denaturation of the protein. This transition leads to the same $\Delta G°$ as that determined from CD measurements and from the shift in maximum fluorescence wavelength (Table 1).

The dependence of the fluorescence intensity upon low denaturant concentrations (below 0.5 M Gdn-HCl) for PGK Δ404−415 is higher than that reported for the wild-type enzyme (Missiakas *et al.*, 1990). It has been previously reported that the tryptophan fluorescence yield of the yeast PGK is very low (around 5%; Nojima *et al.*, 1976) and might be due to the existence of internal quenching, mainly for Trp 308 which is close to a negative charge of an aspartate (Asp 287; Privat *et al.*, 1980). One possible explanation of the large solvent effect on the fluorescence of the native protein could be the consequence of an increase in flexibility

of the protein triggered by the denaturant, moving the tryptophan away from the charge and therefore suppressing the fluorescence quenching.

The structural properties of the truncated protein are very similar, at least qualitatively, to the properties of the isolated domains as indicated the reactivity of the Cys 97 and the NMR measurements. The stricking similarity between the C-domain (Missiakas *et al.*, 1990) and the truncated protein fluorescence transition, although with a different C_m, is close enough to postulate that it represents the same phenomenon. Taking into account the similar profiles of the fluorescence transition curves observed for the truncated protein and the C-domain, it seems more likely that the first part of the C-domain fluorescence transition curve does not correspond to a conformational transition but a strong Gdn-HCl effect on a native-like protein in which the internal quenching is removed by the increase in internal molecular motions.

The similar properties of the isolated domains and truncated protein are probably the consequence of the absence of contacts between helix XIV and the N-domain. Although the covalent link between the two domains, i.e., helix V with residues 185−199, is essential for enzyme activity and stability as previously reported (Fairbrother *et al.*, 1989a), its presence is not sufficient to maintain the stability and activity of the enzyme. Indeed, the loss of helix XIV is sufficient to destabilize the two domains, leading in the truncated protein to domains that have the same flexibility as when they are isolated.

It is well-known that the catalytic properties of an enzyme are very sensitive to any small structural changes in the structure of the catalytic site, such variations being possibly induced by internal motions. It can be supposed that the loss of enzyme activity is a consequence of a higher flexibility induced by the absence of helix XIV. In order to check this hypothesis, the effect of the missing peptide 404−415 binding on protein activity was studied. The addition of this peptide leads to a 40-fold increase in enzymatic activity (Figure 9) at saturation, which occurs spontaneously, during the dead time of manual mixing. It must be noted that the dissociation constant (80 μM) measured from the saturation curve corresponds to the binding of the peptide in the presence of the substrates. However, the presence of ligands is not mandatory since peptide binding was also observed by TRNOESY experiments, which were performed in the absence of ligands (see following paper: Andrieux *et al.*, 1995).

The effect of the deletion on the increase in flexibility of the N-domain can be easily understood in terms of the absence of interactions with helix XIV. However, the effect of the deletion on the activity cannot be interpreted as a direct perturbation between these residues and the substrates. A close examination of the structure around residues 404−415 (Figure 10) shows that residue Leu 405 is in tight interaction with helix XIII. This residue, which is conserved in PGK from different species, is included in a hydrophobic pocket including residues Ala 395 and Leu 399. A possible interpretation of the effect of deletion upon protein activity is that the interaction between Leu 405 and residues Ala 395 and Leu 399 maintains helix XIII in a correct position for the catalytic process. As reported for pig muscle PGK (Harlos *et al.*, 1992), residues 395, 396, and 397, which form the beginning of helix XIII, are completely conserved and are involved in the binding of 3-phosphoglycerate through

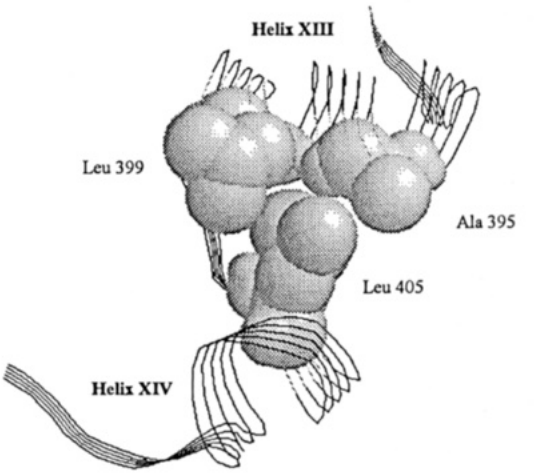

FIGURE 10: Interactions between helix XIV and helix XIII.

a structural water molecule. By deleting residue Leu 405, this structural feature is suppressed, impeding helix XIII to take part in the catalytic process.

The experimental data presented in this paper indicate that the C-terminal helix is crucial for both the stability and activity of yeast PGK. This result must be compared with other experimental data obtained for various proteins. For example, it has been proposed that the C-terminal helix of cytochrome *c* is essential for both folding and stability (Kuroda, 1993); moreover, in cytochrome *c*, the N-terminal segment of the protein interacts with the C-terminal helix as for yeast PGK. In cytochrome *c*, truncation of the C-terminal helix also abolishes enzymatic activity (Fredericks & Pielak, 1993). The same observations have been made with staphylococcal nuclease, which is destabilized by the deletion of the last 13 amino acids (Shortle & Meeker, 1989; Flanagan *et al.*, 1992). The C-terminal helix of the catalytic chain of aspartate transcarbamoylase seems essential for both its folding and stability (Peterson & Schachman, 1991), and the deletion of the C-terminal helix of the ribonucleotide reductase drastically reduces the enzymatic activity (Climent *et al.*, 1992).

In summary, PGK deleted of the C-terminal amino acids (PGK Δ404−415) folds in a conformation very similar to the conformation of the wild-type protein. However, it cannot achieve the increase in rigidity and stability characteristic of the native protein. It appears that the C-terminal part is not necessary for most of the initial folding steps but acts to lock the C-domain on the N-domain. Without this sequence, the protein has the sum of the properties of the two isolated domains. This study illustrates the function of a structural feature common to many proteins and which should be considered for protein design.

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