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Detection of Intermediates in the Unfolding Transition of Phosphoglycerate Kinase Using Limited Proteolysis†

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ABSTRACT: The accessibility of peptide bonds to cleavage by *Staphylococcus aureus* V8 protease bound on a Sepharose matrix was used as a conformational probe in the study of the unfolding–folding transition of phosphoglycerate kinase induced by guanidine hydrochloride. It was shown that the protein is resistant to proteolysis below a denaturant concentration of 0.4 M. The transition curve, determined by susceptibility toward proteolysis, was similar to that obtained following the enzyme activity [Betton et al. (1984) *Biochemistry 23*, 6654–6661]. Proteolysis under conditions where the folding intermediates are more populated, i.e., 0.7 M Gdn·HCl, gave two major fragments of M_r 25K and 11K, respectively. The 25K polypeptide fragment was identified as the carboxy-terminal domain. Its conformation was similar to that of a folding intermediate trapped at a critical concentration of denaturant, and in this form, it was not able to bind nucleotide substrates [Mitraki et al. (1987) *Eur. J. Biochem. 163*, 29–34]. From the present data and those previously reported, we concluded that the intermediate detected on the folding pathway of phosphoglycerate kinase has a partially folded carboxy-terminal domain and an unfolded amino-terminal domain.

In relatively large globular proteins, the polypeptide chain folds in distinct structural regions called domains (Edelman, 1970). These domains have been considered as structural, genetic, and functional units (Janin, 1979; Rossmann & Argos, 1981). Several methods for establishing the presence of domains in proteins from X-ray structures have been developed (Ooi & Nishikawa, 1973; Rossmann & Liljas, 1974; Rose, 1979; Wodak & Janin, 1980). It was suggested that the domains in such proteins result from gene fusion or insertion during evolution. Correlation among exons in genes and protein structural units, which permits the production of new proteins by bringing together the corresponding segments, has been discussed (Gilbert, 1978; Blake, 1978; Gô, 1981).

Wetlaufer (1973) proposed that the early stages of the protein folding process could occur independently in each of these domains. According to this point of view, particular segments of an unfolded polypeptide chain first refold to form individual domains, which then associate and interact to give the final tertiary structure, as do subunits in oligomeric proteins. The folding of polypeptide fragments corresponding to structural domains has been reviewed by Wetlaufer (1981), Ghélis and Yon (1982), and Jaenicke (1987). The major implication of this model is that the portions of the protein corresponding to domains in the whole protein are expected to fold into a nativelike structure independently from the rest of the polypeptide chain. Moreover, as emphasized by Jaenicke (1987), folding units as domains "may be obligatory inter-

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mediates on the folding path", even when these intermediates are unstable and therefore sparsely populated under equilibrium conditions. Once domains have folded, they assemble. Their assembly may subsequently induce structural readjustments which generate the functional properties of the protein. In most cases of multidomain proteins, such as phage T4 lysozyme (Desmadril & Yon, 1984), penicillinase (Mitchinson & Pain, 1985), thermolysin (Fontana et al., 1983), γ-II crystallin (Rudolph et al., 1986), and octopine dehydrogenase (Jaenicke, 1987), intermediates have been detected.

Phosphoglycerate kinase is a monomeric enzyme, built of two structural domains of approximately the same size. These domains correspond to the N- and C-terminal parts of the molecule, as revealed by the three-dimensional structures of the horse muscle (Banks et al., 1979) and the yeast (Watson et al., 1982) enzymes. The separation between the domains lies between helix α7 and segment βF. The nucleotide substrates bind to the C-terminal domain, but the binding site of phosphoglycerate substrates remains unknown. The folding–unfolding transition of horse muscle phosphoglycerate kinase (PGK),[1] induced by guanidine hydrochloride has been investigated by equilibrium and kinetic measurements (Betton et al., 1984, 1985). It has been shown to deviate significantly from a two-state process, indicating the presence of intermediates even under equilibrium conditions. These intermediates have been trapped at a critical concentration of guanidine hydrochloride, and it was found that, in these intermediates, the C-terminal domain is incorrectly or incompletely folded (Mitraki et al., 1987).

In several multidomain proteins, such as elastase (Ghélis et al., 1978), thioredoxin (Slaby & Holmgren, 1979; Reutiman et al., 1981), tryptophan synthase (Högdberg-Raibaud & Goldberg, 1977a,b; Zetina & Goldberg, 1983), and thermolysin (Fontana et al., 1983; Vita et al., 1984), domains have been isolated from the native protein by limited proteolysis. In contrast, native PGK is very resistant to proteases (Betton, 1987). Limited proteolysis as a conformational probe has been widely applied in studies of the unfolding–refolding of proteins (Ooi et al., 1963). This approach requires that the native protein must be resistant to proteolysis so that the cleavage of the peptide bonds reveals the unfolding process. Horse muscle PGK fulfills this requisite.

In this paper, limited proteolysis was used as a conformational probe to study the folding process of phosphoglycerate kinase and to identify directly the intermediates in the folding path. We describe the isolation of a major fragment corresponding to the C-terminal domain, following proteolysis at the critical concentration of guanidine hydrochloride, and we compare its spectroscopic properties with those of the intact phosphoglycerate kinase and phosphoglycerate kinase folding intermediates.

Experimental Procedures

Enzyme Preparation. Horse muscle phosphoglycerate kinase (PGK, EC 2.7.2.3) was prepared by the procedure of Scopes (1969) as modified by Blake et al. (1972).

Protease Immobilization. Staphylococcus aureus V8 protease (V8 protease) was immobilized onto CNBr-activated Sepharose Cl-6B (Pharmacia–LKB Biotechnology Inc.) according to March et al. (1974). The protease (10 mg) was coupled to 10 mL of beads with an efficiency greater than 90% as determined by the enzyme activity of the supernatant (see below).

Denaturation and Digestion Procedures. Experiments were carried out at 12 °C in a 50 mM phosphate buffer, pH 7.5, containing 1 mM EDTA and 1 mM DTT (buffer A), unless otherwise indicated. Stock solutions of PGK were diluted to the final concentration of guanidine hydrochloride (Gdn·HCl) from 0 to 1.6 M and incubated at 12 °C. After 24 h, 1-mL samples of the denaturation solution containing 2.5 mg of protein were mixed with 50 μL of sedimented V8 protease gel equilibrated in the same solution at 12 °C, and the mixture was rotated end over end. After 4 h, the mixture was spun to eliminate the protease, and 10 mM diisopropyl fluorophosphate (DFP) was added. Samples were dialyzed to remove the Gdn·HCl and analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli (1970) with a 12% acrylamide slab gel of 0.75-mm thickness. The gels were stained with Coomassie blue and scanned with a Vernon PHI 4 scanner.

Purification of the 25K Fragment. For preparative purposes, 10 mL of PGK (20 mg) was denatured for 24 h at 12 °C in buffer A containing 0.7 M Gdn·HCl, then applied to a column (1 × 15 cm) of V8 protease–Sepharose Cl-6B (1 mg of enzyme) equilibrated with the same buffer, and recirculated at 20 mL/h for 4 h at 12 °C. The column was then eluted. In order to prevent further proteolysis, DFP (10 mM final concentration) was added to the proteolytic digest, which then was concentrated by ultrafiltration using Centricon 10 microconcentrators (Amicon). The resulting concentrate was diluted to 3 mL by addition of 6 M Gdn·HCl in buffer A and loaded onto a Sephacryl 100 HR (Pharmacia–LKB Biotechnology Inc.) column equilibrated at 1 mL/min with the same denaturation solution at 10 °C. Fractions (1 mL) were analyzed by SDS–PAGE. Those fractions containing the 25K fragment (the main peak according to spectrophotometric detection at 280 nm) were pooled, concentrated to 1 mL (Centricon 10), and equilibrated in a 20 mM Tris-HCl buffer, pH 8.3, containing 1 mM DTT and 5 M urea (buffer B) by Sephadex G25 (Sigma) gel filtration. The partially purified fraction (1 mL), containing the 25K fragment, was applied to a Mono Q HR 5/5 FPLC column (Pharmacia–LKB Biotechnology Inc.) equilibrated with buffer B at a flow rate of 0.5 mL/min. The column was eluted with a 30-mL gradient of 0–0.5 M NaCl in buffer B. Peak fractions were pooled, and the purity of the 25K fragment was assessed by SDS–PAGE.

Amino Acid Analysis. Amino acid analysis was performed on a Biotronik LC 5001 amino acid analyzer using a single column procedure (Hummel, 1959). Samples containing 0.4 nmol of the 25K fragment were hydrolyzed in vacuo in 0.1 mL of 6 N HCl for 15, 48, and 72 h at 110 °C (Spackmann et al., 1958). For the exact determination of tyrosine, hydrolysis was carried out for 15 h in the presence of 0.2% phenol. Methionine was determined as methionine sulfone after periodic acid oxidation according to Hirs (1967). Cysteine was estimated as cysteic acid after performic oxidation.

Amino-Terminal Sequence Analysis. The amino-terminal sequence of 5 nmol of the 25K fragment was determined by using the manual 4-(dimethylamino)azobenzene-4′-isocyanate/phenyl isocyanate double-coupling technique. The resulting 4-(dimethylamino)azobenzene-4′-isocyanate derivatives were identified by both TLC and HPLC methods as described by Chang (1983).

[1] Abbreviations: CD, circular dichroism; DFP, diisopropyl fluorophosphate; Gdn·HCl, guanidine hydrochloride; PGK (EC 2.7.2.3), phosphoglycerate kinase; V8 protease, *Staphylococcus aureus* V8 protease; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

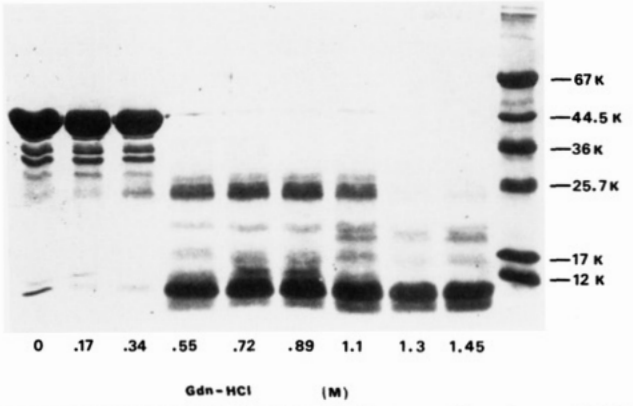


FIGURE 1: SDS–PAGE of the digest by *S. aureus* V8 protease of PGK denatured in various Gdn·HCl concentrations. PGK denatured for 24 h in various Gdn·HCl concentrations was digested at pH 7.5 in the same Gdn·HCl concentrations at 12 °C for 4 h by protease–Sepharose 6B; the protease/PGK molar ratio was 1/20. The digestion products were separated on an SDS–polyacrylamide (12%) gel and stained by Coomassie blue. Final Gdn·HCl concentrations are indicated at the bottom of the gel. Markers (right) show the position of molecular weight standards.

Spectrophotometric Measurements. Concentrations of the 25K fragment were determined by using $\epsilon^{0.1\%}(280\ nm) = 1.05$. This value was calculated on the basis of the amino acid composition of the 25K fragment (Hardy et al., 1981) and by using the molar extinction coefficients at 280 nm for tyrosine and tryptophan residues (Edelhoch, 1967). The concentrations thus determined were found to be in good agreement with those calculated from the amino acid analysis.

Fluorescence spectra were recorded on a Perkin-Elmer MPF 44B spectrofluorometer. Circular dichroic spectra were obtained from a Dichrograph Mark V, Jobin and Yvon. Both instruments were equipped with thermostatically controlled cell holders. Analyses of the secondary structure content derived from far-ultraviolet CD spectra were carried out by the method of Yang et al. (1986).

RESULTS

Susceptibility toward Proteolysis of PGK along the Equilibrium Unfolding Transition. To overcome the resistance of native PGK toward proteases (Betton, 1987), proteolysis was performed under conditions which destabilize the protein, i.e., in the presence of denaturant. To avoid destabilization of the protease and to readily remove it from the reaction mixture, V8 protease was coupled onto Sepharose CL-6B. Enzymatic assay of the immobilized V8 protease was performed spectrophotometrically at 412 nm using carbobenzoxy-L-phenylalanyl-L-leucyl-L-glutamic acid 4-nitroanilide (Boehringer Mannheim GmbH) according to Houmard (1974). Control experiments showed that there was no change in the catalytic rate constant under denaturing conditions up to 1.8 M Gdn·HCl. Thus, in the concentration range from 0 to 1.45 M Gdn·HCl used in our experiments, the activity of the immobilized protease was not affected by the denaturant, and it was possible to study the susceptibility toward proteolysis of intermediates in the unfolding equilibrium transition of PGK.

The electrophoretic pattern of denatured PGK proteolyzed for 4 h by V8 protease–Sepharose in the same Gdn·HCl concentrations as previously used for PGK denaturation consisted of two major fragments of M_r 25K and 11K (Figure 1). Three ranges of Gdn·HCl concentration were characterized by changes in the relative peak areas of the intact PGK and its fragments (Figure 2). Between 0 and 0.4 M Gdn·HCl,

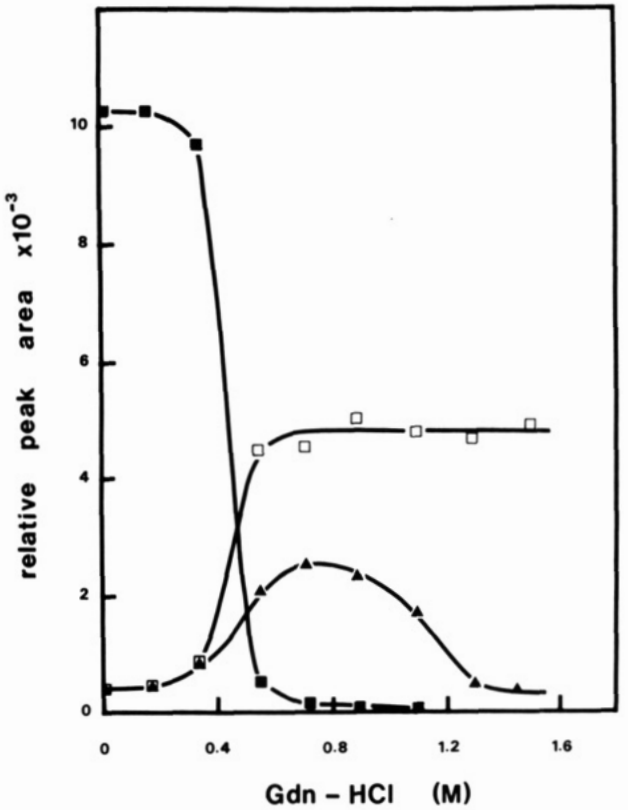


FIGURE 2: Dependence of the relative amount of digestion products upon Gdn·HCl concentration. Relative peak areas of intact PGK (■), 25K fragment (▲), and 11K fragment (□) were obtained by densitometric analysis of the Coomassie-stained SDS–polyacrylamide gel shown in Figure 1.

no intermediate states were present under equilibrium conditions, since the protein has a native conformation resistant to proteolytic attack. Between 0.5 and 1 M Gdn·HCl, corresponding to the transition range as shown by other studies (Betton et al., 1984), both proteolytic fragments appeared. Between 1 and 1.6 M Gdn·HCl, the relative amount of 25K fragment decreased, but the 11K fragment appeared to be quite resistant to further proteolytic digestion.

From a structural point of view, the most interesting facts were the following: first, the susceptibility to proteolysis of intact PGK as a function of Gdn·HCl concentration followed closely upon the transition curve as established by the loss of enzyme activity (Betton et al., 1984); second, a 25K fragment was formed. This molecular weight corresponds to half of the intact molecule, as expected for one domain. The fact that the 25K and 11K fragments were obtained in good yield indicated that the folding–unfolding equilibrium was not significantly perturbed during proteolysis. Such fragments presumably represent compact folded units resistant toward further proteolytic attack.

The results shown in Figure 2 clearly indicate that the formation of the 25K fragment was optimal in 0.7 M Gdn·HCl, i.e., the critical concentration of denaturant (Mitraki et al., 1987).

Limited Proteolysis of PGK in 0.7 M Gdn·HCl. The preceding proteolytic data indicated that limited proteolysis is a convenient method for investigating unfolding equilibrium intermediates. However, aggregation of PGK due to a partially unfolded intermediate on the folding pathway occurs at 23 °C (Mitraki et al., 1987) and may complicate the interpretation of the proteolysis experiments. At low temperature, the aggregation can be prevented, and the intermediate species is present as a monomer. The temperature of proteolysis

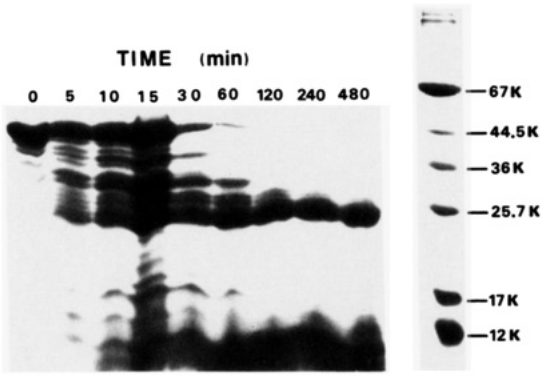


FIGURE 3: SDS–PAGE of the products obtained following *S. aureus* hydrolysis of PGK denatured at "the critical concentration" of Gdn·HCl. PGK denatured in 0.7 M Gdn·HCl for 24 h at 12 °C was hydrolyzed with V8 protease–Sepharose 6B at the same denaturant concentration at pH 7.5 12 °C with a protease/PGK molar ratio of 1/25. At selected time intervals, samples (1 mL) were taken and analyzed by SDS–polyacrylamide gel (15%) electrophoresis is described under Experimental Procedures. The time is indicated at the top of the gel. Markers (right) show the position of molecular weight standards.

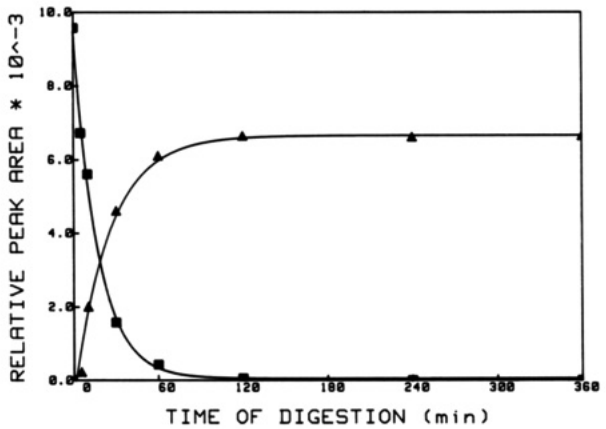


FIGURE 4: Time curve for the hydrolysis of PGK by *S. aureus* V8 protease at the "critical concentration" of Gdn·HCl. Relative peak areas for intact PGK (■) and the 25K fragment (▲) were obtained by densitometric analysis of the Coomassie-stained SDS–polyacrylamide gel shown in Figure 4. The solid lines were calculated according to a first-order kinetic reaction scheme.

experiments (12 °C) was chosen to minimize the extent of aggregation. Nonetheless, the concentration of denaturant corresponding to the midpoint transition was the same at 4, 12, and 23 °C (Betton, 1987).

The kinetics of proteolysis in 0.7 M Gdn·HCl were determined by using the same conditions as in the preceding experiments with the exception of the molar V8 protease/PGK ratio. The corresponding gels are shown in Figure 3. The analysis of the gels indicated that the decrease of the intact PGK band as a function of time correlated with the increase of the 25K band (Figure 4). The corresponding first-order rate constants were found to be identical (5×10^{-2} min^{-1}). The analysis of the 11K band was not possible using the same procedure, since this band was a mixture of several fragments of very similar molecular weights. The yield of proteolytic fragments was found to be strongly dependent upon the molar V8 protease/PGK ratio. Furthermore, the degree of staining by Coomassie blue of these fragments was too different to allow an unambiguous determination of the yield.

These results showed that the 25K fragment represented a stable and homogeneous entity.

Purification of the 25K Fragment. In order to define the sites of proteolytic cleavage and to study the conformational properties of the resulting fragments, it was important to first purify the fragments. Proteolysis of PGK was carried out therefore on a preparative scale. Attempts to isolate the fragments under nondenaturing conditions using a variety of chromatographic procedures were unsuccessful. These results indicated that the two fragments, bound together by noncovalent interactions, aggregated after removal of the denaturant. Thus, the large-scale V8 protease digest (4 h) was transferred to 6 M Gdn·HCl before being applied to a Sephacryl 100 HR column equilibrated with the same denaturant solution (Figure 5A). The main peak from the gel filtration column was equilibrated at pH 8.3 in a Tris-HCl buffer containing 5 M urea and loaded onto a Mono Q column equilibrated with the same buffer (Figure 5B). After elution with a linear gradient of NaCl (0–0.5 M), one main peak was obtained; it corresponded to the 25K fragment, as shown by SDS–PAGE (Figure 5C). This fragment was renatured by dilution to 0.1 mg/mL in a phosphate buffer at 23 °C with rapid mixing. The 11K fragment, partially purified by using a Sephacryl 100 HR column, gave a turbid solution after the renaturation step. In contrast, the 25K fragment remained soluble.

The ultraviolet spectrum of the 25K fragment under denaturing conditions (6 M urea), indicating the presence of tryptophan residues, suggests its localization in the C-terminal domain of PGK. Indeed, the horse muscle enzyme contains 4 tryptophan (310, 335, 344, 382), 4 tyrosine (75, 160, 195, 323), and 16 phenylalanine residues. All tryptophans are located in the C-terminal domain whereas there are tyrosines and phenylalanines in both domains. The partially purified 11K fragment, corresponding to the second peak eluting from Sephacryl 100 HR, which was soluble under denaturing conditions, was also examined. Its UV spectrum indicated primarily the presence of tyrosine. Considering this observation, the size of the fragment, and the amino acid sequence of PGK, two possibilities could be assumed for the origin of the 11K fragment. First, it might represent a limit peptide, no more peptide bonds susceptible to cleavage by V8 protease being present in its sequence; in this case, the only candidate should be the polypeptide Ser1–Glu102 of the N-terminal domain. Second, this fragment might be generated from either the N- or the C-terminal domain. Whatever its origin may be, its size is smaller than that of a domain. Anyway, the aggregation in the absence of denaturant prevented further analysis.

Amino-Terminal Sequence Analysis. Manual microsequence analysis of 5 nmol of the 25K fragment gave the sequence Leu-Asn-Tyr-Phe-Ala. This sequence is beginning at residue 193 in PGK and is preceded by the following sequence: Met-Lys-Lys-Glu (Hardy et al., 1981). Thus, the cleavage site of V8 protease occurred at an internal Glu–Leu peptide bond generating the 25K fragment. This peptide bond belongs to helix $\alpha 7$ which connects the two domains in the hinge region (Banks et al., 1979).

Amino Acid Analysis of the 25K Fragments. Amino acid analysis of the 25K fragment was found to be in reasonable agreement with that expected from the C domain of horse muscle PGK, with cleavage at position Glu192–Leu193 (Table I), i.e., the sequence from Leu193 to Val416. Therefore, no further cleavage had occurred in the C-terminal domain.

Spectroscopic Studies of the 25K Fragment. In order to determine the secondary structure of the 25K fragment, CD spectra in the far-ultraviolet region were examined in pH 7.5 phosphate buffer (Figure 6). The spectrum showed negative ellipticity in the region from 200 to 250 nm, with two typical minima centered at 208 and 222 nm, indicating that the 25K

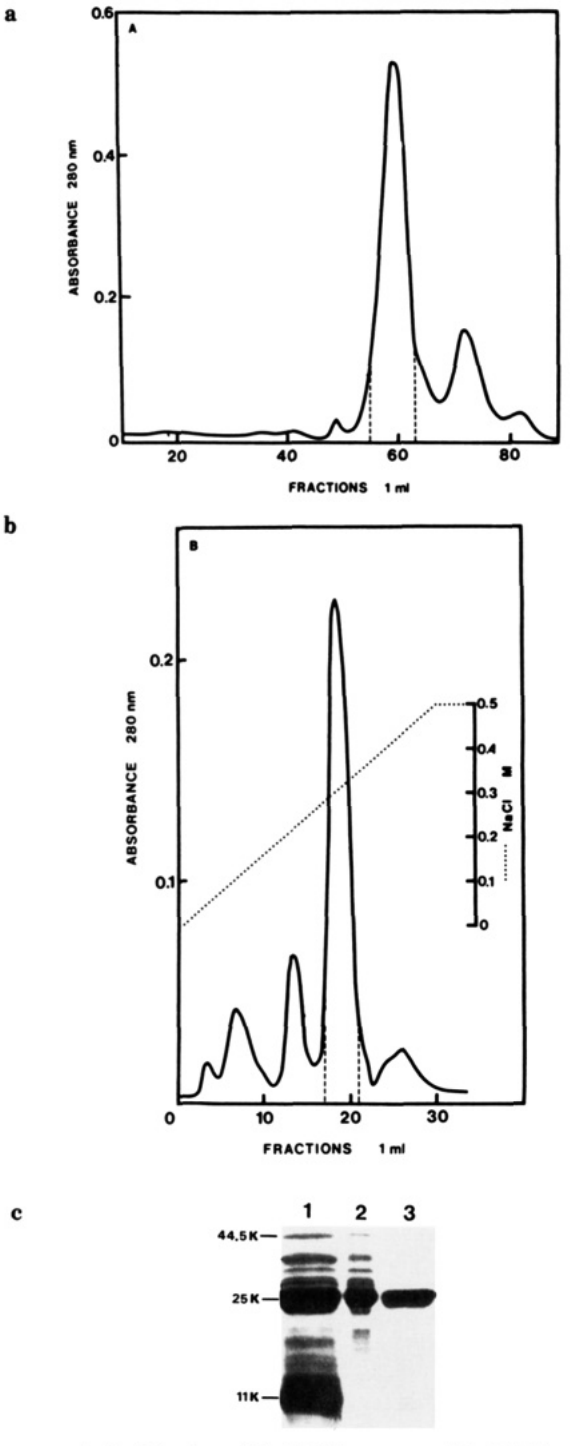


FIGURE 5: Purification of the 25K fragment. (A) Gel filtration at 1 mL/min, 12 °C, of digestion products in 50 mM phosphate buffer, pH 7.5, containing 1 mM EDTA, 1 mM DTT, and 6 M Gdn·HCl on a Sephacryl 100 HR column (1.6 × 95 cm). The indicated fractions, 55–63 (9 mL), were pooled. (B) Ion-exchange chromatography at 0.5 mL/min of the pooled fractions of (A) equilibrated in 20 mM Tris-HCl buffer, pH 8.3, containing 1 mM DTT and 5 M urea. The Mono Q HR 5/5 column was equilibrated at 12 °C in the same buffer. The 25K fragment was eluted with 30 mL of a linear gradient from 0 to 0.5 M NaCl in the same buffer. (C) SDS–PAGE of the pooled chromatographic fractions. Samples were analyzed on an SDS–polyacrylamide (15%) gel and stained by Coomassie blue. (1) 4-h V8 protease digest of PGK; (2) pooled peak fractions from Sephacryl 100 HR; (3) pooled peak fractions from Mono Q HR 5/5.

fragment contained a substantial amount of α-helical structure. Quantitative analyses of the secondary structures from the far-ultraviolet CD spectra (Table II) were carried out by using

Table I: Amino Acid Composition of the 25K Fragment

amino acid	observed[a]	expected[b]
Cys	3.5	4
Asx	21.7	22
Thr	13.2	14
Ser	11.7	11
Glx	18.4	19
Phe	5.5	6
Gly	26.3	25
Ala[c]	25.0	25
Val	18.4	19
Met	4.2	7
Ile	13.0	13
Leu	18.0	18
Pro	2.1	2
Tyr	2.4	2
His	19.5	20
Lys	8.7	9
Arg	2.5[d]	4

[a] Average value of five analyses. [b] Calculated from the amino acid sequence of PGK (Hardy et al., 1981) for the polypeptide 193–416. [c] Arbitrarily taken as reference value. [d] Arg content was systematically underestimated in these analyses.

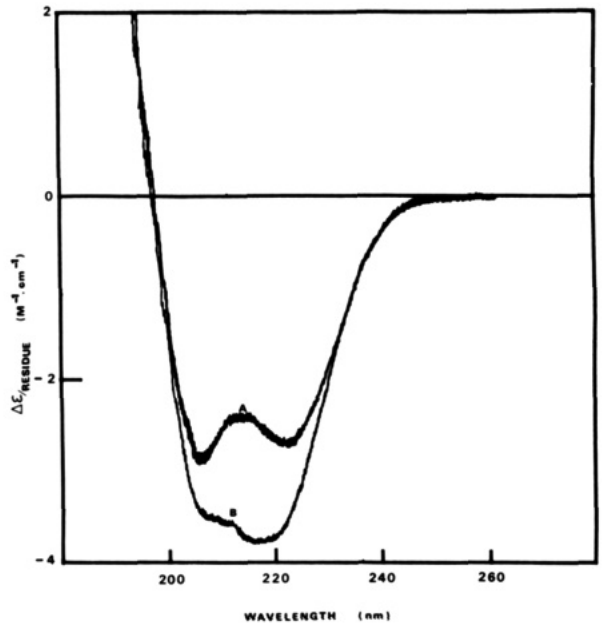


FIGURE 6: Circular dichroic spectrum of the 25K fragment. Spectra were recorded at 20 °C in 50 mM phosphate buffer, pH 7.5, 1 mM EDTA, and 0.5 M DTT and subtracted from the buffer base line. (A) 25K fragment (45 μg/mL); (B) native PGK (40 μg/mL).

the method reported by Yang et al. (1986) and compared with those of the C-terminal domain calculated from the X-ray structure of PGK (Banks et al., 1979). The observed helicity of the 25K fragment (Leu193–Val416) is somewhat lower than expected for a nativelike structure. The CD analysis of PGK incubated for 24 h in a critical concentration of Gdn·HCl and then for another 24 h in the phosphate buffer, pH 7.5, is given for comparison (Mitraki et al., 1987).

The fluorescence emission spectrum of the 25K fragment (Figure 7) showed an increase in fluorescence yield in comparison with PGK, with a small blue shift in the maximum wavelength (λ_{max} = 332 nm). This shift between the two proteins indicated that most of the fluorophores (i.e., tryptophans) are in a more apolar environment in the 25K fragment than in the native protein. The same shift was observed for the renatured intermediate species trapped at a critical concentration of Gdn·HCl (Table II). Both the far-ultraviolet CD and the fluorescence data support a close structural re-

Table II: Estimation of Ordered Structures of Native, Denatured, and Intermediate Species and Isolated C-Terminal Domain of PGK

protein	method	fraction of structures f_α	f_β	f_c	fluorescence, λ_{max} (nm)
native PGK	X-ray data[a]	0.42	0.25	0.33	
	CD[b]	0.41	0.21	0.38	336
PGK denatured in 0.8 M Gdn·HCl[b]	CD	0.18	0.16	0.66	342
PGK denatured in 0.8 M Gdn·HCl and renatured in regeneration mixtures[b]	CD	0.29	0.21	0.50	332
C-terminal domain	X-ray data[a]	0.46	0.23	0.31	
25K fragment	CD	0.26	0.18	0.56	332

[a] From Banks et al. (1979). [b] From Mitraki et al. (1987).

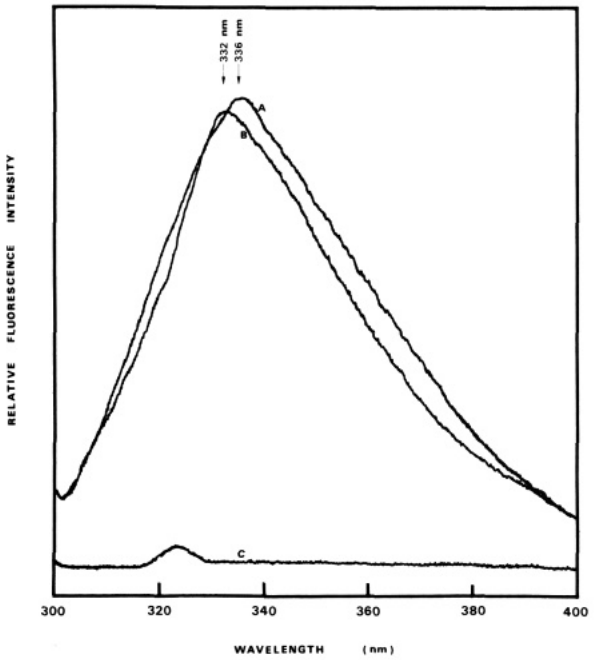


FIGURE 7: Fluorescence emission spectrum of the 25K fragment. Spectra were recorded at 20 °C in 50 mM phosphate buffer, pH 7.5, 1 mM EDTA, and 0.5 mM DTT. The excitation wavelength was 292 nm. (A) Native PGK (40 μg/mL); (B) 25K fragment (45 μg/mL); (C) phosphate buffer base line.

lationship between these species and the 25K fragment. Furthermore, this fragment was not retained on an ADP–Sepharose column as was previously observed for the intermediate species on the folding pathway of PGK (Mitraki et al., 1987).

Discussion

Horse muscle phosphoglycerate kinase was found to be very resistant to proteases under native conditions. Under nondenaturing conditions, no nicked enzyme molecule was obtained (Betton, 1987), contrary to that reported for the pig muscle enzyme (Jiang & Vas, 1988). Cleavage of peptide bonds occurred only during the unfolding process. Thus, the accessibility to V8 protease bound on a Sepharose matrix was used as a conformational probe to follow the folding–unfolding equilibrium of the molecule as induced by Gdn·HCl. The transition was found to coincide with that obtained following the enzyme activity (Betton et al., 1984). No proteolytic cleavage occurred up to a concentration of 0.4 M Gdn·HCl. By increasing the Gdn·HCl concentration, two fragments of M_r 25K and 11K, respectively, are formed, the yield of the first one reaching a maximum around 0.7 M Gdn·HCl.

Taking advantage of this finding, it was possible to produce proteolysis of PGK and determine that the 25K polypeptide resulted from the cleavage of the Glu192–Leu193 peptide bond located in the hinge region. This fragment, which contained tryptophans, was identified, by amino acid analysis and partial sequencing of its amino-terminal end, as polypeptide Leu193–Val416. Figure 8 indicates the peptide bond cleaved by V8 protease. Thus, it was possible to conclude that the 25K fragment represented the C-terminal domain of the protein.

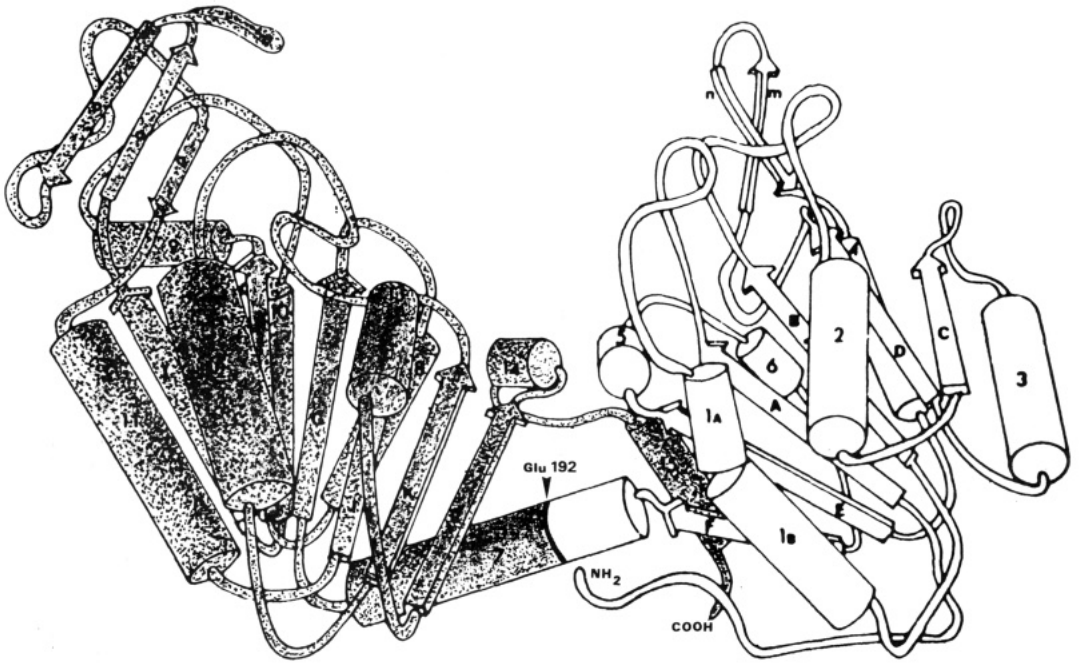
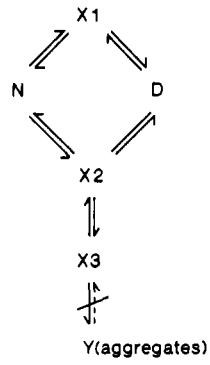


FIGURE 8: Localization of the 25K fragment in the PGK three-dimensional structure. *S. aureus* V8 protease cleavage at glutamic acid residue 192 is indicated by an arrow. The 25K fragment, i.e., the C-terminal domain, is shadowed. [Structure from Banks et al. (1979).]

Scheme I



The number of peptide bonds theoretically susceptible to V8 protease hydrolysis is approximately equal in both domains, with 10 Glu–X bonds in the N-terminal domain and 16 in the C-terminal domain. In the C-terminal domain, 15 of these remained entirely inaccessible to the protease. This fragment probably represents a compact folded unit within the intermediates along the folding pathway of PGK. From the spectroscopic characteristics, i.e., circular dichroism and fluorescence spectra, we conclude that the isolated C-terminal domain is partially folded, having all of the β-sheets present in the native protein but only about 50% of the helical content. This analysis and the blue shift observed in the fluorescence emission spectrum strongly suggest that the C-terminal domain obtained under these conditions is very similar to the intermediate trapped under the critical concentration of denaturant (Mitraki et al., 1987). Furthermore, as found for this intermediate, the 25K fragment is not able to bind nucleotide substrates. According to the data previously reported (Betton et al., 1985; Mitraki et al., 1987), Scheme I has been proposed for the folding pathway of phosphoglycerate kinase. X1 and X2 represent intermediates; X3 and Y are formed from X2 in a side reaction. At a critical concentration of denaturant, X2 and X3 are significantly populated, the aggregation being prevented at low temperature. The spectroscopic characteristics (fluorescence maximum and CD spectrum) of the isolated 25K domain are very similar to those of X2 species as shown by the data presented in Table II. Our results and those previously reported (Betton et al., 1984; Mitraki et al., 1987) suggest that, in this intermediate, the N-terminal domain seems to be devoid of ordered structures.

The attempt to obtain separate domains by limited proteolysis of PGK was not possible since the native protein has a rather compact structure and since helix α15 of the C-terminal domain strongly interacts with the N-terminal region. Only a destabilization at a critical concentration of denaturant has allowed the isolation of a polypeptide fragment (193–416) which presented all characteristics of the stable intermediates detected under equilibrium conditions in the unfolding–refolding transition of the whole PGK. The recent isolation by genetic engineering of each structural domain from the yeast PGK (Minard et al., unpublished data) will help to elucidate the mechanisms by which a two-domain protein folds to generate an active enzyme.

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Photomodification of a Serine at the Active Site of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase by Vanadate†

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ABSTRACT: Irradiation of ribulose-1,5-bisphosphate carboxylase/oxygenase from spinach in the presence of vanadate at 4 °C resulted in rapid loss of carboxylase activity. The inactivation was light and vanadate dependent. When the enzyme was irradiated in the presence of the substrate ribulose 1,5-bisphosphate or an analogue such as fructose 1,6-bisphosphate, the inactivation was greatly reduced. Sodium bicarbonate and phosphate also protected against inactivation. No additional protection was observed in the presence of Mg^{2+} nor did Mg^{2+} alone protect. Carboxylase activity could be partially restored by treatment with $NaBH_4$, and the photomodified protein could be tritiated with NaB^3H_4. Amino acid analysis showed that the tritium had been incorporated into serine. The data suggest that an active-site serine is photooxidized by vanadate to an aldehyde which results in activity loss. Irradiation in the presence of vanadate also resulted in cleavage in the large subunit of the enzyme which was subsequent to inactivation.

The enzyme D-ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO)[1] catalyzes the carboxylation of ribulose 1,5-bisphosphate (RuBP). This is the initial step in carbon fixation [for a review, see Miziorko and Lorimer (1983)]. The enzyme also catalyzes the oxygenation of RuBP, which leads to photorespiration (Andrews & Lorimer, 1978), resulting in a net loss of carbon.

The enzyme in higher plants is a hexadecamer consisting of eight large 55-kDa subunits and eight small 15-kDa subunits. The active site is located on the large subunit, while the role of the small subunit is unknown. In light of the importance of RuBisCO to virtually all life, the active site of the enzyme has been extensively studied. Various affinity labels and protein modification reagents have suggested that two lysines, a cysteine, histidine, tyrosine, arginine, methionine, and a glutamyl or aspartyl residue (Valle & Vallejos, 1974) are in the active-site domain (for a review, see McFadden and Small 1988). However, there have been no reports implicating serine. A serine has been identified in the active site of skeletal myosin using orthovanadate (V_i) trapped at the active site by ADP and Mg^{2+} (Cremo et al., 1988). Upon irradiation of the myosin·V_i·ADP·Mg^{2+} complex, myosin is ultimately modified twice. The first modification results in the oxidation of an active-site serine to the corresponding aldehyde. If V_i is then retrapped at the active site and the complex is exposed to UV light, a second modification occurs resulting in cleavage of the peptide backbone, apparently at the oxidized serine (Grammer et al., 1988).

We now present evidence to support UV-induced, V_i-dependent oxidation of a serine at the active site of spinach RuBisCO to an aldehyde which results in activity loss, and subsequent cleavage of the enzyme.

Experimental Procedures

Materials. Sodium salts of RuBP and FBP were purchased from Sigma Chemical Co. Orthovanadate (V_i) was prepared from V_2O_5 as described by Goodno (1982). Spinach RuBisCO was prepared from fresh spinach leaves (Berhow et al., 1982) and stored at −70 °C in the nonactivated state. NaB^3H_4 and $NaH^{14}CO_3$ were obtained from Dupont–NEN and ICN, respectively. CABP was synthesized as described by Pierce et al. (1980).

Photomodification. Unless otherwise indicated, irradiation was performed in the presence of 0.3 mM V_i in 50 mM MOPS buffer at pH 7.5. The protein concentration was 0.3 mg/mL (4.3 μM active sites) at the time of irradiation, which was performed on ice with a Hanovia 450-W medium-pressure Hg lamp (Ace Glass) at a distance of 9 cm. A glass filter was used to prevent surface heating and to screen out radiation below 330 nm. The samples were kept on ice during irradiation. The initial rate of inactivation was determined by

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[1] Abbreviations: CABP, 2-carboxy-D-arabinitol 1,5-bisphosphate; DTT, DL-dithiothreitol; FBP, D-fructose 1,6-bisphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; P_i, orthophosphate; RuBisCO, D-ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, D-ribulose 1,5-bisphosphate; SDS, sodium dodecyl sulfate; UV, ultraviolet; V_i, orthovanadate ($H_2VO_4^-$).

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