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Limited proteolysis of 3-phosphoglycerate kinase without loss of enzymic activity

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During tryptic digestion of pig muscle 3-phosphoglycerate kinase in the presence of 3-phosphoglycerate both the decrease of enzymic activity and the release of trichloroacetic acid-soluble peptides occur after a pronounced lag period. During this lag phase the native enzyme molecule is split into two fragments with molecular masses of about 30 and 18 kDa, as detected by SDS-PAGE. Under non-denaturing conditions, however, these fragments are held together by non-covalent forces and constitute an active, nicked enzyme molecule. In the absence of substrates or in the presence of MgATP the kinetics of tryptic digestion is apparently a single first order reaction leading to the formation of peptides with molecular masses of less than 10 kDa.

Tryptic digestion; 3-Phosphoglycerate effect; 3-Phosphoglycerate kinase; (Pig muscle)

1. INTRODUCTION

The molecule of the monomeric 3-phosphoglycerate kinase (PGK) consists of two domains of about equal size, connected by a 'waist' region [1–4]. A large scale hinge-bending structural motion was assumed to bring together the two domains, as a basic requirement of catalysis [1,2]. Although there are physicochemical data [5–9] and chemical modification experiments [10–12] in agreement with this hypothesis, the structure of the active enzyme-substrate complex has not been determined at high resolution. There are speculations about the mechanism of hinge-bending motion [2,4,13] and experimental approaches to explore it [14–17], but the role of the substrates in the functionally important structural motion is not yet specified. Especially little is known about the binding mode of 3-phosphoglycerate (3-PG) and about the details of accompanying structural changes.

There is a high degree of structural homology between PGKs from various species, especially the secondary and tertiary structures are very much conserved [1–4]. Among the mammalian PGKs, even at the level of the primary structure, the homology is greater than 96% [18]. In the present work the classical method of tryptic digestion, a sensitive probe for the conformation of the protein substrate [19], was applied in the case of pig muscle PGK the enzyme we investigated previously [10–12]. Similar attempts on chemically modified yeast PGK resulted in inactive fragments larger than one of its domains [20]. Amino acid sequences of mammalian PGKs contain several residues throughout the molecule involving the hinge region [18] that are susceptible to tryptic attack.

The results described here show a considerable

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Abbreviations: PGK, 3-phosphoglycerate kinase (EC 2.7.2.3); 3-PG, 3-phosphoglycerate; BAEE, benzoyl-L-arginine ethyl ester; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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degree of protection by 3-PG against tryptic digestion of PGK. In the presence of 3-PG limited proteolysis occurs producing two large fragments of non-equal size, i.e. the cleavage site is within one of the two structural domains.

2. MATERIALS AND METHODS

Pig muscle PGK was isolated and assayed as described [11]. The specific activity of the enzyme varied between 600 and 700 kat/mol. Before the experiments microcrystals of the enzyme were dissolved and dialysed either in 0.1 M glycine buffer, pH 8.1, containing 1 mM 2-mercaptoethanol or in 0.1 M ammonium bicarbonate containing the same additive.

Trypsin (bovine pancreatic, Boehringer) had a specific activity of 33 TU_{BAEE}/mg dry wt. Stock solutions of ~10 mg/ml were stored at 5°C in 1 mM HCl. Trypsin activity was assayed with the substrate BAEE (Merck) according to [21].

Trypsin inhibitor (soy bean, specific activity 26 TIU_{BAEE}/mg dry wt), PMSF and Coomassie blue R-250 were Sigma preparations, SDS, acrylamide, ammoniumpersulfate, *N,N'*-methylene bisacrylamide and *N,N,N',N'*-tetramethylenediamine were purchased from Serva. The electrophoresis molecular mass markers were obtained from Pharmacia. 3-PG was a Boehringer product, ATP-Na and NADH were Reanal (Budapest) preparations. All other chemicals were reagent-grade commercial preparations.

Digestion of PGK was carried out at 37°C in either of the solutions used for dialysis. If glycine buffer was used, its concentration was increased up to 0.5 M. Digestion was initiated by the addition of trypsin of different concentrations (from about 1 to 10 TU_{BAEE}/ml digestion mixture).

At different time intervals aliquots were withdrawn to (i) test PGK-activity (the buffer used for dilution contained 2 mM PMSF); (ii) precipitate the protein with ice-cold trichloroacetic acid (final concentration 25%); and (iii) analyse the digestion products by SDS-PAGE. Before addition of SDS and 2-mercaptoethanol the sample was preincubated for 2 min with trypsin inhibitor (about 2 TIU/TU).

Trichloroacetic acid precipitates were centrifuged and washed twice with 25% trichloroacetic acid and once with 2 mM PMSF and dissolved in 2% SDS. The protein concentration of these solutions was determined according to Peterson [22]. The absorbances of the supernatants were read at 280 nm. This value is proportional to the amount of Tyr and Trp containing peptides solubilized during digestion.

SDS-PAGE was carried out in 15% gel according to [23].

Gel chromatography was performed at 8°C on a Sephadex G-75 column (0.9 × 47 cm) equilibrated with 0.1 M glycine buffer, pH 8.0, containing 1 mM 2-mercaptoethanol, 1 mM EDTA and 2 mM PMSF. A sample of the trypsin inhibitor-treated digestion mixture was loaded onto the column in a volume of 0.35 ml. A constant flow rate of 8.7 ml/h was maintained by a peristaltic pump and effluent fractions of 0.7 ml were collected. The column was calibrated with blue dextrane 2000 (Pharmacia), ovalbumin (Reanal, molecular mass 43 kDa), chymotrypsinogen (Sigma, molecular mass 25 kDa), yeast ribonuclease A (Reanal, molecular mass 13.4 kDa) and ATP.

3. RESULTS AND DISCUSSION

The time course of digestion of PGK was followed by: (i) measuring the change of PGK activity (fig. 1), (ii) determining the amount of remaining protein in the precipitate formed with TCA (fig.2), and (iii) analysing the digestion products by SDS-PAGE (fig.3).

Digestion of substrate-free PGK or the binary complex with MgATP proceeds according to an apparently single first-order reaction, as illustrated by the semilogarithmic plot in fig.1. During this process the molecule of native PGK (molecular mass 47–48 kDa) completely decomposes into peptides smaller than 10 kDa. These peptides are too small to be detected by SDS-PAGE and therefore only the intensity decrease of the native PGK band can be observed during incubation with trypsin (fig. 3A). Thus, most of the possible cleavage sites within the PGK molecule (about 40 lysines and 10 arginines) seem to be split by trypsin at about the same rate.

3-PG considerably protects PGK against tryptic decomposition. Both the decrease of enzymic ac-

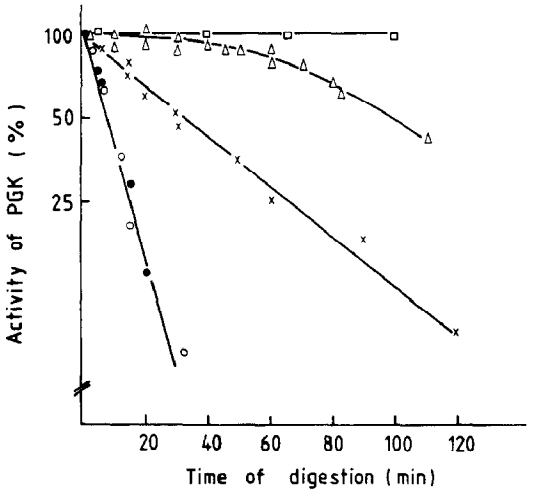

Fig.1. Semilogarithmic plot of the decrease of PGK activity during tryptic digestion. 50 μM substrate-free PGK was digested with different concentrations of trypsin: 10.9 TU_{BAEE}/ml (○—○) and 2.2 TU_{BAEE}/ml (×—×). The experiment with 10.9 TU_{BAEE}/ml trypsin was repeated in the presence of either 6.6 mM 3-PG (△—△) or 6.6 mM MgATP (●—●). As a control, 50 μM PGK was incubated under the same conditions without trypsin (□—□). From the slopes of the straight lines apparently first-order rate constants, $k = 0.021$ min^{-1} and $k = 0.098$ min^{-1} were calculated for trypsin concentrations of 2.2 and 10.9 TU_{BAEE}/ml, respectively.

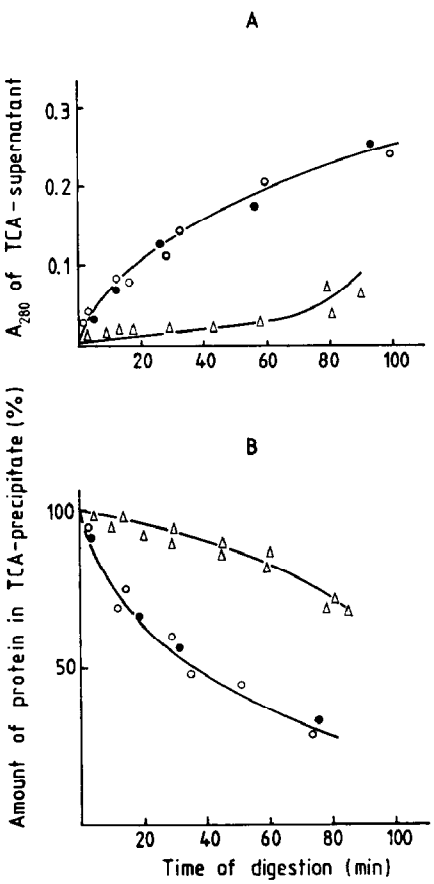

Fig.2. Increase of trichloroacetic acid soluble peptides during digestion. 0.5 mM PGK was digested in the absence of substrates (○—○) or in the presence of 6.6 mM MgATP (●—●) with 2.2 TU_{BAEE}/ml trypsin. The same concentration of PGK in the presence of 6.6 mM 3-PG was digested with 10.9 TU_{BAEE}/ml trypsin (△—△). At different time intervals the digestion was stopped by trichloroacetic acid (TCA). The supernatants (A) and the precipitates (B) were analysed as described in section 2.

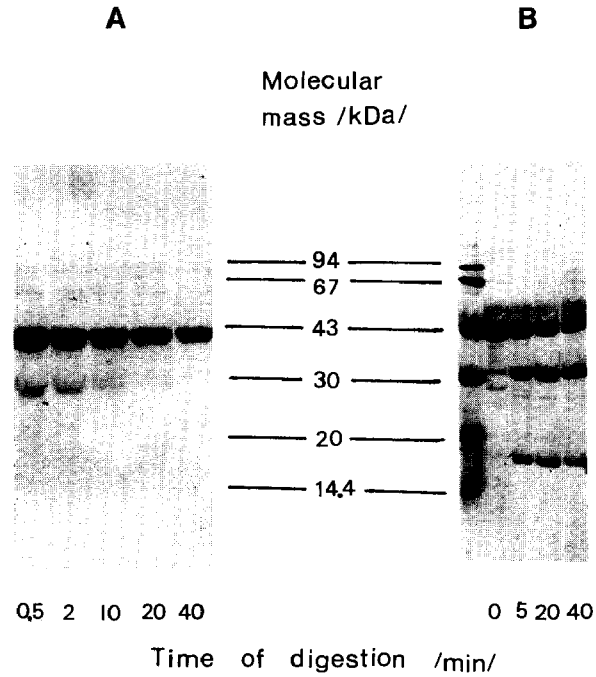

Fig.3. SDS-PAGE analysis of the digestion mixtures. 0.3 mM PGK was digested with 10.9 TU_{BAEE}/ml trypsin in the absence of substrates (A) and in the presence of 6.6 mM 3-PG (B). At different time intervals the digestion was stopped as described in section 2.

tivity and the release of trichloroacetic acid-soluble peptides start only after a relatively long lag phase (figs 1 and 2). However, during this lag phase two large intermediate peptides (molecular masses of about 30 and 17–18 kDa) are already formed, as indicated by SDS-PAGE (fig. 3B).

Gel chromatographic analysis of the same reaction mixture under non-denaturating conditions revealed that both intermediate peptides were eluted together with the intact PGK, i.e. no separation according to size could be achieved (fig. 4). Since the specific PGK-activity of the eluted protein corresponds to the activity of the native enzyme, it follows that the two fragments together constitute an active, nicked PGK molecule. Thus, the fragments are held together in the active conformation by non-covalent forces.

Considering the sizes of the fragments obtained and the known X-ray structure of horse muscle PGK [1,2] there are two alternative candidates for the tryptic cleavage site. This site should be within either of the two domains (either in/around helix 4 or in/around helix 10 according to the numbering of the structure of horse muscle PGK). Splitting between the domains, i.e. in the hinge region, would result in two fragments of about equal size (22–23 and 24–26 kDa) and possibly a small (about 1 kDa) additional fragment, which is not consistent with the present experimental findings. Although it is difficult to judge the site of limited proteolysis [24,25], it is often at a polypeptide chain segment that either connects two compact globular domains or loops out from a compact fold of a domain [24]. Neither of these seems to occur in the case of PGK complexed with 3-PG. The structure of this binary complex at high resolution together with the identification of the cleavage site

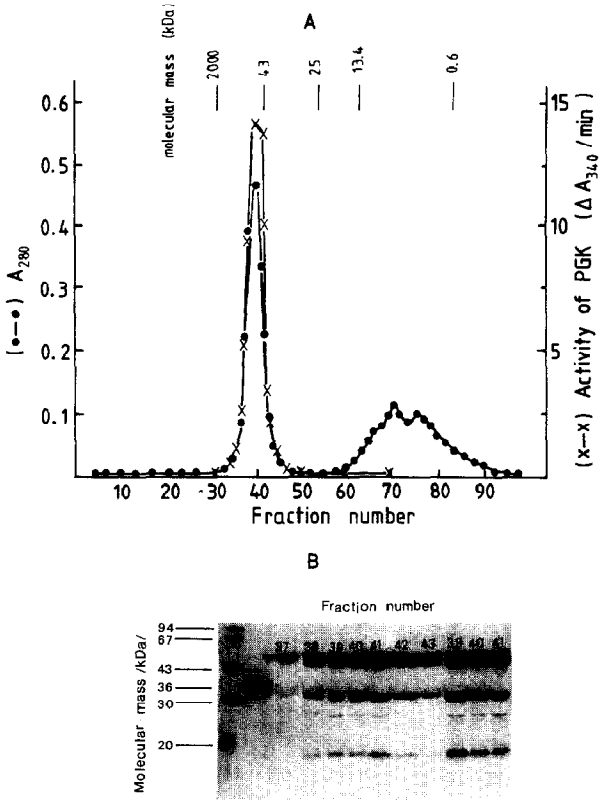

Fig.4. Gel chromatographic analysis of the digestion mixture. 0.5 mM PGK was digested in the presence of 6.6 mM 3-PG with 10.9 TU_{BAEE}/ml trypsin. After 60 min digestion a sample was withdrawn and treated and loaded onto the column as described in section 2. A. Elution profile detected at 280 nm (•—•) and by measuring PGK-activity (×—×). B. SDS-PAGE analysis of the eluted fractions.

will give the unambiguous answer. The latter is underway in our laboratory.

At any rate, as the cleavage site is within either of the domains, the conclusion can be drawn that in the presence of 3-PG neither the hinge region of PGK nor numerous other possible cleavage sites, even far away from the 3-PG binding site, are exposed to trypsin. In the literature there are observations indicating a pronounced effect of 3-PG on PGK conformation [5,10,11,26,27]. Therefore, the protection by 3-PG against tryptic decomposition of PGK seems to be due to its effect on enzyme conformation, rather than to direct steric hindrance at a crucial tryptic cleavage site.

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