Limited Proteolysis at the Carboxy End Modifies Interactions between the Subunits of *Escherichia coli* Phosphofructokinase†

Gisèle Le Bras and Jean-Renaud Garel*

Unité de Biochimie Cellulaire, Institut Pasteur, 75724 Paris Cedex 15, France

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ABSTRACT: The limited proteolysis of *Escherichia coli* phosphofructokinase by subtilisin involves the removal of a segment of 40–50 residues at the C-terminal end of each polypeptide chain [Le Bras, G., & Garel, J. R. (1985) *J. Biol. Chem. 260*, 13450–13453]. The time course of proteolysis has been followed by the appearance of shorter chains, the loss of allosteric inhibition by phosphoenolpyruvate, and the weakening of the tetrameric structure in the absence of fructose 6-phosphate. It is found that with only one shorter chain out of four the stability of the tetramer is altered so that it is no longer stable in the absence of fructose 6-phosphate. Also, the reduction in size of only two chains is sufficient to render the enzyme insensitive to allosteric effectors, albeit the protein still possesses the ability to bind such an effector (at least partially); the cleavage of all four chains is needed to lose all the effector binding ability. The C-terminal segment therefore plays an important role in subunit interactions as seen from the gradual changes in structural and functional properties which follow its removal from one, two, or four chains.

The limited proteolysis of the allosteric phosphofructokinase (PFK)[1] from *Escherichia coli* by subtilisin yields a homogeneous derivative in which a segment of 40–50 amino acids has been removed from the C-terminal end of each of the four polypeptide chains (Le Bras & Garel, 1982, 1985). This derivative, called PFK* from hereon, has the following properties (Le Bras & Garel, 1982, 1985): (i) PFK* remains tetrameric in the presence of its substrate fructose 6-phosphate (F6P). (ii) The tetrameric state of PFK* is enzymatically active, with the same maximum velocity and half-saturating concentrations for ATP and F6P as intact PFK; the saturation of PFK* by F6P is still cooperative. (iii) The activity of the tetrameric state of PFK* is insensitive to the presence of allosteric effectors, whether activator like GDP or inhibitor like phosphoenol pyruvate (PEP). (iv) PFK* is less stable than PFK toward irreversible thermal denaturation; both are protected by F6P, but PFK* is not protected by GDP, suggesting that no effector binding site remains. (v) In the absence of F6P, PFK* dissociates spontaneously from a tetrameric into a dimeric form which is devoid of enzyme activity. (vi) The addition of F6P to dimeric and inactive PFK* restores the active and tetrameric structure provided that proper precautions are taken.

PFK and PFK* differ in several functional and structural properties, and these differences reflect the role of the C-terminal segment in the intact structure. The conversion of PFK into PFK* involves the cleavage of at least four peptide bonds (i.e., one on each chain) and thus does not probably take place in a single step. The present work investigates the kinetics of the production of PFK* from PFK following the changes in some of their different properties. It is found that all these changes do not occur at the same rate, which shows that the conversion of PFK into PFK* is indeed a multistep process: in particular, the dissociation into dimeric species and the absence of allosteric regulation are not correlated to the cleavage of four polypeptide chains.

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Materials and Methods

All the chemicals used for buffers, activity assays, and electrophoresis were of analytical grade and obtained from Sigma, Merck, or Boehringer. The preparation of PFK, the measurement of its activity, and polyacrylamide gel electrophoresis (PAGE) were performed as described previously (Le Bras & Garel, 1982, 1985).

To study the kinetics of proteolysis, the amount of subtilisin and the temperature were lowered as compared to previous procedures (Le Bras & Garel, 1982, 1985) so as to slow down the reaction rate. The conditions for limited proteolysis were as follows: 0.2–0.5 mg/mL PFK and 0.5% (w/w) subtilisin in 0.1 M Tris buffer, 1 mM $MgCl_2$, 7 mM β-mercaptoethanol, and 2 mM F6P, at pH 8.2 and 27 °C, unless specified. When needed, proteolysis was stopped by the addition of phenylmethanesulfonyl fluoride to a final concentration of 10 mM.

PAGE analysis in nondenaturing conditions was carried out in the absence or in the presence of 1 mM F6P; in the latter, F6P was present in both the gel and the electrophoresis buffer. It was checked that successive dilutions and the conditions of PAGE were sufficient to get rid of any interference from residual F6P (Le Bras & Garel, 1985), when the absence of substrate was needed. The proteolysis was stopped by using phenylmethanesulfonyl fluoride before any PAGE analysis.

Results

From the known differences between PFK and its proteolyzed derivative, PFK*, the time course of proteolysis should be accompanied by changes in several properties such as the loss of sensitivity to allosteric effectors, the decrease in the molecular weight of the polypeptide chains, the increased tendency of the tetrameric structure to dissociate, etc. (Le Bras

[1] Abbreviations: PFK, phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11); PFK*, homogeneous proteolytic derivative of PFK described by Le Bras and Garel (1982, 1985); F6P, D-fructose 6-phosphate; PEP, phosphoenolpyruvate; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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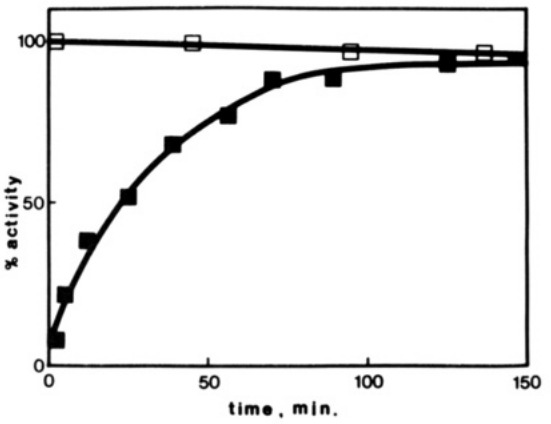

FIGURE 1: Changes in the activity of PFK upon proteolysis by subtilisin. Samples were withdrawn from the proteolysis mixture and assayed immediately in the absence (□) or in the presence (■) of 4 mM PEP. Proteolysis conditions are 0.5 mg/mL PFK and 0.5% (w/w) subtilisin in 0.1 M Tris buffer, 2 mM F6P, 1 mM $MgCl_2$, and 7 mM β-mercaptoethanol at pH 8.2, 27 °C.

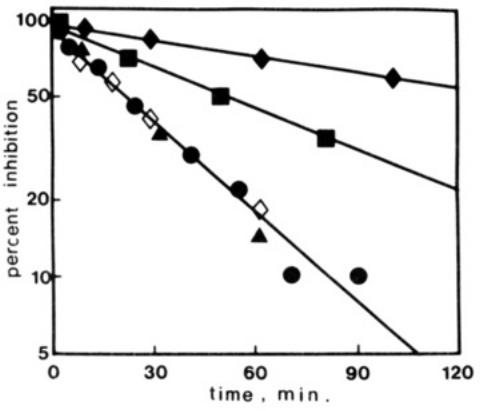

FIGURE 2: Changes in PEP inhibition of PFK activity upon proteolysis in different conditions. Inhibition is expressed as the ratio $[(A_0 - A_I)/A_0] \times 100$, where A_0 and A_I are respectively the activities measured in the absence and presence of 4 mM PEP for the same sample after a given time of proteolysis; inhibition is shown on a logarithmic scale to point out the first-order kinetics of its disappearance. Proteolysis conditions are as given in Figure 1 (●) or with one difference: (◇) no F6P is present; (▲) [PFK] is only 0.025 mg/mL [the amount of subtilisin is unchanged and is therefore 10% (w/w)]; (■) 4 mM PEP or (♦) GDP was present. The greater protection afforded by GDP as compared to PEP is due to its better binding to PFK and thus to a slower cleavage (Le Bras & Garel, 1982). Inhibition values below 10% are not shown because of their inaccuracy. Inhibition of the original enzyme by 4 mM PEP is 90–95%.

& Garel, 1982, 1985). Three methods were used to follow the kinetics of proteolysis of PFK by subtilisin: (a) activity measurements which are related to the catalytic and regulatory properties; (b) direct measurements of the cleavage reaction using PAGE in the presence of SDS; and (c) measurements of the tetramer to dimer dissociation by PAGE analysis in nondenaturing conditions.

Proteolysis of PFK As Followed by Activity Measurements. Figure 1 shows that the activity remains constant when assayed in the absence of effector, while it increases when assayed in the presence of the inhibitor PEP; also, the activity assayed at a nonsaturing concentration of F6P in the presence of the activator GDP decreases (result not shown). As proteolysis proceeds, the allosteric inhibition by PEP (or activation by GDP) becomes less and less efficient; this loss of sensitivity to effectors follows first-order kinetics with a half-life of about 20–30 min (Figure 2). Similar kinetics are obtained when the PFK concentration during proteolysis is lowered by a factor of 20 while that of subtilisin is unchanged (Figure 2); this

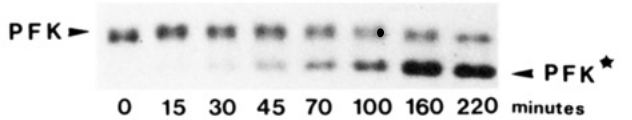

FIGURE 3: Time course of the reaction of cleavage of PFK by subtilisin. Samples are withdrawn from the same proteolysis mixture as that utilized for activity measurements of Figure 1 at the indicated time, treated with phenylmethanesulfonyl fluoride to inactivate subtilisin, then denatured by boiling in 1% SDS, stored in ice until the end of proteolysis, and analyzed by PAGE in the presence of SDS as described by Le Bras and Garel (1982). The arrows show the positions where the chains of original PFK and completely proteolyzed PFK* are found; they correspond respectively to molecular weights of 37 000 and 32 000 (Le Bras & Garel, 1982).

suggests that proteolysis of PFK is not linked to dissociation and indeed occurs in the tetramer. Figure 2 also shows that the rate of loss of the sensitivity to allosteric effectors is the same whether F6P is present or not during proteolysis but is lower when PEP or GDP is present. Occupation of the regulatory sites of PFK by an effector, inhibitor as well as activator, renders proteolysis much slower; the cleavage by subtilisin appears as a conformational probe related to the "bound" or "free" state of the regulatory sites, and not to an "active" or "inactive" conformation of the enzyme, in agreement with previous conclusions (Le Bras & Garel, 1982). Although F6P has no influence on this particular reaction, its presence protects markedly the intact enzyme (Le Bras & Garel, 1982) and its proteolyzed derivative (Le Bras & Garel, 1985) from further degradation, and it was included in the standard proteolysis conditions.

Proteolysis of PFK As Followed by the Cleavage Reaction. The cleavage reaction can be directly measured by the changes in the relative amounts of chains with different sizes, as determined by PAGE in the presence of SDS (Figure 3). Only two bands are present, corresponding respectively to PFK and PFK*, which suggests that proteolysis does not involve intermediate length chains; the 40–50 amino acids removed from each chain do not appear as a single peptide. If such a peptide exists, it is rapidly degraded to small pieces by the rather large amount of subtilisin used in the proteolysis of PFK; it is also possible that the conversion from the PFK chain into the PFK* chain implies successive cuts, although no stable intermediate is observed. Therefore, PAGE in the presence of SDS does not measure the actual number of peptide bonds which have been split; it allows only to distinguish between "long" and "short" chains, the latter being obviously more proteolyzed than the former.

The extent of cleavage visible on a gel cannot be lower than that in the proteolysis mixture at the time when the samples were taken; rather, it can be higher if some further proteolysis has taken place during the preparation of the PAGE samples, in which case the cleavage reaction would appear on the gel as faster than it really is. Comparing the time courses of cleavage (Figure 3) and of loss of PEP inhibition (Figures 1 and 2) suggests that the former is slower than the latter. For instance, around 30 min the protein has lost half of its inhibition by PEP, whereas it still possesses a majority of long chains. Also, around 70 min the protein has apparently similar amounts of long and short chains, while almost all of the inhibition by PEP has disappeared. A detectable fraction of long chains is still present at 160 or 220 min, when the enzyme is completely insensitive to PEP, even at high concentrations above 10 mM. It seems then as if the loss of sensitivity to allosteric effectors is not related to the reduction in size of all four chains upon proteolysis; visual inspection of the gels such as that shown in Figure 3 suggests that the size of only half

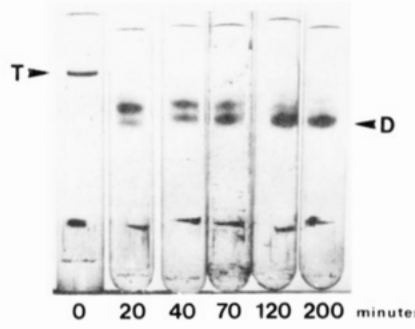

FIGURE 4: PAGE analysis of the proteolysis of PFK by subtilisin in nondenaturing conditions in the absence of F6P. Gels contain 10% acrylamide. It has been checked that residual F6P does not interfere with electrophoresis. Indeed, the same migration is obtained when no F6P is present during the proteolysis; also, under these conditions PFK* migrates as a dimeric species (Le Bras & Garel, 1985) at the position corresponding to the arrow labeled D. The arrow labeled T corresponds to the migration of the tetrameric structure of either PFK or PFK* (Le Bras & Garel, 1982).

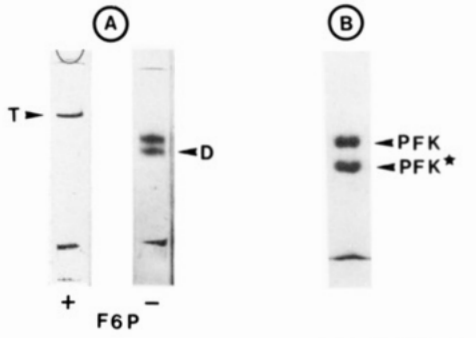

FIGURE 5: Intermediate state of the proteolysis of PFK. Proteolysis was stopped when the PEP inhibition of PFK activity was about 10% by adding phenylmethanesulfonyl fluoride (dissolved in dioxane); the protein was then filtered through an ACA 34 Ultrogel column as described by Le Bras and Garel (1985) for the preparation of PFK*. The intermediate state thus obtained was not inhibited by 4 mM PEP within experimental errors (inhibition ≤10%). (A) PAGE in nondenaturing conditions in the presence (+) or absence (−) of 1 mM F6P in both the gel and the electrophoresis buffer. (B) PAGE in the presence of SDS. The arrows labeled T, D, PFK, and PFK* have the same meaning as in the legends of Figures 3 and 4.

of the chains needs to be reduced for losing the allosteric regulation of the activity.

Proteolysis of PFK As Followed by the Dissociation into Dimers in the Absence of F6P. In the presence of F6P, both PFK and PFK* are tetrameric (Le Bras & Garel, 1982, 1985); in the absence of F6P, PFK remains tetrameric, while PFK* dissociates into dimers (Le Bras & Garel, 1985). This dissociation of PFK* can be observed by PAGE in nondenaturing conditions depending on whether F6P is present or not during electrophoresis (Le Bras & Garel, 1985). The tetrameric states of PFK* and PFK have the same electrophoretic migrations, and indeed no change is visible throughout the proteolysis reaction when PAGE is performed in the presence of F6P (not shown). However, PAGE without F6P shows that protein dissociation takes place from the beginning of proteolysis (Figure 4). It was checked that subtilisin is completely inhibited by phenylmethanesulfonyl fluoride and that no residual proteolysis occurs during the PAGE run; indeed, the zero time in Figure 4 corresponds to a sample PFK to which phenylmethanesulfonyl fluoride and subtilisin were added in this order at the same time (i.e., within a few seconds): no dissociation is visible. The most striking feature is the rapid disappearance of the band corresponding to a tetrameric structure; indeed, this band is no longer visible at a time of 20 min when the enzyme still shows about half of its PEP inhibition and a majority of long chains (compare with Figures 1–3). Since intact PFK migrates the same whether F6P is present or absent (Le Bras & Garel, 1985), this shows that no intact PFK molecules remain at this time of 20 min. Proteolysis is carried out in the presence of 2 mM F6P (see Materials and Methods), where all species between PFK four intact chains) and PFK* (no intact chain) are tetrameric; the earliest change observed during proteolysis of PFK seems then to be the disappearance of the ability to remain under a tetrameric association state in the absence of F6P.

The two bands on the gels in Figure 4 are related to dimeric species with different degrees of cleavage. The lower band can unambiguously be assigned to a dimer with two short chains by comparison with PFK*. The higher band could correspond to both dimers with two long chains (which could not reassociate during electrophoresis) and dimers with one long chain and one short chain; this is also suggested by the fact that the relative ratio of these two bands does not change very much between 40 and 70 min, whereas the cleavage reaction proceeds to a significant extent (Figure 3). However, any conclusion about the various dimeric species produced by dissociation of partially proteolyzed PFK seems rather tentative as long as the detailed mechanism of cleavage has not been elucidated.

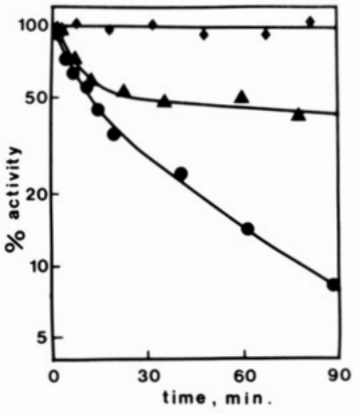

FIGURE 6: Thermal inactivation of the intermediate state of the proteolysis of PFK. First-order representation of the irreversible loss of activity upon heating at 55 °C in 0.1 M Tris buffer, 1 mM $MgCl_2$, and 7 mM β-mercaptoethanol, pH 8.2 (●), and in the presence of 10 mM GDP (▲) or 10 mM F6P (♦). The kinetics of inactivation in the absence of ligand could be biphasic, either because of some residual F6P (Le Bras & Garel, 1985) or because of intrinsic heterogeneity (see text); the inactivation in the presence of GDP is clearly biphasic.

Some Properties of the Intermediate Species Possessing an Average of Two Long and Two Short Chains. In some experiments, the proteolysis was stopped when the PEP inhibition had reached about 10% (as compared with the original level of over 90% for the original PFK) in the presence of 4 mM PEP, i.e., when the sensitivity to allosteric effectors could hardly be measured. PAGE analysis of this protein shows that it is composed of about equal amounts of long and short chains (Figure 5B) and that dissociation in the absence of F6P yields about equal amounts of two different dimers (Figure 5A). This protein could indeed correspond to a mixture of different species with one, two, or more cleaved chains; it could also be considered as an "intermediate state" between PFK and PFK* for some of its properties.

The presence of GDP protects PFK against irreversible thermal inactivation, whereas it has no such effect in the case of PFK* (Le Bras & Garel, 1982). Figure 6 shows that this intermediate state is partially protected by GDP: about half of the initial activity is lost rapidly upon heating whether GDP

is present or not, and about half remains only when GDP is present. This biphasic loss of activity in the presence of GDP probably arises from the heterogeneity of this intermediate state at high temperature: only a fraction of the protein molecules still has a GDP binding site. For this fraction, the binding of GDP occurs, as seen from its protective effect, but does not influence the enzymatic activity; indeed, the bulk of this intermediate state is no longer sensitive to allosteric effectors. Thus, during proteolysis of PFK, the effector sites seem to be destroyed only after the ability of these same sites to regulate the activity has disappeared. Figure 6 also shows that the bulk of the intermediate state is protected by F6P against thermal inactivation, as expected from the known same property of PFK and PFK* (Le Bras & Garel, 1982).

Both PFK and PFK* show cooperative saturations for F6P, when measured by the activity at saturating ATP, albeit with different Hill coefficients of 4 for PFK and 2 for PFK*. The intermediate state has a cooperative saturation by F6P with a Hill coefficient of 2 (result not shown), but since this state could be a mixture of species, this result only indicates that partially proteolyzed molecules behave like PFK* rather than like PFK.

Discussion

Some of the functional and structural properties of PFK are modified upon limited proteolysis by subtilisin (Le Bras & Garel, 1982, 1985). The study of the time courses of these modifications and of the properties of an intermediate state, taken as a snapshot of the proteolytic reaction, shows that all the properties are not affected at the same rate. One can indeed distinguish between the properties of native PFK according to the rate of their disappearance upon proteolysis: (i) *rapid disappearance*, the ability to maintain a tetrameric structure in the absence of F6P (Figure 4); (ii) *medium rapid disappearance*, the sensitivity to allosteric effectors of the enzymatic activity (Figures 1 and 2); (iii) *slow disappearance*, the average number of intact chains (Figure 3) and the presence of a GDP binding site (Figure 6). Some properties are not affected by proteolysis, such as the catalytic activity (Figure 1) and the tetrameric structure in the presence of F6P (Le Bras & Garel, 1982, 1985).

PAGE in the presence of SDS separates long and short chains (Figure 3) but gives only their average relative amount and does not in principle reflect the composition of individual protein molecules. However, the different time courses observed must reveal the formation and/or the disappearance of different protein species; following the disappearance of native PFK, the probable order of appearance on stage is as follows: (i) a (set of) species with only one short chain, the tetrameric structure of which is no longer stable in the absence of F6P; (ii) a (set of) species with still long chain(s) and effector binding site(s) albeit insensitive to allosteric regulation of its activity; (iii) PFK*, with only short chains and without any effector binding site. Thus, removing the C-terminal segment from more and more chains gradually alters the properties of the protein.

The sequence of PFK from *E. coli* is homologous to that of the enzyme from *Bacillus stearothermophilus* (P. R. Evans and H. Hellinga, personal communication), so their three-dimensional structures should also resemble each other. In the PFK from *B. stearothermophilus*, the C-terminal segment, which is removed by proteolysis, is close to the effector binding site and belongs to one of the contact areas between two subunits (Evans & Hudson, 1979; Evans et al., 1981). Therefore, some of the changes observed upon proteolysis of PFK are not unexpected in view of the structural data; their order, however, was difficult to predict. In addition, the enzyme from *E. coli* has 21 more residues at its C-terminal end than that from *B. stearothermophilus* (P. R. Evans and H. Hellinga, personal communication), and the detailed conformation of the two proteins may differ somewhat in this region.

The C-terminal segment seems to have an important role in the interactions between the subunits of PFK. Indeed, it must be present on all four chains for a tetrameric structure to be stable in the absence of F6P; removal of one such segment out of four renders the protein "F6P dependent" for its tetrameric state and therefore for its enzymatic activity. Also, when this C-terminal segment is present on only two chains out of four (on the average) as in the intermediate state, there is a marked alteration in the influence exerted by a given site on the others: allosteric effectors can still bind (at least partially) but without affecting the properties of the active sites, and similarly, the cooperativity for saturation by F6P between the active sites is reduced by half. The C-terminal segment could then be considered itself as a covalently bound effector the presence of which would tighten the tetrameric structure, thus making it more stable, more cooperative, and able to be regulated; this "built-in" effector also shows some cooperativity since its major effects are related to the presence of three or four such segments. Limited proteolysis acts as the progressive dissociation of this effector and allows partial separation of the functional and structural importance of this region of the polypeptide chain.

Acknowledgments

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Registry No. PFK, 9001-80-3.

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