A Proteolyzed Derivative of *Escherichia coli* Phosphofructokinase Is No Longer Sensitive to Allosteric Effectors and Still Shows Cooperativity in Substrate Binding†

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ABSTRACT: Limited proteolysis of *Escherichia coli* phosphofructokinase by subtilisin yields a homogeneous derivative. This proteolyzed protein is composed of four polypeptide chains, with a molecular weight of 32000 as compared to 37000 for the original enzyme. Removal on each chain of about 5 kdaltons maintains the enzymatic activity and does not change the apparent affinity for the substrates ATP and fructose 6-phosphate. Limited proteolysis, however, affects the cooperativity of fructose 6-phosphate binding: the Hill coefficient is reduced from almost 4 in the native enzyme to only 2 in its proteolyzed derivative. Also, the proteolyzed protein is no longer sensitive to allosteric effectors, activator, or inhibitor. These changes in regulatory properties upon proteolysis are apparently due to the destruction of the effector binding site. The allosteric effector GDP protects phosphofructokinase against proteolysis and irreversible thermal inactivation; GDP is, however, inefficient in protecting the proteolyzed protein against thermal denaturation. These results suggest that phosphofructokinase may function as a dimer of dimers, in which homotropic and heterotropic allosteric effects are not mediated by the same sets of quaternary interactions.

Phosphofructokinase (PFK)[1] catalyzes the reaction

ATP + fructose 6-phosphate → ADP + fructose 1,6-bisphosphate

In many organisms, this enzyme is highly regulated and corresponds to an important control point of the glycolytic pathway. The enzyme from *Escherichia coli* K12 has been purified and extensively studied from the point of view of both its structure and its regulatory properties. Native PFK is a tetramer of molecular weight around 140000 composed of four polypeptide chains of 35000 each (Blangy, 1968; Kotlarz & Buc, 1977). Although their amino acid sequence is not known, several arguments suggest that these four chains might be identical: (i) These chains have the same size and electrical charge (Kotlarz, 1980). (ii) Given the total content in lysine and arginine of PFK, the number of tryptic peptides is consistent with the existence of four identical chains (Thornburgh et al., 1978). (iii) Equilibrium dialysis experiments show the presence of one site per chain for either the substrate ATP or the effector GDP (Blangy, 1971). (iv) There is a strong immunochemical resemblence between the enzymes extracted from *E. coli* and *B. stearothermophilus* (Kotlarz, 1980), in which case the identity of the chains has been established by amino acid sequence determination (Hudson et al., 1979).

E. coli PFK shows a hyperbolic saturation curve for its first substrate, ATP, and a markedly cooperative saturation curve for its second substrate, F6P. Under some conditions, the cooperativity index (or Hill coefficient) almost reaches the value of 4, i.e., the number of subunits, indicating a strong interaction between the F6P binding sites. In addition, the enzyme is allosterically activated by the purine nucleoside diphosphates, ADP or GDP, and inhibited by PEP. Most of the enzymatic properties of *E. coli* PFK are compatible with a concerted allosteric mechanism; the enzyme would exist under two conformational states having the same affinity for ATP and catalytic efficiency and differing in their affinities for F6P and for the allosteric effectors (Blangy et al., 1968).

Among the various tools used in the study of proteins, limited proteolysis has often been successful in pointing out some of the relationships between structure and function. Its general strategy rests upon the transformation of a protein into (a) simpler species, the properties of which being then compared to those of the original material. In the case of *E. coli* PFK, limited digestion with subtilisin yields a homogeneous derivative in which the same piece (about 5 kdalton) is removed from each polypeptide chain. This proteolyzed PFK still possesses a tetrameric structure, albeit with a reduced stability toward dissociation into dimers or thermal denaturation. The shortening of each chain has no effect on enzymatic activity: when saturated, proteolyzed PFK is as active as the original enzyme, with the same apparent affinity for ATP. Proteolyzed PFK still shows a cooperative saturation for F6P binding, but with a reduced cooperativity index as compared to the uncleaved enzyme. The greatest difference between native PFK and proteolyzed PKF is that the latter is completely insensitive to the presence of allosteric effectors; limited proteolysis abolishes the ability of the enzyme to be activated by GDP or inhibited by PEP. The absence of regulatory properties in proteolyzed PFK contrasts with the significant cooperativity observed in F6P binding; this suggests that different subunit interactions are responsible for homotropic and heterotropic allosteric effects. Recent determination of the three-dimensional structure of another bacterial PFK, that from *B. stearothermophilus* (Evans & Hudson, 1979), indeed shows that the F6P and effector (GDP or PEP) binding sites do not involve the same contacts between polypeptide chains.

Materials and Methods

Materials

The chemicals used for buffers, enzymatic activity measurements, and electrophoresis were all of analytical grade and were purchased from Sigma, Merck, or Boehringer. Subtilisin was protease type VIII from Sigma. PFK was prepared from

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[1] Abbreviations: PFK, phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11); F6P, D-fructose 6-phosphate; PEP, phosphoenolpyruvate; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

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E. coli K12, strain Tir8, using the procedure of Kotlarz & Buc (1977).

Methods

PFK enzymatic activity was measured by using the coupled assay described by Kotlarz et al. (1975). Standard conditions for activity measurements were 10^{-3} M in both substrates, F6P and ATP, in 0.1 M Tris buffer and 10^{-2} M $MgCl_2$, at pH 8.2, 25 °C. Some of the activity measurements, especially those at low substrate concentration, were also performed at a constant ATP concentration in the presence of an ATP-regenerating system (creating phosphate and creatine phosphokinase). PFK concentration was determined according to Kotlarz & Buc (1977), from either absorbance measurements using an extinction coefficient of 0.6 L g^{-1} cm^{-1} or activity assays using a specific activity of 190 units/mg.

Limited Proteolysis of PFK. Limited proteolysis of PFK by subtilisin was carried out under the following conditions: 0.2–0.5 mg/mL PFK, 3–6% (w/w) subtilisin, in 0.1 M Tris buffer, 10^{-3} M $MgCl_2$, 7×10^{-3} M β-mercaptoethanol, and 2×10^{-3} M F6P, at pH 8.2, 37 °C, for 2 h. Proteolysis was then stopped by the addition of phenylmethanesulfonyl fluoride (dissolved in dioxane) to a final concentration of 10^{-2} M. The proteolyzed derivative prepared in this way is stable for several days, as seen from its enzymatic activity, subunit structure, and chain size, provided it is kept in a concentrated state (such as an ammonium sulfate precipitate) and in the presence of its ligands F6P and/or GDP.

Polyacrylamide Gel Electrophoresis. Electrophoresis in the presence of $NaDodSO_4$ was carried out on slab gels at 10% acrylamide. Gels were stained with Coomassie Blue type R and destained by 7.5% acetic acid/30% methanol (v/v).

Electrophoresis under nondenaturing conditions was performed on disc gels at variable acrylamide concentrations. Gels were stained by Coomassie Blue type G in 3.5% perchloric acid and destained by 5% acetic acid.

Thermal Denaturation of PFK. PFK or its proteolytic derivative was heated at a given temperature in the same buffer as used for limited proteolysis, except for the absence of F6P. After a given time, the residual enzymatic activity was measured in standard conditions.

Results and Discussion

When native PFK is subjected to a limited digestion by subtilisin under the conditions given above, the protein is rapidly modified into a new species which is quite resistant to further proteolysis. This first rapid cleavage step is not accompanied by a change in enzymatic activity when assayed under standard conditions. This new species, called proteolyzed PFK from here on, appears as homogeneous when analyzed by electrophoresis on polyacrylamide gels under both native and denaturing conditions (Figure 1). Finding a unique band on a $NaDodSO_4$ gel shows that proteolyzed PFK is composed of chains of the same size and, hence, that limited proteolysis has taken place to the same extent on all four original chains of PFK. Under the conditions used here, the formation of this homogeneous proteolyzed PFK is rapid, and no partially cleaved intermediates have been observed. Further studies are now in progress to analyze the sequence of events leading to the formation of proteolyzed PFK, but the present report gives only the properties of this derivative. Proteolyzed PFK can be further degraded, albeit very slowly, with a concomitant loss of enzymatic activity. This further degradation does not yield fragments of a defined size and probably corresponds to an extensive digestion of each polypeptide chain. Proteolyzed PFK seems then to be the only stable intermediate species formed during this reaction, at least under the present conditions.

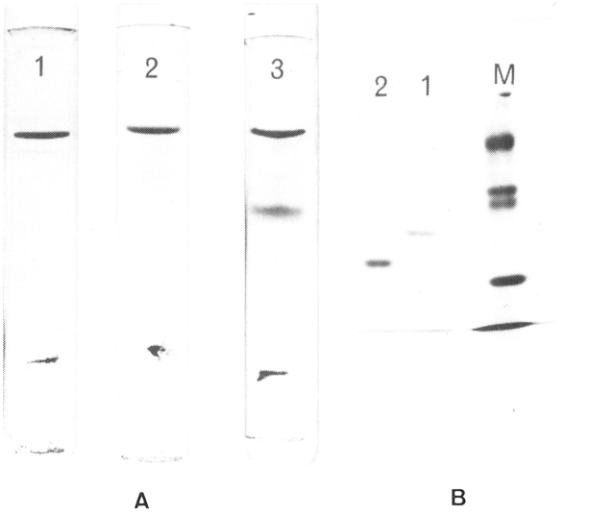

FIGURE 1: Polyacrylamide gel electrophoresis of PFK and its proteolyzed derivative. (A) Electrophoresis under nondenaturing conditions on a gel containing 10% acrylamide. (1) Native PFK; (2) proteolyzed PFK; (3) proteolyzed PFK partially dissociated upon aging for several days. (B) Electrophoresis under denaturing condtions. (1) Native PFK; (2) proteolyzed PFK, whether tetrameric or partially dissociated into dimers; (M) molecular weight markers.

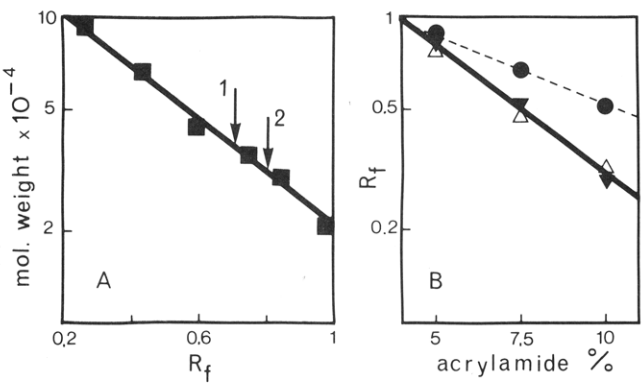

FIGURE 2: Determination of the molecular weight and subunit structure of proteolyzed PFK. (A) electrophoresis under denaturing conditions in the presence of $NaDodSO_4$. Arrows 1 and 2 correspond to native and proteolyzed PFK, respectively. The molecular weight markers (■) are the following: phosphorylase *b*, 94 000; serum albumin, 67 000; ovalbumin, 43 000; lactate dehydrogenase, 35 000; carbonic anhydrase, 30 000; soybean trypsin inhibitor, 20 000. The molecular weight of 37 000 obtained for the PFK chain is in agreement with other determinations (Blangy, 1968; Kotlarz & Buc, 1977). (B) Electrophoresis under native conditions of native (▼) and proteolyzed (△) PFK in polyacrylamide gels of different concentrations in acrylamide. Also shown is the mobility of the lighter component obtained from proteolyzed PFK after partial dissociation (●). The slope of the dashed line is about half of that found for tetrameric species and thus apparently corresponds to dimeric species (see text).

Subunit Structure of Proteolyzed PFK. Proteolyzed PFK is composed of chains of a unique size; the molecular weight of these chains is around 32 000 as determined from a calibrated $NaDodSO_4$ gel (Figure 2A). Limited proteolyzed has shortened each of the PFK chains by about 15%, decreasing the molecular weight from 37 000 to 32 000. Because of the cooperativity exhibited by proteolyzed PFK in its saturation by one of its substrates, F6P, this derivative is composed of at least two subunits (see below). The actual number of chains which are present in proteolyzed PFK was investigated by gel filtration and polyacrylamide gel electrophoresis, in order to determine whether this number is two or four. Gel filtration on an 80 × 1.5 cm column of Sephadex G-150 or Sephacryl

S-300 does not clearly separate native and proteolyzed PFK (the two proteins can be differentiated by their regulatory properties as shown below). This indicates that the hydrodynamic volumes of these proteins are not markedly different; indeed, the same column separates completely native PFK, with a molecular weight of 140 000, from bovine serum albumin, with a molecular weight of 67 000. Native PFK and proteolyzed PFK were also compared by electrophoresis on gels of different acrylamide concentrations. These proteins have very similar electrophoretic mobilities (Figure 1), independent of the acrylamide concentration of the gel between 5 and 10% (Figure 2B). Such behavior also indicates that the sizes, and hence the molecular weights, of native PFK and its proteolyzed derivative are close to one another (Hedrick & Smith, 1968). It appears then that proteolyzed PFK, like the native enzyme, is composed of four polypeptide chains. The conclusion is therefore that, besides leaving intact the enzymatic activity, the limited proteolysis of PFK does not affect the tetrameric structure of the protein.

The tetrameric structure of proteolyzed PFK is less stable than that of the native enzyme. When kept as a dilute protein solution and in the absence of its ligands, proteolyzed PFK slowly dissociates into smaller species. These smaller species can be detected by their electrophoretic mobility (Figures 1 and 2B). Their apparent size is about half of that of proteolyzed PFK, as seen from experiments using gels of different acrylamide concentrations (Figure 2B). This dissociation of proteolyzed PFK takes place without further change in either the chains size or the enzymatic activity. The cooperativity observed for the saturation curve with F6P is not changed by this dissociation, suggesting that the dissociated species are dimers. The possibility that dissociated species could reassociate during the activity measurements seems unlikely: the addition to the electrophoresis buffer or either substrate, F6P or ATP, does not change the mobility of these species. This slow dissociation of proteolyzed PFK can be a consequence of limited proteolysis; it occurs, however, so slowly (on the time scale of days) that the proteolyzed derivative can be easily studied under its tetrameric state, and all the following results are related to this tetrameric state. Also, finding that proteolyzed PFK can dissociate into species which show cooperative saturation by F6P, and are therefore dimers, confirms the above conclusion about the tetrameric state of the proteolyzed derivative.

Although the actual site(s) of cleavage is (are) not known, finding that proteolyzed PFK has chains of a unique size and maintains a tetrameric structure suggests not only that each chain has to be cleaved to the same extent but also that the same segment(s) has (have) been removed from each chain. If it is so, and if native PFK is actually composed of four identical chains, the same conclusion would also apply to proteolyzed PFK, namely, that its chains would be identical.

Enzymatic Properties of Proteolyzed PFK. The conversion from native to proteolyzed PFK is not accompanied by changes in enzymatic activity, when assayed in standard conditions. Figure 3 gives the saturation curves of both native PFK and proteolyzed PFK for ATP at high F6P concentration. These two curves are superimposable, which shows that limited proteolysis has not changed the apparent affinity for ATP; both proteins exhibit a hyperbolic saturation, with a half-saturating concentration, around 7×10^{-5} M ATP, in close agreement with that given by Blangy et al. (1968).

Figure 4 shows the saturation curves for F6P at high ATP concentration of both native PFK and proteolyzed PFK. The native enzyme exhibits a highly cooperative saturation, corresponding to a Hill coefficient of 3.8 ± 0.2 (Figure 4) in agreement with other results (Blangy et al., 1968). The saturation curve of proteolyzed PFK for F6P is still clearly cooperative (Figure 4), but the corresponding Hill coefficient is only 2 ± 0.2 (Figure 4). The half-saturating concentrations are also quite similar, about 5×10^{-4} and 4×10^{-4} M F6P for native and proteolyzed PFK, respectively. Thus limited proteolysis has not apparently changed the apparent affinity for F6P, nor has it abolished the ability of the F6P binding sites to interact with one another. In native PFK, a Hill coefficient of 3.8 indicates that the four F6P binding sites operate in a highly concerted way; the reduction of 2 of this coefficient upon proteolysis suggests that the concertation between F6P binding sites involves only two sites at a time or, in other words, that proteolyzed PFK behaves as a dimer of concerted dimers during its saturation by F6P. This interpretation is further supported by the observation that proteolyzed PFK is able to dissociate into two smaller species without a change in the shape of the F6P saturation curve (see above).

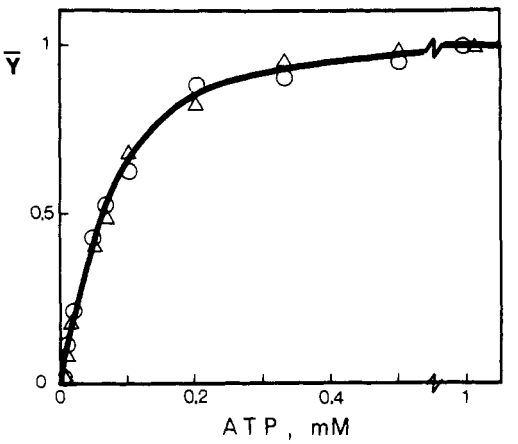

FIGURE 3: Dependence on ATP concentration of the enzymatic activity of PFK (○) and proteolyzed PFK (△). Conditions: 10^{-3} M F6P, 0.1 M Tris buffer, 10^{-2} M $MgCl_2$, pH 8.2, 25 °C. Activity is measured at 340 nm by using a coupled assay (Kotlarz et al., 1975) to follow the formation of fructose 1,6-bisphosphate. Activity is expressed at the fraction of saturation, i.e., relative to the maximum activity.

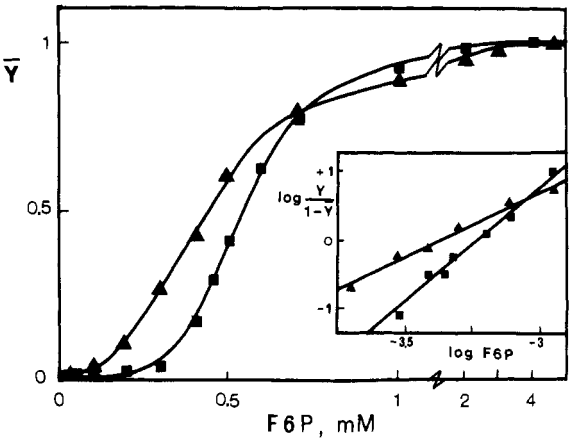

FIGURE 4: Dependence on F6P concentration of the enzymatic activity of PFK (■) and proteolyzed PFK (▲). Conditions: 10^{-3} M ATP, 0.1 M Tris buffer, 10^{-2} M $MgCl_2$, pH 8.2, 25 °C. The curve of PFK is very close to that of Blangy et al. (1968). Activity values correspond to initial rate measurements and were the same with or without an ATP-regenerating system. Activity is expresed as the fraction of saturation, $\bar{Y}$, i.e., of maximal velocity (Monod et al., 1965). Hill representation of the data shown in this figure for the saturation of PFK (■) and proteolyzed PFK (▲) by F6P at an ATP concentration of 10^{-3} M.

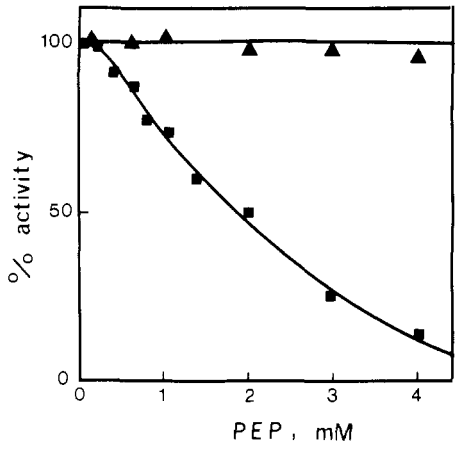

FIGURE 5: Influence of the presence of PEP on the activity of PFK (■) and proteolyzed PFK (▲). Activity is measured in standard conditions, at 10^{-3} M F6P and ATP, and is expressed relative to that in the absence of PEP.

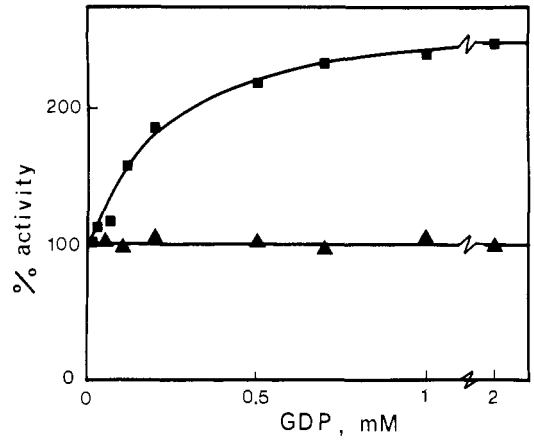

FIGURE 6: Influence of the presence of GDP on the activity of PFK (■) and proteolyzed PFK (▲). Activity is measured at 10^{-3} M ATP and 5×10^{-4} M F6P and is expressed relative to that in the absence of GDP. The F6P concentration, 5×10^{-4} M, corresponds to a partial saturation of PFK and its proteolyzed derivative, which is necessary to observe the activation by GDP; for native PFK, the activity at 5×10^{-4} M F6P is only 40% of the maximum value (Figure 4).

Limited proteolysis has occurred to the same extent (and presumably at the same sites) on each of the four original chains of PFK; it has probably not introduced a structural asymmetry into the protein molecule. Therefore, native PFK could also work as a dimer of dimers, even though this is not reflected in the Hill coefficient for F6P saturation. This conclusion is indeed supported by the structural properties of another bacterial PFK, that of *B. stearothermophilus*, as determined by crystallography (Evans & Hudson, 1979). If this property of being a dimer of dimers pertains to *E. coli* PFK as its seems, then two sets of quaternary interactions exist within the protein, one between the dimers and the other within each dimer. The existence of these two sets is masked in native PFK, whereas it becomes apparent in its proteolyzed derivative. Therefore limited proteolysis seems to have affected only one of these sets of interactions, the result being a cooperativity for F6P binding reduced by a factor of 2.

Regulatory Properties of Proteolyzed PFK. Native PFK is allosterically activated by GDP and inhibited by PEP (Figures 5 and 6; Blangy et al., 1968). In contrast, the enzymatic activity of proteolyzed PFK is not modified by the presence of either effector, GDP or PEP, even at a concentration well above that which saturates the native enzyme (Figures 5 and 6). Limited proteolysis has therefore abolished

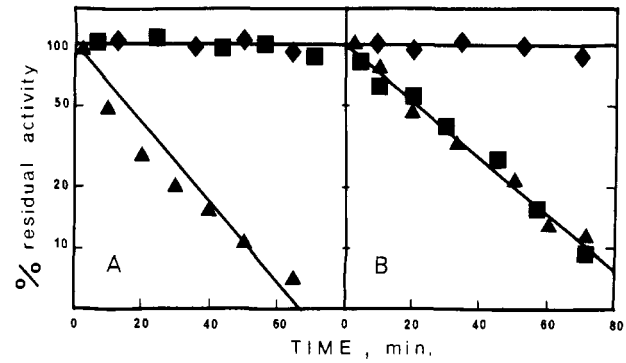

FIGURE 7: Thermal inactivation of PFK and proteolyzed PFK. (A) First-order representation of the rate of irreversible denaturation of PFK at 57 °C in 0.1 M Tris buffer, 10^{-3} M $MgCl_2$, 7×10^{-3} M β-mercaptoethanol, at pH 8.2 (▲), and in the presence of 10^{-2} M F6P (♦) or GDP (■). Although the kinetics of inactivation of PFK in the absence of ligand may correspond to a biphasic process, no detailed investigation was carried out, and the straight line drawn here represents an average first-order kinetics. (B) First-order representation of the rate of irreversible denaturation of proteolyzed PFK at 40 °C under the same conditions as those given in part A. No ligand (▲), 10^{-2} M F6P (♦), or 10^{-2} M GDP (■).

the ability of the enzyme to be regulated, by either activation or inhibition, i.e., has abolished the heterotropic allosteric effects, without abolishing all the homotropic effects in F6P binding. Such behavior is not expected from a strictly concerted allosteric mechanism; indeed, if the same structural transition between two states is responsible for both homotropic and heterotropic cooperative effects, then both should disappear together (Monod et al., 1965). It is, however, possible that the absence of regulatory properties in proteolyzed PFK results from the destruction of the effector binding site by limited proteolysis. The same site is apparently able to bind GDP or PEP in the PFK from *B. stearothermophilus* (Evans & Hudson, 1979), and destruction of this site would obviously abolish the heterotropic effects because of the absence of effector binding. An allosteric transtion could still take place in proteolyzed PFK, even though each subunit is now devoid of effector site; it would be this transition which is reflected in the homotropic cooperative binding of F6P (Figure 4). As shown below, the absence of protection against irreversible thermal denaturation in the presence of GDP suggests that proteolyzed PFK has indeed lost its effector sites.

Stability of Proteolyzed PFK against Irreversible Thermal Denaturation. Proteolyzed PFK is less stable than the native enzyme toward irreversible thermal denaturation (Figure 7): similar rates of denaturation are obtained for the native enzyme at 57 °C and its proteolyzed derivative at 40 °C. The presence of F6P stabilizes native PFK against this denaturation (Figure 7A and Blangy, 1971); it also protects the proteolyzed protein (Figure 7B), which shows that F6P binds to proteolyzed PFK and that the F6P–protein complex is more stable than the free protein. The presence of GDP also protects native PFK against thermal denaturation (Figure 7A), but it has no effect on the stability of the proteolyzed protein (Figure 7B). Although this absence of protection by GDP of proteolyzed PFK does not prove that GDP does not bind to the protein, this lack of binding seems to be the simplest explanation. The loss of sensitivity to GDP upon proteolysis might be due to the destruction of the effector sites, and GDP would no longer bind to proteolyzed PFK, hence, the absence of both allosteric activation (Figure 6) and protection against irreversible thermal denaturation (Figure 7B). Thus a simple explanation of the properties of proteolyzed PFK is that proteolysis has destroyed the effector binding sites.

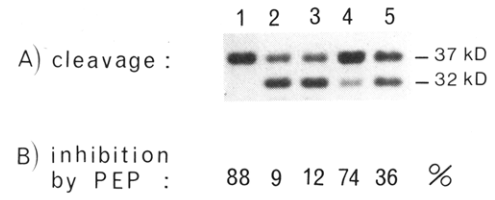

FIGURE 8: Influence of the presence of F6P or GDP on the limited proteolysis of PFK. Proteolysis was carried out under standard conditions for 1 h, and its extent was measured by (A) the changes in chain size, as revealed by electrophoresis on polyacrylamide gels in the presence of $NaDodSO_4$, and (B) the regulatory properties, as measured by the inhibition of enzymatic activity by 4×10^{-3} M PEP. (1) Native PFK (control); proteolysis in the presence of (2) no added ligand, (3) 10^{-2} M F6P, (4) 10^{-2} M GDP, and (5) 10^{-2} M PEP.

Effect of PFK Ligands on the Protein Proteolysis. The standard conditions used here to prepare the proteolyzed derivative of PFK include the presence of 2×10^{-3} M F6P. Proteolysis of PFK was also studied under the same conditions of pH, temperature, PFK and protease concentrations, etc. (see Methods), and in the presence of various ligands at a concentration of 10^{-2} M, proteolysis was monitored by the changes in both regulatory properties and polypeptide chain size (Figure 8). Depending on the ligand present, only two cases were observed after the same time of proteolysis: either PFK is not cleaved and remains regulated or it is proteolyzed with the loss of regulatory properties and concomitant shortening of the chain length. The presence of F6P gives the same result as for the free enzyme. The presence of GDP retards the conversion of native into proteolyzed PFK. PEP also protects PFK from being proteolyzed, but less efficiently than GDP at the same concentration of 10^{-2} M. In the presence of PEP, PFK is transformed in a mixture of long (37 kdaltons) and short (32 kdaltons) chains, with a concomitant partial loss of regulatory properties. Thus it seems that binding at the active site has no effect on the proteolytic attack, whereas binding at the effector site protects the polypeptide chain from being cleaved and of course leaves the regulatory properties unaffected. This result indicates that the susceptibility of PFK to limited proteolysis is not controlled by a conformational effect such as the allosteric transition; if it were so, F6P and GDP should have similar effects because binding of either ligand favors the active conformation, and PEP, which stabilizes the inactive conformation, should not behave as GDP. Rather, this suggests that the proteolytic attack indeed takes place in the vicinity of the effector binding site. Therefore filling this site would protect PFK from being cleaved. This interpretation is consistent with that proposed above that proteolysis actually destroys this effector site. Such a proposal could be experimentally tested, provided that the amino acid sequence and tridimensional structure of *E. coli* PFK are known, by locating the site(s) of cleavage within the polypeptide chain. Assuming that the enzymes from *E. coli* and *B. stearothermophilus* resemble each other suggests that cleavage could have taken place within the carboxyl-terminal region of chain (Evans & Hudson, 1979).

Conclusion

Limited proteolysis of *E. coli* PFK yields a derivative in which each of the four polypeptide chains has been cleaved to the same extent. This derivative, proteolyzed PFK, remains fully active and is able to maintain a tetrameric structure. It is, however, less stable than the native enzyme with regard to its thermal denaturation or dissociation into dimeric species. Proteolyzed PFK is no longer sensitive to allosteric regulation, either activation by GDP or inhibition by PEP, and this loss of regulatory properties seems to be due to a destruction of the effector binding sites upon proteolysis. It even seems that proteolysis occurs within or very close to this site, as seen from the protection against cleavage afforded by binding either effector, GDP or PEP. Although it no longer shows allosteric heterotropic effects, proteolyzed PFK still exhibits cooperativity in homotropic F6P binding. This cooperativity apparently involves only two subunits at a time within the tetramer and suggests that native PFK could indeed function as a dimer of dimers. In this case, limited proteolysis would have specifically affected the quaternary interactions related to only one or two of the symmetry axes of the protein molecule.

Acknowledgments

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