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Retention of Allosteric Properties in an Inactive, Proteolyzed Form of Phosphofructokinase†

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ABSTRACT: Treatment of rabbit skeletal muscle phosphofructokinase by subtilisin resulted in inactivation as a consequence of the cleavage of an approximately 10000 dalton equiv from each protomer of the enzyme. Although the initial cleavage rapidly removed a 12 amino acid peptide from the carboxyl terminus without loss of activity [Kemp, R. G., Foe, L. G., Latshaw, S. P., Poorman, R. A., & Heinrikson, R. L. (1981) *J. Biol. Chem. 256*, 7282–7286], most or all of the residual cleavage was from the amino terminus. The cleaved products did not remain associated with the remaining protein. The inactive, 74000-dalton protein remained tetrameric and displayed much of the association–dissociation behavior of the native enzyme. The inactive protein associated to higher polymers in the presence of fructose 1,6-bisphosphate and dissociated in the presence of high concentrations of citrate, but not with low concentrations of citrate plus MgATP as seen with native enzyme. The inactive, digested protein also underwent ligand-induced conformational changes as indicated by changes in the reactivity of thiol groups with 5,5′-dithiobis(2-nitrobenzoic acid). The subtilisin-digested protein retained all of the reactive thiol groups and, as observed with native enzyme, AMP blocked the reactivity of two highly reactive thiol groups. On the other hand, fructose 6-phosphate was not capable of blocking these thiol groups as observed with native phosphofructokinase. MgATP did not block the reactivity of the thiol groups of phosphofructokinase as observed with native enzyme. These data suggested that the inactive protein retained binding sites for AMP, fructose-1,6-P_2, and citrate but had lost binding sites for the two substrates and for MgATP at the inhibitory site. These conclusions were confirmed by direct binding studies which showed only one binding site for adenine nucleotides in contrast to the three observed with native enzyme. Cyclic AMP was bound by the proteolyzed product with the same affinity as observed with the native enzyme. Fructose-2,6-P_2 enhanced the affinity of both proteins for cyclic AMP. Equilibrium binding studies confirmed the integrity of the fructose-1,6-P_2 binding site and the virtual absence of fructose-6-P binding. The data suggest a relatively discreet domain essential to the catalytic activity but structurally distinct from several allosteric regulatory sites.

Rabbit skeletal muscle phosphofructokinase has a complex regulatory behavior that is the consequence of its interaction with metabolites at specific binding sites [see Uyeda (1979) for review]. Found on the 84000-dalton protomer are a catalytic site for ATP and fructose-6-P,[1] a citrate site that is also capable of binding phosphoenolpyruvate and phosphoglycerates, a sugar bisphosphate site, an adenine nucleotide activating site, an ATP inhibitory site, and an undetermined number of sites for the interaction of inorganic phosphate and several cations. Of further consideration in the overall topography of the enzyme, one must include regions of interaction among protomers for the formation of the active tetramer and for the display of allosteric regulatory behavior, regions that interact in the formation of higher polymers, and the site of phosphorylation (Kemp et al., 1981). In an earlier study of limited proteolysis of phosphofructokinase (Riquelme & Kemp, 1980), we have shown that the enzyme is inactivated upon subtilisin cleavage of an approximately 10000 dalton

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NaDodSO_4, sodium dodecyl sulfate; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; fructose-6-P, fructose 6-phosphate; fructose-2,6-P_2, fructose 2,6-bisphosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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equiv from each protomer of the enzyme but that the remaining protein retains its tetrameric structure. In the present study we show that this protein retains relatively intact many of the above-mentioned ligand binding sites and sites of protomeric interaction. The data suggest a relatively discreet domain essential to the catalytic activity but structurally distinct from other allosteric sites.

Materials and Methods

Enzyme Preparation. Crystalline phosphofructokinase was prepared from fresh rabbit muscle as described by Kemp (1975). Second crystals were collected by centrifugation and dissolved in the indicated buffer. For removal of ammonium sulfate, the phosphofructokinase solution was dialyzed with one change against 100 volumes of the same buffer, at room temperature. When it was necessary to remove bound ATP, the enzyme solution was passed through a charcoal–cellulose (1:1 w/w) column (4.5 × 20 mm). A 280:260 absorbancy ratio greater than 1.6 was taken as an indication that ATP had been essentially removed from the protein (Parmeggiani et al., 1966).

Binding Experiments. Determinations of the binding of effectors of phosphofructokinase were performed by two different methods: the Hummel–Dreyer method and the fast-flow equilibrium dialysis method. Briefly, the Hummel–Dreyer technique (1962) was as follows: A Sephadex G-50 column (1.5 × 30 cm) was equilibrated at room temperature with a pH 6.95 buffer consisting of 25 mM β-glycerophosphate, 25 mM glycylglycine, 1 mM EDTA, 1 mM dithiothreitol, 5000 cpm/mL labeled ligand, and the indicated concentrations of unlabeled ligands. One-half milliliter of enzyme solution (0.3 mg/mL), equilibrated with the column buffer, was layered onto the column. The column was developed at a rate of 15 mL/h.

The fast-flow equilibrium dialysis method of Colowick & Womack (1969) was used to determine the dissociation constant for cyclic [2,8-^{3}H]AMP (Amersham, Arlington Heights, IL) and [γ-^{32}P]ATP (Amersham) with a pH 7.0 buffer containing 25 mM β-glycerol-P, 25 mM glycylglycine, 1 mM EDTA, and 1 mM dithiothreitol as previously described by this laboratory (Gottschalk & Kemp, 1981). Phosphofructokinase at concentrations of 0.5–2.0 mg/mL was employed in the cylindrical dialysis chambers.

Thiol Titrations. Titrations of phosphofructokinase sulfhydryl groups with 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma) were performed as previously described by Kemp & Forest (1968). The reactions were carried out at 20 °C in a pH 7.0 buffer containing 25 mM β-glycerol-P, 25 mM glycylglycine, and 1 mM EDTA with approximately 0.2 mg/mL phosphofructokinase in a final volume of 3 mL. The reaction was initiated by the addition of 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma). A millimolar extinction coefficient of 13.6 (Ellman, 1959) at 412 nm for the thionitrobenzoic acid ion was used and the reaction was followed spectrophotometrically.

Polyacrylamide Gel Electrophoresis. The polyacrylamide gel electrophoresis was carried out by using the system described by Laemmli (1970). The lyophilized samples were dissolved in 50 mM borate (pH 9.2) containing 1% $NaDodSO_4$ to a protein concentration of 500 μg/mL, aliquots were taken and diluted with water, and an equal volume of sample buffer was added. The sample buffer was 120 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 2% 2-mercaptoethanol, 20% glycerol, and 0.003% bromophenol blue. After boiling for 3–5 min, aliquots were applied to the slab consisting of 7.5% polyacrylamide gel (0.15 × 10 cm). Standards used were myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, and ovalbumin. The slabs were run at 25 mA/slab, fixed for 30 min with 25% 2-propanol and 10% acetic acid, stained for 1 h with 0.1% Coomassie blue R-250 in fixing solution, and destained overnight against 10% 2-propanol and 10% acetic acid. For determination of radioactivity, the gels were sliced, digested with H_2O_2 (Balhorn & Chalkley, 1975), and counted in a scintillation counter.

Chromatographic Procedures. Affinity chromatography of native and digested phosphofructokinase was performed by a modification (Riquelme et al., 1978) of the procedure of Ramadoss et al. (1976). The protein in 10 mM Tris–PO_4 (pH 8.0), 0.2 mM EDTA, and 1.0 mM dithiothreitol was pumped through a 0.4 × 5 cm column of ATP–Sepharose prepared essentially as described by Lindberg & Mosbach (1975) and equilibrated with the Tris–PO_4 buffer. Elution of phosphofructokinase was carried out with a sequential washing of the column by buffer, ADP, and fructose-6-P and final elution of the enzyme with a solution of 50 mM Tris–PO_4 (pH 8.0), 0.2 mM EDTA, 1.0 mM dithiothreitol plus 0.2 mM ADP, and 0.2 mM fructose-6-P.

Anion-exchange chromatography was performed with a 1.5 × 30 cm column of CM-52 cellulose previously equilibrated with 50 mM NaTes (pH 7.0), 0.2 mM EDTA, 0.1 mM ATP and 1 mM dithiothreitol, at room temperature. After 12 mg of digested phosphofructokinase was absorbed onto the column, the column was washed with the same buffer until no further protein was detected in the effluent. The enzyme was eluted from the column with the pH 7.0 buffer containing 0.15 M NaCl. Three-milliliter fractions were collected at a rate of 20 mL/h.

Enzyme Assays. Assays to determine phosphofructokinase activity were performed at 30 °C with a recording spectrophotometer (Kemp, 1975). The assay at pH 8.2 for total activity contained 25 mM β-glycerol-P, 25 mM glycylglycine, 1 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 0.2 mM NADH, 3 mM $(NH_4)_2SO_4$, 1 mM ATP, 1 mM fructose-6-P, 0.6 unit/mL aldolase, 0.3 unit/mL glycerol-P dehydrogenase, and 0.3 unit/mL triose-P isomerase. The three auxiliary enzymes were obtained from Sigma Chemical Co. (St. Louis, MO). Prior to its addition to the assay, phosphofructokinase was diluted with 25 mM β-glycerol-P, 25 mM glycylglycine (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM ATP. Pyruvate kinase was determined from the rate of oxidation of NADH in a coupled assay with lactic acid dehydrogenase. The assay at pH 7.5 contained 50 mM NaTes, 0.15 M KCl, 10 mM $MgCl_2$, 0.5 mM NADH, 1 mM phosphoenolpyruvate, 1 mM ADP, and 0.6 unit of lactic acid dehydrogenase.

Labeling the Class I Thiol Group. Iodo[^{14}C]acetic acid (49.5 cpm/pmol) was added at a final concentration of 0.25 mM to 16 mg of phosphofructokinase in 1.5 mL of solution containing 25 mM β-glycerol-P, 25 mM glycylglycine, 1 mM EDTA, and 2 mM fructose-6-P, all at pH 7.1. Under these conditions the class II thiol groups are blocked, and only a single thiol group, the class I, reacts at a significant rate (Kemp & Forest, 1968; Walker et al., 1977). The reaction proceeded for 100 min in the dark and under helium. The solution was then passed through a column of Sephacryl S-200 previously equilibrated with 50 mM Tris–PO_4 (pH 8.0), 0.1 mM EDTA, 0.1 mM ATP, and 1.0 mM dithiothreitol. The labeled protein that eluted contained 395 cpm/μg which corresponds to 0.67 mol incorporated/mol of phosphofructokinase protomer.

Other Methods. Protein concentration was determined by the Coomassie dye assay as described by Bradford (1976) with ATP-free phosphofructokinase as a standard or spectropho-

tometry at 290 nm in 0.1 N NaOH by using an absorption coefficient of 1.09 mL mg^{-1} cm^{-1} or at 280 nm, pH 7.0, using an absorption coefficient of 1.01 mL mg^{-1} cm^{-1} (Paetkau & Lardy, 1967).

Hydrolysis of peptides to amino acids was performed for 22 h in sealed tubes with constant boiling HCl. The hydrolysates were analyzed on a Glenco custom modular amino acid analyzer (Glenco Scientific, Houston, TX).

Fructose-2,6-P_2 was prepared as described previously (Gottschalk et al., 1982).

Results and Discussion

Site of Cleavage by Subtilisin. We have shown previously (Riquelme & Kemp, 1980) that a limited subtilisin digestion of PFK in the native state proceeds in two fairly discreet steps: first to an 81 000-dalton polypeptide product and then to a 74 000-dalton polypeptide. The second step parallels loss of enzyme activity. The first cleavage removes at least 12 residues from the carboxyl terminus (Kemp et al., 1981), but it is not known whether the inactivation step proceeds by the removal of a single fragment from either end of the molecule or by cleavages at both ends of the molecule. There are two pieces of information that provide an approach to this problem. First, Emerk & Frieden (1975) have shown that a limited trypsin digestion cleaves the molecule into approximate halves of around 40 000 daltons without a significant change in activity. A sequential treatment with trypsin and subtilisin thus should show reduction in molecular weight from 40 000 daltons either of both halves or of only one of the halves depending upon the mechanism of the subtilisin inactivation step. The second observation which contributes to a resolution of the problem is the conclusion of Ogilvie (1980) that the class I thiol group, which can be easily labeled, is located in the amino-terminal half of phosphofructokinase. We have observed that the thiol group that reacts with cyanothionitrobenzoate as described by Olgivie (1980) shows all of the reaction properties such as inhibition by MgATP as does the thiol group highly reactive with iodoacetic acid (S. Bazaes, S. Latshaw, and R. Kemp, unpublished data).

Therefore, for determination of the site of subtilisin cleavage, phosphofructokinase was labeled at the class I thiol group with iodoacetate as described under Materials and Methods. This enzyme at 3.6 mg/mL was digested with bovine trypsin at a phosphofructokinase to trypsin ratio of 100:1 at 30 °C in 50 mM Tris–PO_4 (pH 8.0), 0.1 mM EDTA, 0.1 mM ATP, and 1.0 mM dithiothreitol. The digestion was stopped after 2 h by the addition of a 3-fold excess of soybean trypsin inhibitor, and the cleaved enzyme was passed through a Sephacryl S-200 column equilibrated with 50 mM KTes (pH 7.5), containing 0.1 mM EDTA, 0.1 mM ATP, and 1.0 mM dithiothreitol. This column removed the protease, its inhibitor, and the small fragments cleaved from the terminus of phosphofructokinase (Kemp et al., 1981) and changed the buffer in preparation for the next step. The enzyme at this stage had not lost significant activity due to the trypsin digestion. The enzyme pool was now containing 0.24 mg/mL was then digested with subtilisin in a phosphofructokinase to protease ratio of 1000:1, and samples were removed at 10-min intervals for activity assays and for $NaDodSO_4$–polyacrylamide gel electrophoresis analysis. The activity loss is described in Figure 1. Samples were analyzed on $NaDodSO_4$–polyacrylamide gel electrophoresis, and the stained gels were scanned with a densitometer, sliced, and digested for a determination of radioactivity distribution. Several of the gel scans are shown in Figure 2 with an indication of the distribution of the radioactivity. Trypsin digestion produced a broad band corresponding to a molecular weight of 38 000–40 000, indicating that the enzyme had been cut in half. In the 20-min subtilisin digestion sample, it can be seen that a new band appeared at a position corresponding to about 32 000 daltons. At 60 min, the two peaks were approximately equal in size, and no further change in distribution occurred except a gradual diminution of both peaks. This was interpreted as only one of the halves being converted to the 32 000-dalton protein, and this fragment was the amino-terminal half as indicated by the relocation of the radioactivity to that fragment. In the 20-min sample, the peak representing the 32 000 component was about half the size of the 38 000–40 000 component, but it contains two-thirds of the radioactivity. This is the predicted distribution if two-thirds of only one of the two halves of the protein was digested to 32 000 daltons, resulting in a mass ratio of 2 to 1, in favor of the larger, 38 000–40 000-dalton components. Figure 1 shows

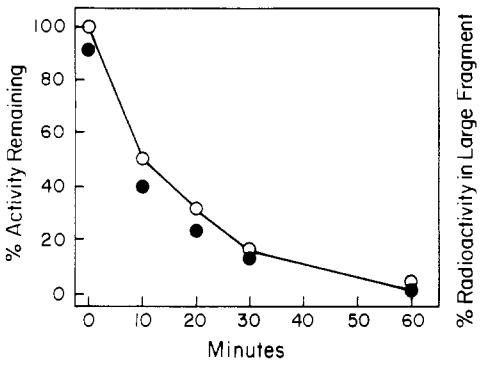

FIGURE 1: Subtilisin digestion of a limited trypsin digest of thiol-labeled phosphofructokinase. Phosphofructokinase, labeled at the class I thiol group with iodo[^{14}C]acetic acid, was digested by trypsin and purified on Sephacryl S-200 as described in the text. The fully active, trypsin-digested enzyme at a concentration of 0.24 mg/mL was digested with subtilisin (1000:1, PFK:protease), and samples were removed for activity assays and $NaDodSO_4$–polyacrylamide gel electrophoresis. Activity loss (open circles) and the relocation of radioactivity (closed circles) to a lower molecular weight component (see Figure 2) are shown.

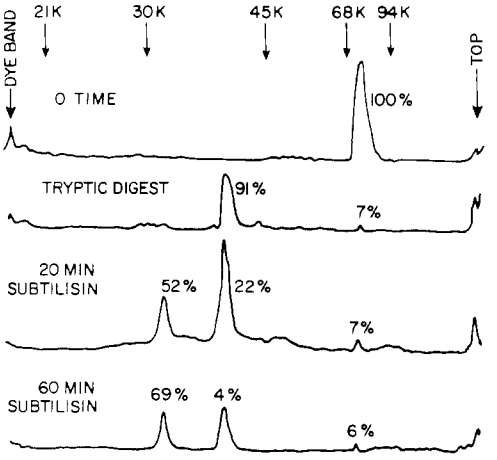

FIGURE 2: Sequential digestion of phosphofructokinase with trypsin and subtilisin. Enzyme was labeled at the reactive thiol with iodo-[^{14}C]acetate and digested as described in the text. Samples of the digest described in Figure 1 were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. The gel was scanned with a densitometer, sliced, and counted. The densitometer scans are shown with the percentages indicating distribution of recovered radioactivity. Approximately 1200 cpm of labeled protein (3 μg) was applied to each channel. Positions of marker proteins are shown at the top. The bottom two tracings represent scans of subtilisin digests of the trypsin-treated protein indicated in the second tracing from the top. Some aggregation occurred with digestion so that the amount of protein and radioactivity that entered the gel at the later time samples was about 75% of the total recovered at zero time.

that the loss of enzyme activity in the digestion directly paralleled the loss of label from the 38 000- to 40 000-dalton protein. The interpretation of the above experiments is that a 6000–8000-dalton domain was removed by subtilisin from the amino terminus of phosphofructokinase and resulted in the loss of enzyme activity.

Properties of the Inactive 74 000-Dalton Protein. Chromatographic Behavior. Native phosphofructokinase with an isoelectric point below pH 6.0 did not bind to CM-cellulose or other anionic chromatographic media under conditions that we have tested. On the other hand, phosphofructokinase digested to the 74 000-dalton fragment did bind to CM-cellulose at pH 7.0 and other conditions described under Materials and Methods. The 74 000 dalton fragment was retained by the column and was eluted upon addition of 0.15 M NaCl to the elution buffer. If a partial digest containing a mixture of the 74 000-dalton and the 81 000-dalton digestion products was applied to the column, enzyme containing some 74 000-dalton peptide but enriched in the larger component was found in the initial effluent, whereas the retained material was almost exclusively 74 000-dalton protein. The presence of 74 000-dalton material in the initial effluent was most likely due to the occurrence of hybrids of 74 000- and 81 000-dalton components as tetramers or higher polymers that were not retained by the anion-exchange medium. Most of the experiments to be described here employed protein that had been purified on CM-cellulose.

Muscle phosphofructokinase has been shown to bind to ATP–Sepharose wherein the ATP was linked to the support phase at N-6 by means of an aminohexylcarbamoylmethyl linkage (Ramadoss et al., 1976). On the basis of its specific elution with the components of the dead-end complex, fructose-6-P and ADP, it has been proposed that the binding occurs at the catalytic site of the enzyme (Ramadoss et al., 1976). We observed that both native phosphofructokinase and the catalytically active 81 000-dalton subtilisin digestion product were capable of binding to ATP–Sepharose and of being specifically eluted by the dead-end complex. On the other hand, the inactive 74 000-dalton digestion product bound to the ATP–Sepharose but could not be eluted with high concentrations of the combination of fructose-6-P and ADP, or by ATP. This is consistent with the loss or the disruption of ATP binding at the catalytic site of phosphofructokinase that has been digested to the 74 000-dalton peptide. The protein could be partially eluted with 0.4 M NaCl and completely removed from the support with a KCl–urea mixture.

Aggregation State. When inactivation by subtilisin digestion was first reported (Riquelme & Kemp, 1980), it was noted that the product existed as a tetramer. The inactive, proteolyzed form was also capable of aggregating to higher polymeric states as well as responding to some of the ligands that influence the polymeric state. The position of elution from Bio-Gel A 1.5 M of native and subtilisin proteolyzed phosphofructokinase was compared at low salt concentrations (the digestion buffer) and in the presence of digestion buffer plus 0.15 M NaCl. Bio-Gel A 1.5 M has an exclusion limit of 1×10^6 daltons, which would exclude superaggregates of phosphofructokinase but retain the lower molecular weight tetramers and dimers. Phosphorylase *a* (390 000 daltons) and aldolase (160 000 daltons) were used as standards in separate runs with the same column. In the low salt buffer, both proteins eluted as aggregates greater than 1.0×10^6 daltons in the void volume. On the other hand, the high salt buffer promoted dissociation of both proteins to tetramers. From a plot of log (molecular weight) vs. the elution volume the extrapolated molecular weight for the tetramer of the digested phosphofructokinase was 295 000. The digested phosphofructokinase did not appear to retain the fragment(s), which accounted for a decrease of 6000–8000 daltons per protomer. The mobilities determined by gel permeation chromatography of the tetramers of native and digested phosphofructokinase differ in accordance with the molecular weights of their respective protomers. The lack of association of peptide products with the 295 000-dalton inactive tetramer was further confirmed by trichloroacetic acid precipitation of the inactive protein that eluted in the void volume of Sephacryl S-200 column. Amino acid analysis of the trichloroacetic acid supernatant and 10% formic acid extracts of the sediment indicated that the total amino acid content of the soluble peptides was in the range 1% of the total protein present. On the other hand, an examination of the low molecular weight region of the elution profile of a subtilisin digest on Sephacryl S-200 demonstrated a great variety of small peptides, indicating that the fragment removed from the amino terminus was rapidly digested to small peptides as one might expect from the action of a fairly nonspecific protease. A survey of the literature shows that in the limited proteolytic cleavage of other enzymes, the cleavage products in some cases remain associated with the major fragment (Botelho et al., 1977), while in other cases the products dissociate (Bergstrom et al., 1978; Dautry-Varsat & Cohen, 1977; Dautry-Varsat et al., 1979).

The influence regulatory ligands on the state of association of native and the inactive, digested phosphofructokinase was also assessed by gradient centrifugation. Sucrose gradient centrifugation was performed with pyruvate kinase (M_r 237 000) as an internal standard, since its molecular weight was not influenced by the addition of various effectors, and because its molecular weight places it directly between that of phosphofructokinase dimers (M_r 168 000) and tetramers (M_r 334 000). All of the 5–20% gradients contained 25 mM β-glycerol-P, 25 mM glycylglycine (pH 7.0), 1 mM EDTA, and 1 mM dithiothreitol, to which was added the ligand to be studied. In the presence of 1 mM ATP, both the digested enzyme and the native enzyme sedimented as tetramers. In the presence of 1 mM fructose-1,6-P_2, the position of sedimentation of both digested enzyme and native enzyme moved to higher densities corresponding to octomers and higher polymers. This demonstrated that the fructose-1,6-P_2 site was intact in the digested enzyme, along with the regions of contact for the formation of higher polymers. The addition of citrate at 5 mM displaced the sedimentation position of both native and digested phosphofructokinase to lower densities. In the presence of low citrate (0.25 mM), no change in the sedimentation was observed. This was consistent with previous studies (Colombo et al., 1975; Colombo & Kemp, 1975) that showed that with native enzyme high concentrations of citrate will promote the formation of inactive dimers, but low citrate concentrations are ineffective. However, in the presence of MgATP, low citrate synergistically acts with MgATP to inhibit phosphofructokinase and promote dissociation. This was demonstrated by sucrose density gradients wherein dissociation of native phosphofructokinase was observed in the presence of low citrate (0.25 mM) and MgATP (1 mM). The digested 74 000-dalton protein was not dissociated by this combination of ligands. The data demonstrate that the citrate site remained essentially intact in the digested protein but that the site of ATP binding that acts synergistically with citrate is not functional.

Ligand Interaction and Thiol Reactivity. The above data provided evidence on the basis of association behavior for the

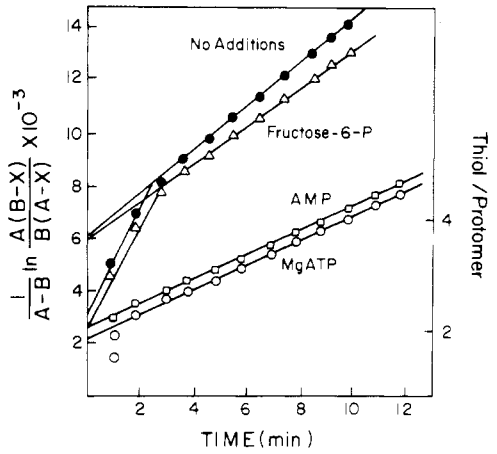

FIGURE 3: Second-order thiol reactivity plot of 74K phosphofructokinase. Digested phosphofructokinase (0.2 mg/mL) was reacted with 33 μM DTNB in the presence of 2 mM effectors and other conditions described under Materials and Methods.

retention of sites of interaction for fructose bisphosphate and citrate, whereas the effect of ATP was not seen. This laboratory has also shown evidence for binding and conformational changes on the basis of the reactivity of thiol groups with dithiobis(nitrobenzoic acid) (DNTB) (Kemp & Forest, 1968; Kemp, 1969). Interaction of MgATP at the inhibitory site blocks the reaction of all of the highly reactive thiols while AMP and fructose-6-P protect against reaction of two thiol groups called class II on the basis of their reactivity (Kemp & Forest, 1968). Thus, with partially proteolyzed 74 000-dalton protein, the integrity of the ATP inhibitory site, the adenine activating site, and the fructose-6-P catalytic site can be determined indirectly by the alteration in phosphofructokinase sulfhydryl reactivity with DTNB in the presence of various metabolic effectors. Figure 3 depicts the second-order rate plots for the reaction of the 74 000-dalton protein with DTNB. In the absence of effectors, four thiols of digested enzyme rapidly titrated, the same result as that obtained with native phosphofructokinase. AMP protected against reaction of the two class II thiols of both native enzyme and digested enzyme. With native enzyme, fructose-6-P also protected the same two thiols as AMP; however, fructose-6-P did not block the titration of the class II thiols of digested protein. With digested phosphofructokinase, MgATP did not block the class I thiol but did protect the class II thiols, apparently due to its weaker interaction with the AMP sites (Mathias & Kemp, 1972). Thus, subtilisin digestion abolished the ATP inhibitory site and the catalytic site, while the adenine nucleotide activating site remained intact.

Ligand Binding Studies. The foregoing studies do not show directly the loss of effector binding sites since only the conformational event associated with binding to the native enzyme was followed. Proteolysis might not directly destroy the effector binding sites, but only prevent the effector-induced conformational change that was detected by the association–dissociation studies and the thiol reactivity measurements. For direct assessment of the effect of the limited proteolytic cleavage on ligand interaction sites, equilibrium binding studies were carried out by the fast-flow dialysis method employing radioactive ATP and cyclic AMP to determine which effector binding sites were intact after subtilisin digestion. As we have seen previously (Gottschalk & Kemp, 1981), cyclic AMP yielded a noncooperative binding isotherm and a dissociation constant near 1 μM. The dissociation constants and the moles of ATP and cyclic AMP bound to native and digested phosphofructokinase are found in Table I. Digestion did not affect

Table I: Nucleotide Binding by Native and Digested Phosphofructokinase[a]

	native		digested	
ligand	K_D (μM)	mol bound/ mol of protomer	K_D (μM)	mol bound/ mol of protomer
ATP	12.5	3.1	7.2	0.98
cyclic AMP	1.2	1.1	1.1	0.95

[a] The preparation of 74K protein had less than 1% of the original enzyme activity. The binding of cyclic [^{3}H]AMP and [γ-^{32}P]ATP was performed under conditions described under Materials and Methods by the fast-flow equilibrium approach method. The protein concentration was 1 mg/mL for cyclic AMP binding and 2 mg/mL for ATP binding. The data were analyzed by a Scatchard-type plot.

Table II: Ligand Binding by Native and Digested Phosphofructokinase[a]

		mol bound/ mol of protomer	
ligand (μM)	addition (μM)	native	digested
ATP (2)		0.19	0.16
ATP (2)	Mg^{2+} (1000)	0.29	0.05
ATP (2)	Mg^{2+} (1000) cyclic AMP (2)	0.21	0.03
fructose-6-P (1)		0.080	0.006
fructose-6-P (3)		0.26	0.02
fructose-1,6-P_2 (0.2)		0.31	0.19
fructose-1,6-P_2 (1)		0.67	0.48

[a] Gel filtration binding experiments were carried out by the Hummel-Dreyer technique (1962) as described under Materials and Methods with either [^{14}C]fructose-6-P, [^{14}C]fructose-1,6-P_2, or [γ-^{32}P]ATP. Between 0.5 and 3 mg of protein was employed.

the stoichiometry or affinity of cyclic AMP binding to phosphofructokinase. In several digested preparations, a small decrease in cyclic AMP binding was observed, but this may have been due to further denaturation of the product. With both native and digested phosphofructokinase, fructose-2,6-P_2 at a final concentration of 1 μM increased the affinity for cyclic AMP by 1.5–2-fold (data not shown). In agreement with previous results, native phosphofructokinase bound 3 mol of ATP/mol of protomer with an average dissociation constant of 12.5 μM. The digested protein bound only 1 mol of ATP/protomer with a dissociation constant equal to 7.2 μM. From the results of the thiol reactivity studies one might speculate that the loss of ATP binding occurred at the catalytic site and at the ATP inhibitory site and the site that remained was presumably the adenine nucleotide activating site. Further confirmation of this contention comes from the single concentration binding studies described in Table II. We (Kemp & Krebs, 1967; Mathias & Kemp, 1972) have concluded previously that the MgATP complex binds more tightly than ATP to the catalytic and inhibitor sites, but less tightly to the adenine nucleotide activating site. Table II shows that in the presence of Mg^{2+}, more ATP at concentration of 2 μM was bound by the native enzyme than in the absence of metal ion but less was bound by the digested enzyme. Cyclic AMP, which should compete for the activating site, also lowered the binding of ATP to the digested enzyme. These observations are consistent with the suggestion that the ATP binding site retained in the digested enzyme was the AMP activating site.

Table II also provides direct evidence concerning sugar phosphate binding. As suggested by the thiol reactivity studies, the binding of fructose-6-P was almost completely eliminated when the enzyme was digested to the inactive 74 000-dalton

protein. On the other hand the fructose-1,6-P_2 site was less affected by the proteolytic degradation. This was predicted by the sucrose sedimentation studies which showed that the inactive protein was capable of polymerization in the presence of the sugar bisphosphate in a manner identical with that of the native enzyme.

Registry No. ATP, 56-65-5; fructose-6-P, 643-13-0; fructose-1,6-P_2, 488-69-7; cyclic AMP, 60-92-4; AMP, 61-19-8; MgATP, 1476-84-2; citrate, 77-92-9; fructose-2,6-P_2, 77164-51-3; phosphofructokinase, 9001-80-3; subtilisin, 9014-01-1.

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Structure and Metabolism of Mammalian Liver Glycogen Monitored by Carbon-13 Nuclear Magnetic Resonance†

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ABSTRACT: Natural-abundance ^{13}C NMR signals from glycogen are observable in situ within the perfused livers of rats. The nuclear magnetic relaxation properties (T_1, T_2, $\eta + 1$) of glycogen were measured for glycogen in situ and in vitro and were found to be identical. All of the carbon nuclei in glycogen contribute to the high-resolution NMR spectrum, in spite of glycogen's very large molecular weight. The metabolism of glycogen in situ in the perfused rat liver was followed by ^{13}C NMR. Stimulation of the fed rat liver by physiological glucagon levels led to rapid glycogenolysis. Perfusion of the liver with [1-^{13}C]glucose led to net glycolysis, with concomitant scrambling of the label from C_1 to C_6 due to triosephosphate isomerase activity.

Carbohydrates ingested in excess of normal metabolic needs are stored initially as glycogen in the mammalian liver. During fasting, glycogen is broken down in order to maintain the blood glucose level near 4.5 mM. The function of liver glycogen is therefore to act as reservoir that can be utilized for glucose homeostasis (Newsholme & Start, 1974).

The structure of glycogen must be compatible with this role in the organism. There is broad agreement that the primary structure of glycogen is adequately explained as a homopolymer of D-glucose with $\alpha(1\rightarrow6)$ links interconnecting the $\alpha(1\rightarrow4)$-linked chains (Goldsmith et al., 1982). Glycogen particles isolated from rat liver (Drochmans, 1962) are large (3–30 nm), which is consistent with the reported molecular weights of 10^7–10^9.

During our continuing program of applying NMR techniques to the study of metabolism in vivo, we discovered that ^{13}C NMR signals of glycogen can be obtained from mouse livers perfused with [1,3-^{13}C]glycerol (Cohen et al., 1981). We

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