Phosphorylation of rabbit skeletal muscle phosphofructokinase by the catalytic subunit of cyclic AMP-dependent protein kinase occurs with a $K_m$ of about 230 $\mu$m and $V_{max}$ approaching that seen with histone as a substrate. The rate of phosphorylation of phosphofructokinase by protein kinase is increased by allosteric activators of phosphofructokinase, whereas inhibitors of phosphofructokinase inhibit the phosphorylation. Inhibitors and activators change $V_{max}$ but not $K_m$.

The site of phosphorylation is a serine residue that is the sixth amino acid from the carboxyl terminus. Limited proteolysis by trypsin releases an octapeptide from the carboxyl terminus and a brief exposure to subtilisin releases a dodecapeptide from the carboxyl end. The sequence of the dodecapeptide is His-Ile-Ser-Arg-Lys-Val. The phosphophosphate is His-Ile-Ser-Arg-Ser(P)-Gly-Glu-Ala-Thr-Val.

Phosphofructokinase isolated from a rabbit injected 18 h prior to killing with $^{32}$PPO contained covalently bound radioactive phosphate. Approximately 80% of the phosphate was released in a trichloroacetic acid-soluble form following limited proteolysis by trypsin, under which conditions the enzyme remained with a monomer size of about 80,000 daltons. The position of elution from Sephadex G-25 of the phosphopeptide was identical with that found following limited trypsin proteolysis of in vitro labeled enzyme. Migration of the phosphopeptides on thin layer cellulose chromatography was also identical. We conclude that at least 80% of the radioactive phosphate introduced within 18 h of an intravenous injection of $^{32}$PPO, is found at the same site as that introduced by phosphorylation with the catalytic subunit of cyclic AMP-dependent protein kinase.

Rabbit muscle phosphofructokinase is a key enzyme in the control of glycolysis. The enzyme is subject to a complex allosteric regulation by a variety of metabolites (see Ref. 1 for review). In the past several years, it has been shown that skeletal muscle phosphofructokinase exists in vivo in a partially phosphorylated state (2-6). Furthermore, the in vitro phosphorylation of the enzyme by the catalytic subunit of cyclic AMP-dependent protein kinase has been demonstrated (7-9). Because of the importance of phosphofructokinase in the control of carbohydrate metabolism there is obvious interest in a potential covalent modulation of the enzyme's activity superimposed upon the well described allosteric regulation phenomena. In the case of the liver isozyme of phosphofructokinase, there have been several recent reports of hormonal regulation of the enzyme by means that may involve a phosphorylation mechanism (10-13).

On the basis of preliminary studies with tryptic peptide maps, Sorensen-Ziganke and Hofer (9) concluded that mouse muscle phosphofructokinase may be phosphorylated at two different peptide sites in vivo. In addition, their data indicated that the same two sites of mouse muscle phosphofructokinase are phosphorylated by the cyclic AMP-dependent protein kinase. In contrast, our studies on rabbit muscle phosphofructokinase have shown that the site of in vitro phosphorylation is localized to a single terminal region of the molecule (14). In the present study we show that in the rabbit the in vitro site of phosphorylation represents a single sequence and that sequence has been determined. Furthermore, most if not all of the phosphate incorporated in vivo is found in the same peptide. In addition, the kinetics of phosphorylation of muscle phosphofructokinase by the catalytic subunit of cyclic AMP-dependent protein kinase have been examined.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit skeletal muscle phosphofructokinase was prepared from fresh muscle as described previously (15). Second crystals of the enzyme were collected by centrifugation. The enzyme was dissolved in 50 mM sodium/Tes buffer containing 1 mM EDTA, 0.1 mM ATP, and 1 mM dithiothreitol, all at pH 7.0, and this solution was either dialyzed against the same buffer overnight or passed through a small Sephadex G-25 column (0.7 x 10 cm) equilibrated with the Tes buffer to remove ammonium sulfate.

The catalytic subunit of cyclic AMP-dependent protein kinase, prepared from beef heart by the method of Sugden et al. (16), was given to us by Dr. Marlene Hosey of our department. The enzyme, which was stored frozen in small batches in 350 mM potassium phosphate buffer, pH 6.8, containing 1 mM EDTA and 20 mM $\beta$-mercaptoethanol, was passed through a small column of Sephadex G-25 equilibrated with 50 mM sodium/Hepes, pH 7.0, containing 1 mM EDTA, 0.1 mM ATP, and 1 mM dithiothreitol.

**Subtilisin** (Sigma type VIII), trypsin, soybean trypsin inhibitor, aldolase, tritose phosphate isomerase, glycerol-P dehydrogenase, phenylmethyl sulfonyl fluoride, and sugar phosphates were obtained from Sigma Chemical Co. (St. Louis, MO). Carboxypeptidase A was purchased from Worthington Biochemical Corp. (Freehold, NJ). Nucleotides were obtained from P-L Laboratories (Milwaukee, WI) and [gamma-32P]ATP was purchased from ICN.

**In Vivo Phosphorylation of Phosphofructokinase**—In experiments designed to study the kinetics of phosphorylation of phosphofructokinase, incubations were carried out in sodium/Hepes buffer at the indicated pH in a reaction mixture containing MgCl$_2$ (5 mM), $[gamma-32P]$ATP (0.8 mM), EDTA (0.5 mM), and dithiothreitol (2.5 mM). The abbreviations used were: Tes, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; SDS, sodium dodecyl sulfate; Fru-P$_x$, fructose 1,6-bisphosphate.
Reaction volume was 60 µl and the reaction was terminated by application of 25-µl samples to Whatman No. 3M paper which was immediately placed in cold 10% trichloroacetic acid and washed as described by Corbin and Reiman (17). The washed papers were then counted in a scintillation counter.

When phosphorylated phosphofructokinase was being isolated for structural studies, the phosphorylation reaction was quenched by cooling and the sample applied immediately to a column (1.5 x 40 cm) of Sephacryl S-200 previously equilibrated with 50 mM sodium/Tes (pH 7.0) containing 0.1 mM EDTA, 0.1 mM ATP, and 1 mM diithiothreitol. The protein peak from the Sephacryl column was precipitated by the addition of 40 g/100 ml of ammonium sulfate. The precipitate was dissolved in 50 mM sodium/Tes (pH 7.0), containing 0.1 mM ATP, 0.1 mM EDTA, and 1 mM diithiothreitol, and the peak of activity was collected. This enzyme preparation was homogenous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and had a specific activity of 178 cpm/mg. The specific enzyme activity was 165.

Analysis of Peptides from 32P-labeled phosphofructokinase—Peptides from both limited trypsin and limited subtilisin digestion of 32P-labeled phosphofructokinase were separated initially from the bulk proteins by gel filtration on a column (1.5 x 30 cm) of Sephacryl S-200, previously equilibrated with the sodium/Tes buffer. This solution was passed through a column of Sephacryl S-200, previously equilibrated with the sodium/Tes buffer, and the peak of activity was collected. This enzyme preparation was homogenous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and had a specific activity of 178 cpm/mg. The specific enzyme activity was 165.

Gel Electrophoresis—Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out in 7.5% acrylamide slab gels (3 mm x 10 cm) with buffers and solutions prepared according to Laemmli (20). Samples to be analyzed were diluted 1:1 in 120 mM Tris/PO4 (pH 7.2) containing 50% glycerol, 2% mercaptoethanol, 2% sodium dodecyl sulfate, and 0.004% bromophenol blue.

Gels were fixed by shaking in 40% isopropyl alcohol/10% acetic acid for 30 min and then stained for protein by allowing them to stand for 1 h in the presence of 0.1% Coomassie Blue R-250 in 10% isopropyl alcohol/10% acetic acid. Destaining was performed by washing overnight against 10% isopropyl alcohol/10% acetic acid. The gels were sliced, digested with H2O2 (21), and counted in a scintillation counter.

RESULTS AND DISCUSSION

Kinetics of the Phosphorylation Reaction—The initial studies (7) on the phosphorylation of muscle phosphofructokinase by the catalytic subunit of cyclic AMP-dependent protein kinase showed that the rate was only about 2% of that seen with phosphorylase kinase as a substrate under the same conditions. It appeared necessary to define more completely the conditions of phosphorylation, to determine the kinetic parameters of the phosphorylation reaction, and to determine the influence of allosteric regulators of phosphofructokinase on its phosphorylation. The last point was of particular interest because regulatory effectors strongly influence the rate of proteolytic removal of the site of phosphorylation (14), presumably as a result of varying availability or exposure of the terminal peptide that is cleaved. Fig. 1 describes the kinetics of phosphorylation of phosphofructokinase at pH 7.0 in the absence of added ligands and in the presence of 1.7 mM AMP or 1.7 mM citrate. The rate of phosphorylation was increased by the allosteric activator, AMP, and decreased by the inhibitor, citrate. This pattern of increased phosphorylation rate in the presence of activators of the phosphofructokinase reaction and a decreased rate with inhibitors was seen also with other regulatory ligands. It should be noted that the extent of phosphorylation remained the same; that is, about 0.6 to 0.75 mol of phosphate was incorporated/mol of protomer.

Table I provides kinetic parameters for the phosphorylation of phosphofructokinase as compared to those obtained with histone. The observed Km was quite high (230 µM) but the Vmax in the presence of the activators of phosphofructokinase
Phosphorylation of Muscle Phosphofructokinase

Phosphorylation was carried out at pH 7.0, except where indicated, and other conditions were as described under "Experimental Procedures." At pH 7.0, the $K_n$ for histone was 3.6 mg/ml with a $V_{max}$ of 2.3 $\times 10^5$ units/mg. $K_n$ for phosphofructokinase was based on a subunit molecular weight of 80,000.

was similar to that for the histone. The effect of increasing the pH was the same as that seen by adding activators, that is, $V_{max}$ increased with no great variation seen in substrate affinity. Citrate decreased $V_{max}$. This suggests that the active conformation of the enzyme is that most readily phosphorylated. Support for this idea is provided by the data of Table II which describes an experiment in which single time points of phosphate incorporation were measured in the presence of various effectors of phosphofructokinase. The activators, AMP and Fru-P$_2$, increased the rate whereas citrate and 3-P-glycerate decreased the reaction rate. AMP at high concentrations was capable of overcoming the action of the inhibitors, and the activation effects of Fru-P$_2$ and AMP were not additive. One surprising observation was that AMP could reverse the inhibition by citrate, whereas Fru-P$_2$ could not.

Isolation of Phosphopeptides from in Vitro Labeled Phosphofructokinase—Native phosphofructokinase (135 mg) was labeled in vitro with $[^{32}P]P$_4 as described under "Experimental Procedures." The extent of labeling was 0.30 to 0.33 mol/mol of subunit and the radioactive protein was separated from protein kinase and by-products by chromatography on Sephadex G-25 and DEAE-Sephadex A-25. On Sephadex G-25, one of several radioactive peptides accounted for over 85% of the total radioactivity applied to the column. Approximately 90% of the radioactivity emerged as a single symmetrical peak (a). Ion-exchange chromatography of this preparation on DEAE-Sephadex (Fig. 3A) gave a pure $[^{32}P]$-octapeptide, the composition of which is shown in Table III. The peptide labeled b in Fig. 2A was not radioactive but had a composition identical with that of the $[^{32}P]$-labeled trypsin peptide (Table III) except that it lacked Lys and Arg.

Sequence Analysis of the $[^{32}P]$ Phosphopeptides—The subtilisin and trypsin phosphopeptides were subjected to automated Edman degradation and the results are summarized in Fig. 4. Both peptides were excised from the carboxyl terminus of the phosphofructokinase chain and the tryptic octapeptide is contained within the structure of the larger subtilisin dodecapeptide. Approximately 80 nmol of tryptic peptide was analyzed and the yield of Gly and Ala was 30 nmol each. All

| Conditions | $K_n$ | $V_{max}$ relative to histone |
|------------|------|-----------------------------|
| pH 7.0     | 230  | 0.44                        |
| + Fru-P$_2$| 235  | 1.0                         |
| + Citrate  | 220  | 0.33                        |
| pH 8.0     | 200  | 0.53                        |

| pH Conditions | Additions | Kinetic Parameters |
|---------------|-----------|--------------------|
| pH 7.0        | None      | $K_n$ (mM) | $V_{max}$ (nmol/mg) |
|               | Fru-P$_2$ | $134$     | $180$             |
|               | AMP       | $134$     | $180$             |
|               | Fru-P$_2$ + AMP | $134$ | $180$ |
|               | Citrate   | $134$     | $180$             |
|               | Citrate + AMP | $134$ | $180$ |
|               | Citrate + Fru-P$_2$ | $134$ | $180$ |
|               | 3-Phosphoglycerate | $134$ | $180$ |
|               | P$_i$     | $134$     | $180$             |
|               | NH$_4^+$  | $134$     | $180$             |

Amino acid compositions of $[^{32}P]$ peptides generated by cleavage of in vitro labeled phosphofructokinase with subtilisin and trypsin

Samples were hydrolyzed for 24 h in vacuo at 110 °C in 6 N HCl. Under these conditions, P-Ser is hydrolyzed to Ser; no corrections are made for the decompositional losses. Purification procedures for the two peptides are summarized in the text. Numbers in parentheses indicate nearest whole integral residue values.

| Amino acid | Subtilisin | Trypsin |
|------------|------------|---------|
| Thrreonine | 0.89(1)    | 0.94(1) |
| Serine     | 1.68(2)    | 0.83(1) |
| Glutamic acid | 1.13(1) | 1.20(1) |
| Glycine    | 1.09(1)    | 1.15(1) |
| Alanine    | 1.09(1)    | 1.00(1) |
| Valine     | 0.97(1)    | 0.92(1) |
| Isoleucine | 0.91(1)    | 0.00    |
| Histidine  | 1.09(1)    | 0.00    |
| Lysine     | 1.01(1)    | 1.01(1) |
| Arginine   | 1.96(2)    | 0.98(1) |
| Total residues | (12)   | (8)     |
residues were observed by hydrolytic back-conversion and amino acid analysis. Assignments of all residues save Arg were confirmed by gas chromatography and thin layer chromatography. Analysis of 200 nmol of the subtilisin peptide gave yields of Ile and Ser-3 of 175 and 125 nmol, respectively. Basic amino acids His, Lys, and Arg were identified by hydrolysis and amino acid analysis. Identical results were obtained from both peptides following digestion with carboxypeptidase A. The stoichiometric release of Val followed by Thr was observed.

The seryl residue involved in phosphorylation was identified as that 6 residues in from the carboxyl terminus of the enzyme. This is the only Ser in the tryptic peptide and the logical site of substitution. Proof for the assignment rests upon counting data from the analysis of both peptides which indicate a sharp rise in radioactivity upon liberation of the anilinothiazolinone of that serine. Phosphoserine is not completely extracted by the chlorobutane employed for removal of the cyclized derivative but the removal of radioactivity was easily associated with the site of phosphorylation indicated in Fig. 4.

The original preparation of [32P]phosphofructokinase contained only about 0.3 mol of [32P]P04/mol of subunit. It is interesting to note the isolation of a peptide in the tryptic digest (peak b in Fig. 2) that corresponds in composition to the tryptic phosphopeptide lacking Lys and Arg. Presumably, the nonphosphorylated phosphofructokinase is hydrolyzed by trypsin at the Arg-Ser bond and phosphorylation prevents the cleavage. This would be in keeping with the known sluggishness of trypsin in the hydrolysis of such linkages.

Tryptic Phosphopeptide from in Vivo Labeled Phosphofructokinase—In order to determine whether the phosphorylation site identified in phosphofructokinase labeled in vitro is the same as that labeled in vivo, enzyme isolated from a rabbit injected with [32P]P04 was subjected to tryptic digestion under conditions described above. Samples were removed at various times for analysis of trichloroacetic acid-soluble radioactivity and for SDS-polyacrylamide gel electrophoresis. More than 90% of the radioactivity was solubilized in 5 min and approximately 80% was trichloroacetic acid-soluble in 15 min. Gel electrophoretic analysis indicated that at least 90% of the protein remained with a size of about 80,000 daltons and only a small amount (less that 5%) of 40,000 to 42,000 fragments were present. It was shown originally by Emerk and Frieden (26) and confirmed in this laboratory that trypsin will cleave phosphofructokinase into halves under the conditions described above. Complete digestion to half-halves requires about 2 h. Thus, in the short times employed here, very limited cleavage has occurred. To compare the size of the released peptide with that obtained from in vitro labeling, 10 mg of in vivo labeled phosphofructokinase was digested under the conditions described above with 0.01 mg of trypsin for 10 min. The reaction was stopped by the addition of trichloroacetic acid and the protein was removed by centrifugation, leaving approximately 80% of the 32P in the supernatant. Most of the trichloroacetic acid was removed from the supernatant solution by two extractions with ether. The aequous phase was lyophilized and chromatographed on a column of Sephadex G-25 under conditions identical with those described above for the isolation of P-peptide derived from in vitro labeled phosphofructokinase. The elution profile in Fig. 2B shows that almost all of the radioactivity eluted at exactly the same position as that from the tryptic digest of in vitro labeled enzyme (Fig. 2A). No substantial fluoroscane-positive peak co-eluted with labeled material because the content of phosphate in the isolate enzyme is quite low (7). Furthermore, as discussed above, if the protein is not phos-
Phosphorylation, the course of the trypsin digestion is different.

The fraction of the peak of radioactive material was lyophilized, dissolved in H2O, and applied to a thin layer cellulose plate. A sample of the in vitro labeled peptide was applied in parallel and the plate was developed with butanol-acetic acid-water (60:15:25). All of the radioactivity of both samples moved as a single zone with an approximate Rf of 0.3. We conclude that at least 80% of radioactive phosphate introduced (radioactive peak from Fig. 2B) into phosphofructokinase within 18 h of an intravenous injection of 32P is found in the same site as that introduced by phosphorylation with the catalytic subunit of cyclic AMP-dependent protein kinase.

These observations are consistent with the concept that the phosphate found in muscle phosphofructokinase has been introduced by cyclic AMP-dependent protein kinase. The presence of basic amino acid residues several positions toward the NH2 terminus from the serine undergoing phosphorylation appears obligatory for protein substrates of the kinase (27).

With synthetic peptide substrates, Feramisco et al. (28) showed that peptides with the Lys-Arg sequence immediately adjacent the serine acceptor, as we see with phosphofructokinase, gave a K0 that was 30 times higher than peptides with a single spacer residue between the basic amino acids and serine. This probably accounts for the high K0 that the protein kinase shows with respect to phosphofructokinase. The concentration of phosphofructokinase in muscle is about 15 µM if one assumes that the enzyme occupies half the volume of muscle. This would indicate that the phosphorylation occurs in vivo far below half-saturation and thus probably rather slowly, unless undiscovered factors interact with phosphofructokinase to make it a better substrate. Known effectors of phosphofructokinase, however, change the Vmax of phosphorylation and not the K0. It is possible, of course, that the in vitro phosphorylation by cyclic AMP-dependent kinase is fortuitous and that in vivo phosphorylation is achieved with a different kinase. A search for such a kinase in muscle extracts has been unsuccessful.

A likely candidate was the myosin light chain kinase described by Hathaway and Adelstein (29). Using light chain kinase and calmodulin supplied by Adelstein's laboratory, we have been unable to detect phosphorylation of phosphofructokinase under condition where the light chain of myosin is readily phosphorylated. Thus, at the present time the most likely candidate for the protein kinase of muscle phosphofructokinase remains the cyclic AMP-dependent protein kinase.

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