Phosphorylation of Rat Hepatic Fructose-1,6-bisphosphatase and Pyruvate Kinase*

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Fructose-1,6-bisphosphatase from rat liver was phosphorylated with cyclic AMP-dependent protein kinase and [γ-32P]ATP. Brief exposure of the 32P-labeled enzyme to trypsin removed all radioactivity from the enzyme core and produced a single-labeled peptide. The partial sequence of the 17-amino acid peptide was found to be Ser-Arg-Pro-Ser(P)-Leu-Pro-Leu-Pro-Ser(Glu, Pro, Leu, Arg). The kinetics of cyclic AMP-dependent protein kinase-catalyzed phosphorylation of native fructose bisphosphatase were compared with those of rat liver type L pyruvate kinase where the sequence around the phosphoserine is known (Arg-Ala-Ser(P)-Val; Hjelmquist, G., Anderson, J., Edlund, B., and Engstrom, L. (1974) Biochem. Biophys. Res. Commun. 61, 559-563). The $K_m$ for pyruvate kinase (17 μM) was less than that for fructose bisphosphatase (58 μM); the $V_{max}$ was about 3-fold greater with pyruvate kinase as substrate. The relationship between the rates of phosphorylation of these native substrates and the amino acid sequences surrounding the phosphorylated sites is discussed.

The cyclic AMP-dependent protein kinases catalyze the phosphorylation of many proteins (1). It has been shown that the presence of basic amino acid residues on the NH₂-terminal side of the phosphorylated serine or threonine residues is important in determining the specificity of these proteins as substrates for the cyclic AMP-dependent protein kinases (1-5). This is best illustrated with type L pyruvate kinase where the presence of two adjacent basic amino acids near the phosphorylated serine has been shown to be an important determinant for enzyme phosphorylation (5). However, few studies on the phosphorylation of the native enzyme have appeared and its $K_m$ for phosphorylation by protein kinase is unknown.

We have recently shown that in vitro phosphorylation of rat hepatic fructose-1,6-bisphosphatase is catalyzed by the catalytic subunit of the cyclic AMP-dependent protein kinase (6). In vivo phosphorylation of this enzyme has also been demonstrated (6). In the present study, the amino acid sequence around the phosphorylated site in this enzyme was determined. The kinetics of phosphorylation of fructose bisphosphatase were also compared with those of type L pyruvate kinase from rat liver. The studies show that the sequence around the phosphorylated site in fructose bisphosphatase contains only one basic residue just NH₂-terminal to the phosphorylated serine and that the Michaelis constants for the phosphorylation of both pyruvate kinase and fructose bisphosphatase are substantially greater than in vivo concentrations.

METHODS

Purification and Assay of Enzymes—Rat hepatic fructose bisphosphatase was purified by the method of Riou et al. (6). The enzyme was homogeneous as judged by sodium dodecyl sulfate-gel electrophoresis and had a specific activity of 40 to 45 units/mg of protein (6). Fructose bisphosphatase activity was assayed as described earlier (6). Purified fructose bisphosphatase contained from 0.2 to 0.3 mol of endogenous phosphate/mol of tetrameric enzyme (data not shown).

Rat hepatic L-type pyruvate kinase was purified to homogeneity by the method of Riou et al. (7) and had a specific activity of 400 to 550 units/mg of protein. Endogenous phosphate was removed from pyruvate kinase by taking advantage of the fact that the protein kinase reaction can be reversed as described by El Maghrabi et al. (8). Pyruvate kinase activity was assayed as described by Riou et al. (9).

The catalytic subunit of rat liver cyclic AMP-dependent protein kinase was purified by a modification (8) of the method of Sugden et al. (10). The preparation consisted of a mixture of catalytic subunits from the type I and II isozyme forms. The enzyme exhibited a single band on sodium dodecyl sulfate-gel electrophoresis and had a specific activity of 3 to 4 × 10⁶ units/mg of protein where 1 unit is the amount of enzyme which catalyzes the transfer of 1 pmol of 32P from [γ-32P]ATP to histone/min at 30°C. The catalytic subunit preparation was free of fructose bisphosphatase and pyruvate kinase activity.

Protein was determined by the method of Lowry et al. (11) with bovine serum albumin as standard.

Phosphorylation of Pyruvate Kinase and Fructose Biphosphatase—Selected concentrations of a homogeneous preparation of rat liver fructose bisphosphatase (0.1 to 100 μM) or pyruvate kinase (0.1 to 70 μM) were incubated at 30°C in a final volume of 50 μl that contained 20 mM Tris-HCl, pH 7.4, 35 mM potassium phosphate, 10 mM mercaptoethanol, 6 mM MgCl₂, 0.3 mM [γ-32P]ATP (50 to 200 cpm/mol), and various concentrations of catalytic subunit (100 to 10,000 units/ml). Labeled phosphate incorporation into the appropriate enzyme substrate was estimated by the method of Corbin and Reimann (12). The number of moles of 32P incorporated/mol of enzyme was calculated on the basis of molecular weights of 144,000 for fructose bisphosphatase and 228,000 for pyruvate kinase. [γ-32P]Fructose bisphosphatase was freed of catalytic subunit and labeled ATP by passage over a Sephadex G-100 column (1.5 × 90 cm) equilibrated in 5 mM malonate buffer, pH 6.2.

Initial rates of phosphorylation of these two enzymes were measured under conditions where the catalytic subunit was sufficiently

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‡ Investigator of the Howard Hughes Medical Institute.
Phosphatase-Fructose bisphosphatase was phosphorylated with \( [\gamma-\text{P}]\text{ATP} \) and catalytic subunit of cAMP-dependent protein kinase in 5 min, \( K_m \) and \( V_{max} \) values for phosphorylation of these substrates were determined from Lineweaver-Burk Plots.

**Cyanogen Bromide Cleavage of \( ^{32}P \)-Labeled Fructose 1,6-Bisphosphatase—**Fructose bisphosphatase was phosphorylated with \( [\gamma-\text{P}]\text{ATP} \) and the reaction stopped with 10% trichloroacetic acid. The phosphorylated protein was dissolved in NaOH, reprecipitated with trichloroacetic acid, and dissolved in 100 \( \mu \)l of 70% formic acid. Cyanogen bromide (1 to 3 mg) was added and allowed to react at room temperature for 16 h. The mixture was then diluted with water, frozen, and lyophilized. The residue was dissolved in 50 \( \mu \)l of 0.2 M sodium dodecyl sulfate electrophoresis buffer added, and heated again for 20 min.

The cyanogen bromide peptides were subjected to disc gel electrophoresis on 15% polyacrylamide gels (0.7 × 14 cm) in the presence of sodium dodecyl sulfate (14). After electrophoresis, the gels were sliced into 2-mm sections and counted for \( ^{32}P \).

**Analysis of Peptides from \( ^{32}P \)-Labeled Fructose Bisphosphatase—**

Peptides derived from limited trypsin digestion of \( ^{32}P \)-labeled fructose bisphosphatase were separated by means of high voltage electrophoresis and descending paper chromatography. The peptide mixture was applied to Whatman No. 3MM chromatography paper and subjected to high voltage electrophoresis in pyridine/acetate buffer, pH 3.5, as the first dimension, and then to descending chromatography in butanol/acidic acid/water (4:1:5) as the second dimension and \( ^{32}P \)-labeled peptides were located by autoradiography. Peptides were detected analytically with 0.25% ninhydrin and Sakaguchi stain was used to detect arginine-containing peptides (15). Labeled peptides were eluted from paper with 50% acetic acid. An aliquot of the labeled peptide was hydrolyzed at 110°C for 24 h in 6 N HCl containing a small amount of phenol. Amino acid analyses were performed on a Durrum D-900 analyzer and concentrations were converted to the nearest integer values to obtain the composition reported. The purified phosphopeptide (about 35 nmol) was sequenced by automated techniques on a Beckman 890C sequenator using the standard di-line-Carle Place, N. Y.) and their migration is shown at the top.

**RESULTS AND DISCUSSION**

In initial experiments \( ^{32}P \)-fructose bisphosphatase was subjected to cleavage by cyanogen bromide. Only one labeled peptide was observed after the cyanogen bromide-treated enzyme was subjected to electrophoresis in 15% sodium dodecyl sulfate gels with a molecular weight of about 6000. The results suggested that only one site was phosphorylated, but it is possible that more than one site of phosphorylation could be present in the enzyme.

**CONCLUSION**

Phosphorylation of fructose bisphosphatase was investigated by incubating the homogeneous enzyme (12 \( \mu \)m) with 0.33 \( \mu \)m \( [\gamma-\text{P}]\text{ATP} \), 10 \( \mu \)M MgCl\(_2\), and 10,000 units/ml of catalytic subunit for 2 h as described above. The enzyme was freed of \( [\gamma-\text{P}]\text{ATP} \) by passage over a Sephadex G-25 column (2.5 × 40 cm) equilibrated with 5 mM malonate buffer, pH 6.2. An aliquot of the labeled enzyme was incubated with trypsin (fructose bisphosphatase: trypsin = 15:1) at pH 7.0 in 30 mM Tris-HCl and 0.2 mM dithiothreitol. Aliquots (15 \( \mu \)l) were removed at the indicated times and added to tubes containing 250 \( \mu \)l of cold 6% trichloroacetic acid. The tubes were centrifuged at 10,000 rpm for 10 min at 4°C. Aliquots of the trichloroacetic acid supernatant fraction were counted for \( ^{32}P \) radioactivity. The enzyme pellet was redissolved in NaOH and protein content determined by the method of Lowry et al. (11). \( ^{32}P \) radioactivity in the trichloroacetic acid supernatant, \( \Delta \) percentage of enzyme protein present in the pellet.

This result suggests that the brief enzyme digestion and subsequent precipitation resulted in a 10- to 15-fold purification of the phosphopeptide and that fructose bisphosphatase conforms to the pattern of the many other protein substrates of cyclic AMP-dependent protein kinase.

In order to characterize this phosphopeptide further, fructose bisphosphatase (20 \( \mu \)g), isolated from 30 rats, was phos-
The peptide was submitted to automated Edman degradation with the resulting identification of eight NH$_2$-terminal amino acids. The sequence was determined to be:

Ser-Arg-Pro-X-Leu-Pro-Leu-Pro

The recovery of $^{32}$P radioactivity at each cycle of degradation resulted in the subsequent identification of phosphoserine at position 4 in the above sequence. In order to determine if the phosphorylated peptide were located near the NH$_2$ terminus of the enzyme subunit, as is the case for pyruvate kinase (16), the labeled enzyme was digested briefly with subtilisin. Several groups have demonstrated that such treatment removes a 5,000 to 6,000 $M$, fragment (S-peptide) from the NH$_2$-terminal region of fructose bisphosphatase and leaves a 29,000 $M$, fragment (22, 23). Treatment of the labeled rat liver enzyme with subtilisin also generated similar fragments (24). After sodium dodecyl sulfate-disc gel electrophoresis, $^{32}$P radioactivity was found in the 29,000 $M$, fragment while none was present in the 6,000 $M$, fragment indicating that the phosphorylated residue is not located in the NH$_2$-terminal peptide (data not shown). Since the phosphopeptide is not NH$_2$-terminal and since it was isolated from a trypsin digest, it is likely that a basic residue appears NH$_2$-terminal to the above sequence in the intact subunit. The sequence of the subunit near the site of phosphorylation is thus

\[
\text{(Arg)}_2\text{Ser-Arg-Pro-Ser(P)-Leu-Pro-Leu-Pro}
\]

Although low chemical yields precluded determination of the peptide sequence beyond the eighth residue, the repetitive yield was sufficiently high that $^{32}$P-phosphoserine would have been detectable in the remainder of the sequence had it been present. No additional residues of phosphoserine were detected, however, and we conclude the peptide contains a unique site of phosphorylation.

The amino acid sequence in fructose bisphosphatase contains a single arginine residue two residues NH$_2$-terminal to the phosphorylated serine. Results with the pyruvate kinase peptide analog Leu-Arg-Arg-Ala-Ser-Val-Ala (25, 26) have emphasized the important contribution of two adjacent arginine residues in enhancing the rate of enzyme phosphorylation. However, in only a few cases have the kinetics of phosphorylation of native protein substrates been compared where the sequence around the phosphorylated sites are known. Yeaman et al. (27) have compared the rate of phosphorylation of several substrates at a single substrate concentration (5 mM). Under these conditions the $\beta$ subunit of phosphorylase kinase was the best substrate for cyclic AMP-dependent pro-

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>mol amino acid/mol peptide</th>
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<tbody>
<tr>
<td>Aspartic acid</td>
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<tr>
<td>Serine</td>
<td>4.0 (4)</td>
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<tr>
<td>Glutamic acid</td>
<td>1.9 (2)</td>
</tr>
<tr>
<td>Proline</td>
<td>4.7 (5)</td>
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<tr>
<td>Alanine</td>
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<tr>
<td>Leucine</td>
<td>3.0 (3)</td>
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<tr>
<td>Lysine</td>
<td>0.1</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.2 (3)</td>
</tr>
</tbody>
</table>

Table I

Amino acid composition of the trypptic phosphopeptide from $^{32}$Pfructose bisphosphatase

The purified trypptic phosphopeptide was hydrolyzed at 110°C for 20 h in 6 N HCl containing a small amount of phenol. The analysis was performed on the single column Durum amino acid analyzer as described under "Methods". Amino acids not reported below were absent from the hydrolysate. The numbers in parentheses represent the corresponding integer values.

1 S. J. Pilkis, unpublished results.
tein kinase followed by histone 2B, glycogen synthase (site 2), and pyruvate kinase, but no attempt was made to determine \( K_m \) and \( V_{max} \) values for each substrate. In the present studies, we found that pyruvate kinase was phosphorylated at 10 to 100 times the rate of histone (\( V_{max} \) histone 1 to 2 \( \mu \)mol/min/mg, \( V_{max} \) pyruvate kinase 45 \( \mu \)mol/min/mg). It should be noted that all endogenous phosphate in the regulatory sites in pyruvate kinase in the present study was removed by using the protein kinase reaction in the reverse direction with pyruvate kinase as substrate (8). We have shown that such "dephosphorylated" preparations are phosphorylated at very much higher rates than that of the native enzyme which contains 2 to 3 mol of chemical phosphate/mol of enzyme (8).

A comparison of the phosphorylation of fructose bisphosphatase and pyruvate kinase catalyzed by the cyclic AMP-dependent protein kinase is shown in Table II. Initial rates of phosphorylation were determined with preparations of fructose bisphosphatase and pyruvate kinase that contained less than 0.2 mol of phosphate/mol of enzyme in cyclic AMP-dependent sites. The \( K_m \) for pyruvate kinase (17 \( \mu \)M) was less than that for fructose bisphosphatase (55 \( \mu \)M), while the \( V_{max} \) for pyruvate kinase was about 3-fold greater than that for fructose bisphosphatase (Table II). Pyruvate kinase was also about 10 times better than fructose bisphosphatase as a substrate as judged by the ratio of \( V_{max}/K_m \). This result is not unexpected since pyruvate kinase contains two arginine residues on the NH\(_2\)-terminal side of the phosphorylated (25, 26) serine whereas fructose bisphosphatase contains only one.

Although pyruvate kinase appears to be the favored substrate, the estimated in vitro concentration of fructose bisphosphatase is more than an order of magnitude greater than pyruvate kinase (Table I). If the rates of in vitro phosphorylation of the enzymes are compared at their physiological concentrations, the rate of phosphorylation of fructose bisphosphatase is in fact three times greater than that of pyruvate kinase (fructose bisphosphatase, 1.7 pmol/min/\( \mu \)M pyruvate kinase, 0.6 pmol/min/\( \mu \)M). It should also be noted that the concentration of both protein substrates in the hepatocyte is at least 10-fold lower than the concentration of substrate necessary to give a half-maximal rate of phosphorylation. Under these conditions it is reasonable to assume that the rate of phosphorylation of these enzymes will be approximately first order with regard to substrate concentration. It should be noted, however, that the concentration of MgATP used in these in vitro studies, although saturating, is not identical with the in vivo concentrations of free MgATP. These calculations may therefore require some modifications in order to describe the in vivo situation accurately.

The \( K_m \) for native pyruvate kinase was about 17 \( \mu \)M in terms of enzyme concentration or 68 \( \mu \)M in terms of its subunit concentration. This is in contrast to a \( K_m \) of about 10 \( \mu \)M for the pyruvate kinase peptide analog, Leu-Arg-Arg-Ala-Ser-Val-Ala (5). Thus, the native enzyme is not as good a substrate as the peptide analog, a conclusion which differs from that claimed by several investigators (5, 27). However, it should be noted that until now no determination of the \( K_m \) for pyruvate kinase for cyclic AMP-dependent protein kinase had been reported. In previous studies, an exact value could not be obtained since the rate of phosphorylation continued to increase even when micromolar concentrations of pyruvate kinase were used (29). In the present studies, final concentrations of up to 100 \( \mu \)M protein substrate were required to determine the \( K_m \) values for fructose bisphosphatase and pyruvate kinase.

We have characterized some properties of the cyclic AMP-dependent protein kinase-catalyzed phosphorylation of fructose bisphosphatase. The pH optimum for phosphorylation was about pH 7 and differed dramatically from that of pyruvate kinase (Fig. 4). Neither fructose bisphosphatase (0.1 mM) nor AMP (1 to 200 \( \mu \)M) had any effect on the initial rate of phosphorylation (data not shown). The apparent \( K_m \) for MgATP required for cyclic AMP-dependent protein kinase-catalyzed phosphorylation of fructose bisphosphatase was about 50 \( \mu \)M (Fig. 5A), which is similar to that reported for pyruvate kinase (29). The influence of Mg\(^{2+}\) concentration on the protein kinase-catalyzed phosphorylation of fructose bisphosphatase is shown in Fig. 5B. Maximal rates of phosphorylation were obtained between 2 and 8 mM Mg\(^{2+}\) when the concentration of MgATP was 0.3 mM. In the experiments reported in Table II, optimal conditions for phosphorylation (0.3 mM ATP and 6 mM Mg\(^{2+}\)) were employed. Under these conditions, rabbit skeletal muscle phosphorylase kinase did not catalyze phosphorylation of either fructose bisphosphatase or pyruvate kinase in the absence or presence of calcium.

### Table II

<table>
<thead>
<tr>
<th>Comparison of pyruvate kinase and fructose bisphosphatase with regard to phosphorylation</th>
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<tbody>
<tr>
<td><strong>Cyclic AMP-dependent phosphorylation</strong></td>
</tr>
<tr>
<td>Number of phosphorylation sites</td>
</tr>
<tr>
<td>Sequence at phosphorylated sites</td>
</tr>
<tr>
<td>( K_m ) (native enzyme)</td>
</tr>
<tr>
<td>( V_{max} ) (native enzyme)</td>
</tr>
<tr>
<td>Concentration in ( \text{in vivo} )</td>
</tr>
</tbody>
</table>

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Fig. 4. Influence of pH on the rate of phosphorylation of pyruvate kinase and fructose bisphosphatase. Instead of the standard buffer in the reaction mixture described under "Methods," three different buffers were used at a final concentration of 50 mm in each experiment. The buffers were acetate, 2-(N-morpholino)ethanesulfonic acid, and N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid. The amount of protein kinase was 75 units. The concentration of pyruvate kinase was 0.4 \( \mu \)M whereas that of fructose bisphosphatase (FDPhase) was 4.0 \( \mu \)M.
Phosphorylation of Fructose Bisphosphatase

Fig. 5. The effect of MgATP and Mg²⁺ concentration on the protein kinase-catalyzed phosphorylation of fructose bisphosphatase. A, MgATP concentration was varied with a constant Mg²⁺ concentration of 4 mM. B, the Mg²⁺ concentration was varied with a constant concentration of 0.5 mM MgATP. The concentration of fructose bisphosphatase was 5 μM and the concentration of catalytic subunit was 500 units/ml.

Fig. 6. Fluoride inhibition of dephosphorylation of ³²P-labeled fructose bisphosphatase by rat liver phosphoprotein phosphatase. Labeled fructose bisphosphatase was prepared as described in Fig. 1. The enzyme contained 180 cpm/pmol. Rat liver phosphoprotein phosphatase was purified by the method of Brandt et al. (28) through the first (NH₄)₂SO₄ fractionation step. ³²P-Labeled fructose bisphosphatase (40 μg, 50,000 cpm) was incubated with 75 μg of partially purified phosphatase in 50 mM MgCl₂, 2 mM dithiothreitol in a total volume of 50 μl. Aliquots were removed at the indicated times and ³²P radioactivity in fructose bisphosphatase was determined by the method of Corbin and Reimann (12). □, enzyme incubated without MgCl₂; ■, enzyme incubated with 10 mM MgCl₂ in the presence of 0, 50, and 100 mM NaF, respectively.

(data not shown). Also, Fig. 6 shows that ³²P-labeled fructose bisphosphatase, like pyruvate kinase (24), can be dephosphorylated by incubation with a partially purified preparation of phosphoprotein phosphatase from rat liver. Dephosphorylation of the enzyme could be inhibited in a dose-dependent manner by NaF. Thus the phosphorylation of fructose bisphosphatase is readily reversible.

In summary, this study demonstrates that phosphorylation of fructose bisphosphatase catalyzed by cyclic AMP-dependent protein kinase occurs at a single specific serine residue. Phosphate incorporated into this serine residue can be removed by a phosphoprotein phosphatase partially purified from rat liver. The sequence about this residue differs somewhat from the usual pattern seen in proteins phosphorylated by cyclic AMP-dependent protein kinase since it does not contain a pair of basic residues two-to-three residues NH₂-terminal to the phosphorylated serine. The sequence of the phosphorylated site in fructose bisphosphatase contains a proline just NH₂-terminal to the phosphorylated serine. This suggests that proline can be easily accommodated into the binding site for protein kinase, a view also supported by the recent report that the peptide Leu-Arg-Arg-Pro-Ser-Leu-Gly is a good substrate for protein kinase (30). Kinetic studies on enzyme phosphorylation revealed that fructose bisphosphatase was about one-tenth as good a substrate as pyruvate kinase, which is consistent with the hypothesis that two adjacent basic residues are important with regard to the kinetics of phosphorylation of native enzyme substrates (25, 26). However, it appears that two adjacent basic residues are not essential for phosphorylation of native substrates. It is likely that a single arginine residue is important in conferring substrate specificity but that a higher order of structure may also be important in enhancing the kinetics of phosphorylation. A similar conclusion was reached recently by Kemp (31) in the study of phosphorylation of synthetic peptide analogs of rabbit heart troponin inhibitory subunit by the cyclic AMP-dependent protein kinase.

Acknowledgment—We are grateful to Jo Pilkis for excellent technical assistance.

REFERENCES


1 After submission of this report, Humble et al. (1979) Biochem. Biophys. Res. Commun. 90, 1064-1072 reported the sequence around the phosphorylated site in rat liver fructose bisphosphatase. They reported a tyrosine residue at position 3 of the peptide. We have not detected any tyrosine in three separate preparations of the tryptic phosphopeptide.
Phosphorylation of Fructose Bisphosphatase

Phosphorylation of rat hepatic fructose-1,6-bisphosphatase and pyruvate kinase.
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