Role of Enzyme Interactions in the Regulation of Gluconeogenesis: Phosphorylation of Fructose 1,6-Bisphosphatase and Phosphofructokinase by Kidney Protein Kinase†

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ABSTRACT: The phosphorylation of homogeneous swine kidney fructose 1,6-bisphosphatase, phosphofructokinase and glycogen synthetase has been studied using a purified preparation of kidney protein kinase. A maximum of four moles of phosphate were transferred from ATP to each of the enzymes. All of the enzymes have a tetrameric structure and contain four identical subunits, which suggests that only one phosphate is transferred to each subunit. Further evidence for the phosphorylation of these enzymes was obtained by affinity chromatography on cellulose-phosphate and Blue Dextran-Sepharose 4B columns, poly(acrylamide) gel electrophoresis in sodium dodecyl sulfate, and immunoprecipitation with antibodies specific for each of the enzymes. Hydrolysis with acid showed that more than 95% of the ^{32}P incorporated was present as phosphoseryl residues. The phosphorylation of glycogen synthetase was accompanied by a decrease in enzyme activity. Phosphorylation of fructose 1,6-bisphosphatase and phosphofructokinase did not increase or decrease the activities of the enzymes measured by several different assay procedures. The sensitivity of these enzymes to allosteric effectors, including AMP, ATP, fructose 1,6-bisphosphate, and fructose 6-phosphate, was not significantly altered by phosphorylation. The kinetic properties of kidney protein kinase were examined with four different substrates. The apparent Michaelis constants calculated from initial velocity patterns obtained with these substrates were very similar. The constants for fructose 1,6-bisphosphatase, phosphofructokinase, glycogen synthetase, and histone were 53 μM, 55 μM, 50 μM, and 63 μM, respectively. The concentrations of these enzymes in swine kidney have also been determined. This tissue contains 1.45 μM, fructose 1,6-bisphosphatase, 0.08 μM phosphofructokinase, 0.038 μM glycogen synthetase, and 0.26 μM protein kinase. These results suggest that the concentrations of these enzyme substrates would be rate limiting under physiological conditions, and that the rate of phosphorylation of fructose 1,6-bisphosphate by protein kinase would be at least tenfold greater than the other enzymes. These effects may play a role in the regulation of gluconeogenesis by epinephrine and parathyroid hormone in this tissue.

The reciprocal regulation of fructose 1,6-bisphosphatase (D-fructose-1,6-biphosphate 1-phosphohydrolase, EC 3.1.3.11) and phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) during gluconeogenesis in kidney is not yet fully understood. The simultaneous action of both enzymes would lead to "futile cycling of substrates and products" with a concomitant hydrolysis of ATP. Thus, in tissues such as kidney and liver, where both enzymes are present in the cytosol, the activities may be regulated in such a manner that only fructose 1,6-bisphosphatase is active during gluconeogenesis. A number of independent studies have now shown that there is little or no "futile cycling" and very low rates of glycolysis during active gluconeogenesis in these tissues (Soling et al., 1973; Clark et al., 1974; Hue & Hers, 1974a,b).

Epinephrine and parathyroid hormone stimulate gluconeogenesis in kidney and increase the intracellular levels of cAMP (Nagata & Rasmussen, 1970). A possible mechanism for the control of swine kidney fructose 1,6-bisphosphatase following hormonal stimulation of gluconeogenesis was suggested by the early findings of Mendicino et al. (1966) which indicated that epinephrine might act by promoting the phosphorylation of this enzyme. In subsequent studies it was shown that the enzyme could be phosphorylated by incubation with ATP, $MgCl_2$, and particulate preparations from swine kidney (Mendicino et al., 1968; Kratowich & Mendicino, 1970). Recent studies by Brand & Soling (1975) have also shown that phosphofructokinase could be activated by phosphorylation with ATP in the presence of $MgCl_2$ and a rat liver extract. Rat liver fructose 1,6-bisphosphatase was also phosphorylated by a cAMP-dependent protein kinase (Riou et al., 1977). In the present study, we have examined the phosphorylation of homogeneous fructose 1,6-bisphosphatase and phosphofructokinase by a purified homologous protein kinase from swine kidney.

Materials and Methods

Isolation of Homogeneous Protein Kinase, Phosphofructokinase, and Fructose 1,6-Bisphosphatase from Swine Kidney. Large amounts of homogeneous fructose 1,6-bisphosphatase were isolated from kidney by methods described in our previous studies (Kratowich & Mendicino, 1974; Mendicino et al., 1975; Abou-Issa & Mendicino, 1973). The principal step in the purification involved affinity chromatography on cellulose-phosphate columns, in which the enzyme was eluted with μM concentrations of AMP, ATP, and fructose 1,6-bisphosphate (Kratowich & Mendicino, 1974). Aliquots of 3 mL were passed through Sephadex G-25 columns (2 × 31 cm) to remove the last traces of AMP and ATP. A solution containing 25 mM Tris-HCl, pH 7.4, and 0.154 M KCl was used to develop these columns. Fructose 1,6-bisphosphatase was eluted immediately after the void volume and AMP and ATP appeared in later fractions. The enzyme was precipitated by dialysis against saturated ammonium sulfate at pH 7.4, and the precipitate was dissolved and dialyzed

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against 25 mM Tris-HCl (pH 7.4)–0.154 M KCl–0.1 mM dithiothreitol–0.1 mM EDTA. Protein concentration was adjusted to 10 mg per mL. The final homogeneous preparation has a specific activity of 50 units per mg (Mendicino et al., 1975; Kratowich & Mendicino, 1974). Enzyme preparations could be stored at −20 °C for months. Some preparations began to lose activity after several months; however, all of the original activity could be restored by incubation at 30 °C in the presence of 30 mM cysteine, at pH 7.4 (Kratowich & Mendicino, 1970; Mendicino et al., 1972).

Phosphofructokinase was isolated from swine kidney extracts by procedures described previously (Mendicino et al., 1975; Shih & Mendicino, submitted for publication). This enzyme was purified to homogeneity by affinity chromatography on Blue Dextran–Sepharose 4B and cellulose–phosphate columns. The final preparation was also passed through a Sepharose G-25 column to remove small amounts of ATP and fructose 6-phosphate which were used to elute the enzyme from cellulose–phosphate columns. The final homogeneous preparation has a specific activity of 90 units per mg and showed only a single band on gel electrophoresis. This preparation was stored at −20 °C in 30% glycerol, and some preparations which lost activity were reactivated by incubation at 30 °C with 10 mM dithiothreitol and 1 mM ATP.

Glycogen synthetase was extensively purified from swine kidney by the method of Abou-Issa & Mendicino (1973). The homogeneous preparation used in the present studies had a specific activity of 13 units per mg. The enzyme was stored at −20 °C in a buffer containing 0.05 M Tris-HCl (pH 7.4)–0.3 M sucrose–20 mM 2-mercaptoethanol. The enzyme was routinely activated with 30 mM cysteine before addition to phosphorylation assay mixtures. The extent of conversion to the inactive phospho form was measured by determining the decrease in activity in the absence of glucose 6-phosphate as described in our previous studies (Abou-Issa & Mendicino, 1973).

The protein kinase present in both the soluble and particulate fractions of swine kidney homogenates was purified to homogeneity (Leibach et al., submitted for publication). The procedures used for the purification of protein kinase from swine kidney were based, in part, on a more comprehensive method for the sequential isolation of glycogen synthetase, phosphorylase, fructose 1,6-bisphosphatase, and phosphofructokinase from the same crude extract (Mendicino et al., 1975). The principal steps in the isolation of this enzyme involved adsorption to calcium phosphate gel, elution from DEAE-cellulose columns with cAMP and specific elution from Blue Dextran–Sepharose 4B or cellulose–phosphate columns. The final homogeneous preparations had a specific activity of 2.1 to 3.5 μmol per min per mg of protein with a mixture of calf thymus histones as the protein substrate. The preparation was completely free of protein substrates and did not bind cAMP. The enzyme was stored at −20 °C in 30% glycerol. Preparations used in phosphorylation studies were passed through small Sephadex G-25 columns which were equilibrated and developed with a solution containing 40 mM potassium phosphate, pH 7.4, and 0.1 mM dithiothreitol.

Preparation of Antisera to Swine Kidney Phosphofructokinase, Glycogen Synthetase, and Fructose 1,6-Biphosphatase. An emulsion of each enzyme (500 μg) in Freund's complete adjuvant was injected intradermally and into foot pads of young adult rabbits to produce antibody. After 3 weeks each animal received booster injections of 500 μg of enzyme and a month later blood was collected and centrifuged to remove cells. The antibodies were partially purified from the serums (Utsumi & Karush, 1965), and they were stored at −20 °C for months without any loss of anti-fructose 1,6-bisphosphatase, anti-glycogen synthetase, or anti-phosphofructokinase activity. Examination by immunodiffusion against purified enzymes resulted in the appearance of only one precipitin line in each case.

Rabbit antiserum to purified swine kidney protein kinase was prepared by injecting 100-μg samples in complete Freund's adjuvant into hind footpads. Booster injections were given after 3 weeks and antibody levels were assayed by measuring the binding of ^{125}I-labeled protein kinase in a standardized radioimmunoassay (Hansen et al., 1971). Antisera were collected 3 weeks after the secondary immunization, and an enriched immunoglobulin fraction was prepared by precipitation with ammonium sulfate and chromatography on DEAE-cellulose as described by Utsumi & Karush (1965). Purified kidney protein kinase was labeled with ^{125}I by the procedure of Bolton & Hunter (1973).

Immunoprecipitation was performed with a double antibody technique (Capecchi et al., 1974). Goat anti-rabbit serum was added to promote the precipitation of labeled enzyme–antienzyme complex. The method of Kessler (1975) was also used to precipitate the enzyme–antibody complex with A protein on *S. aureus* cells.

Assay of Fructose 1,6-Bisphosphatase Activity. Three different methods were used to determine the activity of fructose 1,6-bisphosphatase. During purification of the enzyme the activity was assayed by measuring the rate of formation of P_i as described previously (Mendicino et al., 1975). The standard reaction mixture was incubated at 37 °C and contained in 9 mL: 100 mM Tris-HCl, pH 7.4, 12 mM $MgCl_2$, 5 mM cysteine, 0.08 mM fructose 1,6-bisphosphate, and enzyme. After 5 min the reaction was stopped by the addition of 1 mL of 5 N H_2SO_4–2.5% ammonium molybdate and the release of P_i was determined. One unit of activity represents the amount of enzyme catalyzing the formation of 1 μmol of P_i from fructose 1,6-bisphosphate per min and specific activity is expressed as units per mg of protein. The activity of fructose 1,6-bisphosphatase was also determined spectrophotometrically by following the rate of formation of fructose 6-phosphate in the presence of NADP and excess phosphohexose isomerase and glucose 6-phosphate dehydrogenase (Mendicino et al., 1975). The incubation mixture contained 40 mM Tris-HCl, pH 7.4, 0.2 mM fructose 1,6-bisphosphate, 10 mM $MgCl_2$, 0.2 mM NADP, 10 mM 2-mercaptoethanol, 2 units each of phosphohexoisomerase and glucose-6-phosphate dehydrogenase, and enzyme in a total volume of 1 mL. In order to examine the effects of very low concentrations of fructose 1,6-bisphosphate on the activity of the phospho and dephospho forms of the enzyme, a second spectrophotometric assay, which contained a fructose 1,6-bisphosphate regenerating system, was employed (Mendicino et al., 1975). The enzyme from kidney has a very low K_m for fructose 1,6-bisphosphate and at high concentrations the substrate inhibits the reaction. In the presence of excess phosphofructokinase, pyruvic kinase, and lactic dehydrogenase, the hydrolysis of fructose 1,6-bisphosphate by fructose 1,6-diphosphatase is accompanied by a stoichiometric oxidation of NADH and fructose 6-phosphate is continuously reconverted to fructose 1,6-bisphosphate by phophofructokinase in the presence of ATP. In this way the initial concentration of fructose 1,6-bisphosphate is maintained and the effect of nearly catalytic quantities of substrate on the activity of the enzyme can be measured. The reaction mixture was incubated at 26 °C and contained 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 154 mM KCl, 0.2 mM NADH, 0.3 mM ATP, 2 units each of lactic dehydrogenase, pyruvic kinase, and phosphofructokinase, and

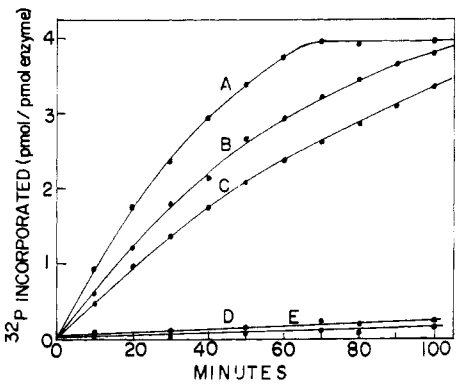

FIGURE 1: Time course of phosphorylation of purified swine kidney fructose 1,6-bisphosphatase, phosphofructokinase, and glycogen synthetase by homogeneous kidney protein kinase. Curve A was obtained with 5 μM glycogen synthetase, curve B with about 5 μM fructose 1,6-diphosphatase, curve C with 3 μM phosphofructokinase, curve D with the same amounts of the three enzymes in the absence of protein kinase, and curve E was obtained with 1.5 μM protein kinase alone. The standard incubation mixture containing 1.5 μM protein kinase and 0.4 mM [γ-^{32}P]ATP (100 cpm/pmol) was used and 0.05-mL aliquots were removed at the indicated times for analysis of ^{32}P covalently bound to protein.

an appropriate amount of fructose 1,6-bisphosphatase. All of the auxiliary enzymes were routinely diluted with a solution containing 10 mM Tris-HCl, pH 7.4, and 0.2 mg per mL of bovine serum albumin. One unit of activity in both of the spectrophotometric assays is defined as the amount of enzyme which catalyzes the hydrolysis of 1 μmol of fructose 1,6-bisphosphate per min.

Assay of Phosphofructokinase Activity. The activity of this enzyme was determined by measuring the rate of formation of fructose 1,6-bisphosphate or ADP in several different assay systems (Mendicino et al., 1975). During purification the activity was measured at 25 °C by following the rate of oxidation of NADH in 1 mL of a reaction mixture containing 30 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM fructose 6-phosphate, 5 mM 2-mercaptoethanol, 0.154 M KCl, 2 mM AMP, 0.3 mM NADH, 1 mg of bovine serum albumin, 0.5 unit of aldolase, 0.5 unit of triosephosphate isomerase, 0.5 unit of α-glycerolphosphate dehydrogenase, and enough phosphofructokinase to yield a rate of 0.04 to 0.1 absorbance unit per min. To examine the effects of small amounts of substrates on the velocity of the reaction another spectrophometric assay which contained a fructose 6-phosphate and ATP regenerating system was used. The reaction mixture contained 30 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 2 mM NADH, 0.5 mM phosphoenolpyruvate, 0.154 M KCl, 1 mg of bovine serum albumin, 1 mM ATP, 0.1 mM fructose 6-phosphate or fructose 1,6-bisphosphate, 1 unit of pyruvate kinase, 1 unit of lactic dehydrogenase, 0.1 unit of kidney fructose 1,6-bisphosphatase, and phosphofructokinase in a total volume of 1 mL. In this assay fructose 1,6-bisphosphate and ADP are continuously reconverted to fructose 6-phosphate and ATP by excess fructose 1,6-bisphosphatase and pyruvate kinase, and the conversion of pyruvate to lactate is accompanied by a stoichiometric oxidation of NADH. The simultaneous accumulation of P_i was measured at intervals by removing aliquots from the incubation mixture. This coupled system is particularly useful in kinetic studies, since both products of the reaction are also allosteric effectors of the enzyme.

Assay for the Phosphorylation of Glycogen Synthetase, Phosphofructokinase, Fructose 1,6-Bisphosphatase, and Histone by Protein Kinase. The phosphorylation of glycogen synthetase was measured by an enzymatic assay based on the conversion of the enzyme to a form which is dependent on glucose 6-phosphate for activity as described in our previous studies (Mendicino et al., 1975; Abou-Issa & Mendicino, 1973). The reaction mixture in a final volume of 0.3 mL contained 20 mM Tris-HCl, pH 7.4, 30 mM cysteine, 5 mM $MgCl_2$, 0.6 mM ATP, 0.25 M sucrose, 2 μM cAMP, 0.02 μM dephosphoglycogen synthetase, and protein kinase. Aliquots of 0.05 mL were removed at various times and the protein kinase reaction was terminated by the addition of 7.5 μmol of EDTA. The extent of conversion of glycogen synthetase to its phosphorylated form was then measured by determining the activity of the enzyme in the absence of glucose 6-phosphate.

The rate of transfer of ^{32}P from [γ-^{32}P]ATP to the different enzymes and histone was estimated by an acid precipitation method described in earlier studies (Abou-Issa & Mendicino, 1973) and by washing samples precipitated on paper with acid (Corbin & Reimann, 1974). The standard reaction mixture in 0.3 mL contained 30 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 12 mM 2-mercaptoethanol, 6 mM $MgCl_2$, 0.2 mM ATP (100 cpm/pmol), 1 mg (280 μM) of histone, and protein kinase. A unit of activity is defined as the amount of protein kinase required to transfer 1 pmol of ^{32}P from [α-^{32}P]ATP to protein per min. The transfer of ^{32}P to fructose 1,6-bisphosphatase, glycogen synthetase, and phosphofructokinase was measured with the same assay system. Because of the high K_m of the enzymes used as substrates in these studies and the small amounts used in routine assays, the reaction kinetics were zero order for only very small conversions to the phospho forms. When more than 10% conversion took place, the reaction rates were calculated from slopes of the linear portions of plots of the activity vs. time.

Procedures and Materials. Purified enzymes purchased as crystalline suspensions in ammonium sulfate were centrifuged and the precipitates were dissolved in a solution containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.154 M KCl, and 0.1% bovine serum albumin. Rabbit muscle phosphofructokinase and fructose 1,6-bisphosphatase were purchased from Sigma. Protein was measured by the method of Lowry et al. (1951). Type II-A histone from calf thymus was obtained from Sigma. [γ-^{32}P]ATP was prepared by procedures described previously (Abou-Issa et al., 1974). Swine kidneys were supplied by the Gold Kist Co., Talmo, Ga.

Results

Phosphorylation of Renal Fructose 1,6-Bisphosphatase, Phosphofructokinase, and Glycogen Synthetase. The influence of the time of incubation on the number of equivalents of ^{32}P incorporated into fructose 1,6-bisphosphatase, phosphofructokinase, and glycogen synthetase is shown in Figure 1. The rate of the reaction was linear with time only for very small conversions of the enzymes to phosphorylated derivatives. The relative amount of protein kinase to enzyme substrate was kept high in these experiments in order to obtain complete phosphorylation. The amounts of enzyme substrates used in these experiments were well below their K_m's; therefore, the rate of the reaction decreased with time. The activity of protein kinase also decreased over long periods of incubation and this effect may also account for part of the decrease in the rate of the reaction at longer times. The rates of phosphorylation of the three enzymes progressively decreased with time, and reached a maximal level after about 90–100 min. Even after 90 min the reactions shown in curves B and C did not reach plateau values. More protein kinase, 500 units, was added at 90 min, but no further phosphorylation was found after periods of up to 3 h. Nearly 3.5 mol of phosphate was incorporated per mol of enzyme, in each case. Little or no ^{32}P was incorporated in the

TABLE I: Activation of Homogeneous Kidney Fructose 1,6-Bisphosphatase, Phosphofructokinase, and Glycogen Synthetase by Incubation with Cysteine.[a]

time of incubation	fructose 1,6-bisphosphatase (units/mg)	phosphofructokinase (units/mg)	glycogen synthetase −Glu 6P (units/mg)	glycogen synthetase +Glu 6P (units/mg)
0	23.5	61.2	8.1	10.2
5	32.7	69.3	9.0	10.8
10	41.3	75.1	10.0	11.4
15	46.5	84.1	10.9	12.3
20	47.1	90.1	11.7	12.7
25	48.8	90.3	12.5	
30	48.3	90.1	12.9	

[a] Purified homogeneous preparations of fructose 1,6-bisphosphatase which were stored frozen at −20 °C for at least a month were adjusted to 30 mM cysteine, pH 7.4, and the mixture was incubated at 30 °C for various times. Aliquots were removed and added to the standard incubation mixture and activity was determined as described in Materials and Methods. The specific activity of the freshly prepared enzyme was about 48 units per mg. Purified preparations of phosphofructokinase stored at 3 °C for 2 weeks were treated in the same manner. Glycogen synthetase stored at −20 °C for a month was incubated with 30 mM cysteine, 0.25 M sucrose, and 10 mM glucose 6-P as indicated. Activity in aliquots removed at various times was assayed as described in the text.

TABLE II: Influence of Phosphorylation on the Activity of Glycogen Synthetase, Fructose 1,6-Bisphosphatase, and Phosphofructokinase.

time (min)	glycogen synthetase ^{32}P incorp. (μmol/μmol)	glycogen synthetase sp act. (units/mg)	fructose 1,6-bisphosphatase ^{32}P incorp. (μmol/μmol)	fructose 1,6-bisphosphatase sp act. (units/mg)	phosphofructokinase ^{32}P incorp. (μmol/μmol)	phosphofructokinase sp act. (units/mg)
0	0	12.9	0	48.3	0	90.1
10	0.95	9.4	0.50	49.1	0.5	85.3
20	1.75	7.2	1.20	48.6	0.97	87.4
30	2.45	5.1	1.80	48.7	1.40	88.1
50	3.40	1.9	2.65	47.3	2.10	86.3

absence of protein kinase (curve D) and no ^{32}P was incorporated into protein kinase when it was incubated with [γ-^{32}P]ATP under identical conditions (curve E). Each of these enzymes has a tetrameric structure and contains four identical subunits (Mendicino et al., 1972, 1975). The results suggest that 1 mol of ^{32}P is incorporated into each subunit of the enzyme under these conditions. Phosphofructokinase and fructose 1,6-bisphosphatase isolated from rabbit muscle and obtained as crystalline preparations were not phosphorylated by purified kidney protein kinase. After treatment with kidney phosphoprotein phosphatase or *E. coli* alkaline phosphatase (Mellgren et al., 1977), they were still inactive as substrates in the standard phosphorylation system.

Influence of Phosphorylation on the Activity and Allosteric Effects of AMP and ATP on Fructose 1,6-Bisphosphatase and Phosphofructokinase. Studies relating the extent of phosphorylation of these enzymes to alterations in their enzymatic or allosteric properties were hampered by their relative instability in the absence of sulfhydryl reagents and stabilizing metabolites. Both enzymes bind some ATP and fructose 1,6-bisphosphate very tightly (Kratowich & Mendicino, 1974; Mendicino et al., 1975), and this effect added to the complications at low concentrations. The criteria used in these studies for activation or inactivation of the enzymes required that the specific activity of the fully dephosphorylated enzyme under stable incubation conditions be altered. Besides using only native unmodified enzymes in these experiments, we have also been careful to use fully active enzymes. It has been observed in our previous studies that stored enzymes which have lost part of their activity can be completely reactivated by incubation with cysteine, as seen in Table I. The activity of fructose 1,6-bisphosphatase increased from 23.5 to 48.3 units per mg on incubation with 30 mM cysteine at pH 7.4. This is the same specific activity as the freshly isolated enzyme. Likewise, the specific activities of phosphofructokinase, 61.2 to 90.1 units per mg, and glycogen synthetase, 8.1 to 12.9 units per mg, were increased by incubation with 30 mM cysteine. These final specific activities are nearly the same as freshly isolated homogeneous enzymes in each case (Mendicino et al., 1975). The fructose 1,6-bisphosphatase did not contain covalently bound phosphate. Glycogen synthetase and phosphofructokinase were completely dephosphorylated with kidney phosphoprotein phosphatase as described in previous studies (Abou-Issa & Mendicino, 1973), and some samples were also treated with *E. coli* alkaline phosphatase. The enzymes were reisolated by affinity chromatography in order to completely remove the phosphatases.

The relationship between the extent of phosphorylation of each enzyme and the specific activity of the resulting phosphorylated species is shown in Table II. It is clear that the activity of kidney glycogen synthetase in the absence of glucose 6-phosphate progressively decreases as the extent of phosphorylation increases. In the presence of 10 mM glucose 6-phosphate no decrease in glycogen synthetase activity was observed. In contrast, the specific activities of phosphofructokinase and fructose 1,6-bisphosphatase determined in several different assay systems described in Materials and Methods were not significantly affected by phosphorylation. The kinetic and allosteric properties of the phosphorylated enzymes were examined. The K_m's for substrates were determined from double-reciprocal plots of kinetic data. The K_m of phosphofructokinase for ATP was 8.3 μM and for fructose 6-phosphate it was 33 μM. The corresponding K_m's of the phosphorylated enzyme were 8.1 and 30 μM. The apparent K_i of the phosphorylated enzyme for ATP, 5.7 μM, was nearly the same as that for the native enzyme, 5.8 μM. The K_m of fructose 1,6-bisphosphatase for fructose 1,6-bisphosphate was 6.1 μM and the phosphorylated form had a K_m of 5.9 μM. The K_i of fruc-

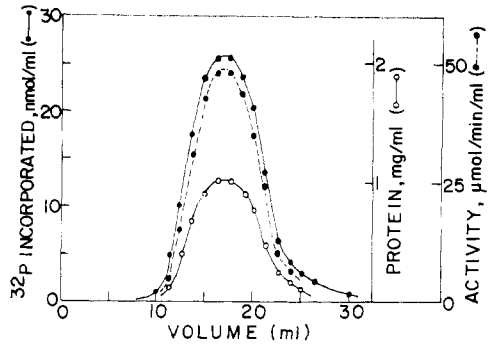

FIGURE 2: Isolation of ^{32}P-labeled fructose 1,6-bisphosphatase by chromatography on cellulose-P. The enzyme was phosphorylated with protein kinase in the standard reaction mixture which contained 3.5 mg (175 units) of fructose 1,6-bisphosphatase and 0.5 mg of protein kinase in a total volume of 1 mL. The enzyme was precipitated twice by the addition of ammonium sulfate to 0.7 saturation at 3 °C. After centrifugation the precipitate was dissolved in a solution containing 0.05 M Tris-HCl, pH 7.4, 10 mM 2-mercaptoethanol, and 0.1 mM EDTA and it was dialyzed against this buffer for 1 h and applied to a cellulose-P column (2.2 × 6 mL). The column was washed with buffer and fructose 1,6-bisphosphatase was eluted with 0.05 M Tris-HCl, pH 7.4, containing 1 mM AMP and 1 mM fructose 1,6-bisphosphate (Mendicino et al., 1972). Aliquots of 1 mL were dialyzed and assayed as described in the text.

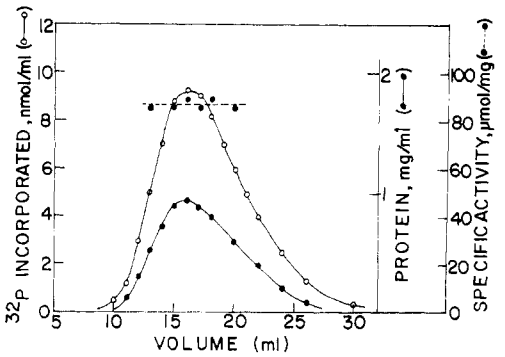

FIGURE 3: Isolation of ^{32}P-labeled phosphofructokinase by chromatography on Blue Dextran–Sepharose 4B. The enzyme was phosphorylated in the standard reaction mixture which contained 4 mg (360 units) of phosphofructokinase and 0.5 mg of protein kinase in a total volume of 1 mL. Afterward the incubation mixture was dialyzed against buffer containing 0.01 M potassium phosphate, pH 7.4, 10 mM 2-mercaptoethanol, 3 mM $MgCl_2$, and 1 mM fructose 6-P and it was applied to a Blue Dextran–Sepharose 4B column (2.2 × 10 mL). The column was washed with this buffer and phosphofructokinase was eluted with buffer containing 3 mM ATP and 3 mM AMP. Fractions of 1 mL were dialyzed and assayed as described in the text.

tose 1,6-bisphosphatase for fructose 1,6-bisphosphate was 8.2 mM and the phospho form had a K_i of 8.0 mM. The K_i of the enzyme for AMP is dependent on temperature. At a concentration of 0.1 mM fructose 1,6-bisphosphate the K_i for AMP was 3 μM at 3 °C, 30 μM at 22 °C and 95 μM at 37 °C. No large alteration in this parameter was observed after phosphorylation of fructose 1,6-bisphosphatase. The K_i was 4 μM at 3 °C, 35 μM at 22 °C, and 100 μM at 37 °C. Phosphorylation did not cause dissociation of the enzyme into subunits as determined by gel filtration on Sephadex G-200. These results suggest that the incorporation of phosphate into these enzymes does not alter their responses to allosteric inhibitors such as AMP or ATP, nor does it change the degree of inhibition by excess substrates such as fructose 1,6-bisphosphate or ATP. Furthermore, no significant alteration in the K_m's or specific activities of these enzymes was observed after phosphorylation by protein kinase.

Characterization of the Phosphorylated Forms of Phosphofructokinase and Fructose 1,6-Bisphosphatase. The enzymes were phosphorylated in a large scale incubation mixture and fructose 1,6-bisphosphatase was reisolated by affinity chromatography on a cellulose-phosphate column. As seen in Figure 2, the profiles of the ^{32}P peak coincided with the protein and activity peaks eluted with ATP and AMP. The specific radioactivities of fractions taken from different parts of the peak were identical. Analysis of the enzyme by gel electrophoresis in sodium dodecyl sulfate showed that all of the radioactivity was present in a single protein band with a molecular weight 34 000 (Figure 4) which corresponded exactly to the subunit of the native enzyme (Mendicino et al., 1972).

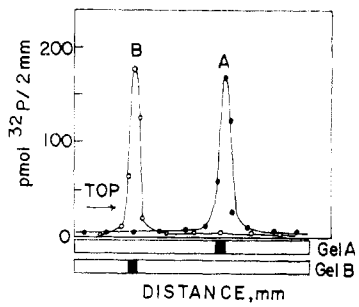

FIGURE 4: Polyacrylamide gel electrophoresis of the phosphorylated forms of swine kidney fructose 1,6-bisphosphatase and phosphofructokinase in 1% sodium dodecyl sulfate. The isolated phospho enzymes were treated with 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol as described previously (Mendicino et al., 1972) and they were applied to gels and analyzed by electrophoresis. Only single bands were obtained with each enzyme (gels A & B). The molecular weight of the protein band obtained with fructose 1,6-bisphosphatase (gel A) was 34 000 and the band obtained with phosphofructokinase (gel B) has a molecular weight of 88 000. Radioactivity profiles were obtained by cutting the gels into slices of 2 mm. Curve A was obtained with fructose 1,6-bisphosphatase and curve B was obtained with phosphofructokinase.

Phosphofructokinase was isolated by chromatography on a Blue Dextran–Sepharose 4B column. The profile of the ^{32}P peak coincided with the protein and activity peaks eluted with ATP, as seen in Figure 3. The specific activities of fractions taken from different areas of the peak were nearly identical. Examination of the phosphorylated enzyme by gel electrophoresis in sodium dodecyl sulfate showed that the covalently bound ^{32}P was present in a single protein band with a molecular weight of 88 000, Figure 4, which migrated at the same rate as the subunits of the homogeneous kidney enzyme (Shih & Mendicino, submitted for publication).

Further confirmation of the phosphorylation of these enzymes was obtained by immunoprecipitation with antibody specific for the enzyme. In each case, the amount of radioactivity found in the isolated enzyme–antibody precipitate was the same as the amount recovered after acid precipitation. The protein kinase isolated from these incubation mixtures by precipitation with specific antibody did not contain covalently bound ^{32}P. The same radioactive profiles as those shown in Figure 4 were obtained when immunoprecipitated fructose 1,6-diphosphatase and phosphofructokinase were dissociated and examined by gel electrophoresis in sodium dodecyl sulfate by procedures described in a previous report (Mendicino et al., 1972).

The covalently bound ^{32}P in both enzymes was completely hydrolyzed to $^{32}P_i$ by treatment with 0.25 M NaOH for 36 h at room temperature. Hydrolysis for 16 h at 105 °C in 6 N HCl yielded $^{32}P_i$. Partial acid hydrolysis of ^{32}P-labeled enzymes isolated by affinity chromatography and concentrated by precipitation with acid as described for the standard protein kinase assay was carried out in 2 mL of 6 N HCl for 4 h or in 2 mL of 2 N HCl for 16 h at 105 °C in evacuated sealed tubes

TABLE III: The Apparent Michaelis Constants for Protein Kinase and the Concentration of Phosphofructokinase, Glycogen Synthetase, and Fructose 1,6-Bisphosphatase in Swine Kidney.[a]

enzyme	Michaelis constant (μM)	concn (μmol/1000 g)	total act. (μmol/(min g)	sp act. (μmol/(min mg))	ref
phosphofructokinase	55	0.08 (360)	2.6	90	Mendicino et al., 1975
fructose 1,6-bisphosphatase	53	1.45 (135)	9.8	50	Kratowich & Mendicino, 1974
glycogen synthetase	50	0.038 (370)	0.18	13	Abou-Issa & Mendicino, 1973
phosphorylase		0.047 (190)	0.27	30	Medicus & Mendicino, 1973
protein kinase		0.26 (42)	0.023	2.1	Reddy et al.,[b]
enolase		1.8 (89)	14.3	90	Oh & Brewer, 1973
histone	63	(12)			Abou-Issa et al., 1974

[a] The total activity in homogenates and the specific activity of homogeneous enzymes were determined using the same standardized assay procedure. The concentrations were also confirmed with specific immunoassays using antibodies to the purified enzymes. The molecular weights, in mg/μmol, of the enzymes used in these calculations are shown in parentheses. [b] S. Reddy, F. Leibach, M. Banner, and J. Mendicino, submitted for publication.

containing 10 μmol of phosphorylserine and 10 μmol of phosphorylthreonine. The hydrolysates were dried by lyophilization, dissolved in 1 mL of 0.1 M formic acid, and applied to a Dowex 1-formate column (2.2 × 4.5 cm). The columns were washed with 50 mL of 0.1 M formic acid and a peak containing ^{32}P-labeled phosphorylserine was eluted with a solution containing 0.25 M formic acid and 0.25 M pyridine. The peak appeared after about 60 mL of this solvent passed through the column. The active samples were combined, concentrated by lyophilization, and subjected to electrophoresis on Whatman paper for 1 h at 2500 V in 2.5% formic acid–7.8% acetic acid, pH 1.9, or in 50 mM pyridine acetate, pH 3.5, with appropriate phosphate and phosphorylserine standards. Amino acids were analyzed and detected on paper with ninhydrin reagents and radioactivity was located by cutting the dried paper into strips and counting in scintillation solvent as described previously (Abou-Issa et al., 1974). Samples of ^{32}P-labeled phosphofructokinase and fructose 1,6-bisphosphatase yielded radioactive phosphorylserine when examined by these procedures.

Influence of the Concentration of Fructose 1,6-Bisphosphatase, Phosphofructokinase, Glycogen Synthetase, and Histone on the Activity of Protein Kinase. The rate of phosphorylation of fructose 1,6-bisphosphatase and phosphofructokinase was directly proportional to the concentration of protein kinase. Incubation of 2 μM fructose 1,6-bisphosphatase with 0.4, 0.8, and 1.2 μM protein kinase for 10 min resulted in the incorporation of 0.21, 0.38, and 0.63 pmol of ^{32}P per pmol of enzyme. The extent of phosphorylation increased to 0.38, 0.74, and 1.19 pmol per pmol when the reaction mixtures were incubated for 20 min. The extent of phosphorylation of 1 μM phosphofructokinase with 0.4, 0.8, and 1.2 μM protein kinase after incubation for 10 min was 0.09, 0.16, and 0.26 pmol of ^{32}P per pmol of enzyme.

The influence of the concentration of several enzyme substrates on the activity of swine kidney protein kinase is shown in Figure 5. The apparent Michaelis constants calculated from initial velocity data by the least-squares fit method for fructose 1,6-bisphosphatase, phosphofructokinase, glycogen synthetase, and histone were 53 μM, 55 μM, 50 μM, and 63 μM, respectively. Protein kinase may recognize only a very small exposed area in the vicinity of the phosphorylated seryl residue in each of these enzymes. The molecular weights of the enzymes, ranging from 380 000 for glycogen synthetase to 12 000 for histone, appear to have little or no effect on the rate of phosphorylation.

The concentration of these enzymes which act as substrates for protein kinase in kidney was determined by enzymatic and immunological procedures. The data summarized in Table III show that the concentrations of all of these substrates are at least an order of magnitude lower than the Michaelis constants determined for the isolated enzymes. It is also clear that the amount of fructose 1,6-bisphosphatase, 1.45 μM, is much higher than phosphofructokinase, 0.08 μM, in this tissue and is nearly equal to the amount of enolase, 1.8 μM, an enzyme which is active during glycolysis and gluconeogenesis. All of the enzymes listed in Table III are substrates for kidney protein kinase, except enolase and phosphorylase. The latter enzyme is phosphorylated by a specific phosphorylase kinase (Mendicino et al., 1975).

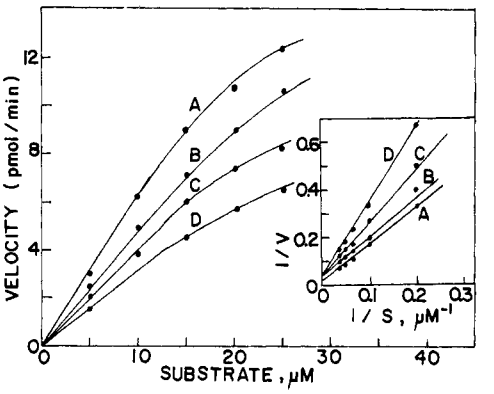

FIGURE 5: Effect of the concentration of fructose 1,6-bisphosphatase, phosphofructokinase, glycogen synthetase, and histone on the rate of phosphorylation. The amounts of each enzyme shown in the figure were incubated with 500 units (0.019 μM) of protein kinase in the standard assay system. Curve A was obtained with fructose 1,6-bisphosphatase, curve B with glycogen synthetase, curve C with phosphofructokinase, and curve D with histone. The inset shows reciprocal plots with $1/V$ in min per pmol.

Discussion

According to current concepts the primary control for glycogenesis and glycogenolysis resides in enzymes which are subject to covalent modification in response to hormonal stimulation (Krebs, 1972). The allosteric sensitivities of glycogen phosphorylase and glycogen synthetase which govern the supply of carbohydrate from stored glycogen and triglyceride lipase which mobilizes stored lipid are directly controlled by the action of protein kinase and phosphoprotein phosphatase. Opposing enzymes, such as fructose 1,6-bisphosphatase and phosphofructokinase which are involved at intermediate stages in the metabolism of carbohydrate, may generate "futile substrate cycles", which lead to hydrolysis of ATP and pro-

duction of heat (Newsholme et al., 1972; Clarke et al., 1973). While this type of mechanism is operative in a highly specialized tissue such as skeletal muscle with contains some fructose 1,6-bisphosphatase but is unable to carry out gluconeogenesis, different control mechanisms may be involved in gluconeogenic tissues such as kidney and liver, since not only the rate but the direction of carbohydrate metabolism must also be regulated in these tissues. The regulation of "futile cycles" has been discussed in several recent reviews (Newsholme et al., 1973; Clark & Lardy, 1976; Utter et al., 1975). As discussed previously very little "substrate cycling" is found at the fructose 6-phosphate–fructose 1,6-bisphosphate level in gluconeogenic tissues (Soling et al., 1973; Hue & Hers, 1974b).

It has been proposed that the activity of fructose 1,6-bisphosphatase and phosphofructokinase might be regulated by changes in the concentration of AMP, ATP, fructose 1,6-bisphosphate or other of the numerous allosteric effectors of these enzymes acting alone or together during gluconeogenesis. However, evidence obtained on the level of these metabolites in kidney and liver during gluconeogenesis and the kinetic properties of the enzymes indicate that an inhibition by AMP and ATP would exist most of the time and that the highest levels of fructose 1,6-bisphosphate found in these tissues would be too low to influence the activity of the enzymes (Williamson et al., 1969).

Hormones and cAMP dramatically increase the rate of conversion of fructose 1,6-bisphosphate to fructose 6-phosphate in kidney and liver during gluconeogenesis (Williamson et al., 1969; Clark & Lardy, 1976; Utter et al., 1975). The phosphorylation of fructose 1,6-bisphosphatase (Mendicino et al., 1966) and phosphofructokinase (Brand & Soling, 1975) suggests that a phosphorylation mechanism might be involved in the control of the enzymes. From the results obtained in the present study, it is possible to conclude that kidney fructose 1,6-bisphosphatase and phosphofructokinase are phosphorylated by protein kinase. The phosphorylation is relatively specific, since only 1 mol of phosphate is incorporated into each subunit of the enzyme and the corresponding enzymes from rabbit muscle are not phosphorylated under identical conditions.

The specific activities of kidney phosphofructokinase and fructose 1,6-bisphosphatase were not altered by phosphorylation with protein kinase. Stored samples of these enzymes were fully activated with 2-mercaptoethanol or dithiothreitol before they were added to the phosphorylation reaction mixture. The activity of rat liver fructose 1,6-diphosphatase was reported to increase about 40% following phosphorylation (Riou et al., 1977). However, the specific activity of the enzyme added to the incubation mixture and that of the phosphorylated form were not reported in these studies. If restoration of the activity of stored preparations of the enzyme occurred during incubation in the reaction mixture containing cysteine, then the observed activation might not be related to phosphorylation of the enzyme. It should be noted that the enzyme phosphorylated with ^{32}P in vivo had a specific activity of 34 units/mg which is nearly the same as the homogeneous enzyme isolated from this tissue, 34–46 units/mg (Riou et al., 1977). A recent study has also shown that phosphofructokinase could also be phosphorylated by protein kinase with a concomitant increase in enzymatic activity (Brand & Soling, 1975). The activation of phosphofructokinase after phosphorylation appeared to be due to an aggregation of monomers to an active protomer complex. It should be noted in this regard that activation of phosphofructokinase by protein kinase is opposite to the kind of response that would be expected from an increase in cAMP levels in a gluconeogenic tissue. The results obtained in the present study carried out with homogeneous undissociated enzyme indicate that no direct alteration in the activity of kidney phosphofructokinase occurs after phosphorylation. The relationship of the phosphorylation of fructose 1,6-bisphosphatase and phosphofructokinase to possible regulation of these enzymes by this process is still unknown. Further studies are needed to determine if the phosphorylation of phosphofructokinase and fructose 1,6-bisphosphatase is involved in the hormonal regulation of these enzymes.

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Fluorescence Energy Transfer between Metal Ions in Thermolysin. Thermal Denaturation Studies†

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ABSTRACT: Thermolysin derivatives have been prepared with one zinc ion, two calcium ions, and one terbium ion. Excitation of this derivative at 280 nm results in emission of visible Tb^{3+} fluorescence at 545 nm. When Co^{2+} is substituted for the Zn^{2+}, the Tb^{3+} emission is quenched due to energy transfer between the Co^{2+} and Tb^{3+}. Distances have been calculated between the two metal ion binding sites assuming a dipole–dipole mechanism for energy transfer (Berner, V. G., et al. (1975) *Biochem. Biophys. Res. Commun. 66,* 763; Horrocks, W. D., Jr., et al. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 4764). We have extended these studies by following the Co^{2+}–Tb^{3+} distances as a function of temperature. We have found a gradual increase in the distance between the two metal ions that ranges from nearly 14 Å at 25 °C to nearly 22 Å at temperatures above 80 °C. Conventional techniques for measuring conformational changes in proteins (tryptophan–tyrosine fluorescence, optical rotation or enzyme activities) show little or no change in the protein structure up to 70 °C. At higher temperature drastic changes in thermolysin properties indicate an extensive unfolding of the protein. In contrast to the optical rotation, fluorescence, and activity measurements, viscosity measurements indicate changes in the protein structure at temperatures well below 70 °C, and these changes correspond rather well with the distance changes between the Co^{2+} and Tb^{3+}. This indicates that both the viscosity and fluorescence energy transfer experiments are detecting changes in the protein structure which are not detectable by the other techniques.

Thermolysin is a neutral metalloendopeptidase isolated from the thermophilic organism *Bacillus thermoproteolyticus* Rokko which has an unusual stability toward thermal denaturation (Endo, 1962; Matsumara, 1967). The amino acid sequence (Titani et al., 1972) and three-dimensional structure have been determined (Matthews et al., 1974, and references therein). The protein consists of two distinct lobes with the active site of the enzyme lying in the cleft formed by these two lobes. A zinc ion, essential for enzyme activity, lies at the base of the cleft while 4 calcium ions presumably provide structural stability.

In recent years, paramagnetic, chromophoric metal ions such as trivalent lanthanide ions have proven useful as a structural probe in the study of structure–function relationships in proteins and enzymes (Darnall & Birnbaum 1970). In the particular case of thermolysin, the calcium ions at sites 1 and 2 have been replaced by a single lanthanide ion while still maintaining calcium ions at sites 3 and 4 (Matthews & Weaver, 1974). Similarly, the zinc ion can be replaced by a cobalt ion without affecting the conformation of the protein (Holmquist & Vallee, 1974). Making use of these substitutions, fluorescence spectroscopy has been used to determine a distance of 13.6 Å between the calcium sites 1 and 2 and the zinc binding site (Berner et al., 1975; Horrocks et al., 1975). These measurements agree well with the distance obtained from X-ray crystallographic measurements. In this present study we have used the same method to monitor the increase in the distance between these two metal sites as a function of temperature. To our knowledge this is the first study where the distance between two sites on a protein can be quantified as the protein is thermally unfolded. Activity, ORD, and viscosity measurements have been carried out to compare the results from fluorescence energy transfer measurements with the more typical measures of conformational changes in proteins.

Methods

Activity. The activity of thermolysin was measured by monitoring the decrease of 3-(2-furylacryloyl)glycyl-L-leucinamide (FAGLA)[1] absorbance at 322 nm at constant temperature (Khan & Darnall, 1978). To obtain a temperature profile, the following procedure was used: 3 mL of FAGLA (5.0×10^{-5} M) in 0.01 M Hepes buffer, 0.01 M $CaCl_2$, 1.0 M NaCl at pH 7.5 was placed in the 1-cm pathlength sample cell at the desired temperature. A similar concentration of FAGLA was placed in the reference cell to partially balance the high initial absorbance of FAGLA at these concentrations. After 10 min, a 50-μL aliquot of a 2×10^{-5} M thermolysin solution which had been incubated at the same temperature for the same length of time was added to the FAGLA solution in the sample compartment. Within 10 s after mixing, the decrease in absorbance at 322 nm was followed as a function of time. A fresh sample of enzyme was allowed to equilibrate for a 10-min period at each particular temperature.

Fluorescence. An Aminco-Bowman ratio spectrophotofluorimeter with a thermostated cell compartment was used for all fluorescence measurements. In a typical experiment, 200 μL of the enzyme solution was placed in a 3-mm fluores-

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[1] Abbreviations used: FAGLA, 3-(2-furylacryloyl)glycyl-L-leucinamide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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