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Rat Liver 6-Phosphofructo 2-Kinase/ Fructose 2,6-Bisphosphatase: A Review of Relationships Between the Two Activities of the Enzyme

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Both the synthesis and the degradation of Fru-2,6-P_2 are catalyzed by a single enzyme protein; ie, the enzyme is bifunctional. This protein, which we have designated 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase is an important enzyme in the regulation of hepatic carbohydrate metabolism since its activity determines the steady-state concentration of fructose 2,6-P_2, an activator of 6-phosphofructo 1-kinase and an inhibitor of fructose 1,6-bisphosphatase. Regulation of the bifunctional enzyme in intact cells is a complex function of both covalent modification via phosphorylation/dephosphorylation and the influence of substrates and low molecular weight effectors.

Recent evidence suggests that both reactions may proceed by two-step transfer mechanisms with different phosphoenzyme intermediates. The enzyme catalyzes exchange reactions between ADP and ATP and between fructose 6-P and fructose 2,6-P_2. A labeled phosphoenzyme is formed rapidly during incubation with [2-^{32}P]Fru-2,6-P_2. The labeled residue has been identified as 3-phosphohistidine. However, it was not possible to demonstrate significant labeling of the enzyme directly from [γ-^{32}P]ATP. These results can be most readily explained in terms of two catalytic sites, a kinase site whose phosphorylation by ATP is negligible (or whose E-P is labile) and a fructose 2,6-bisphosphatase site which is readily phosphorylated by fructose 2,6-P_2. Additional evidence in support of two active sites include: (1) limited proteolysis with thermolysin results in loss of 6-phosphofructo 2-kinase activity and activation of fructose 2,6-bisphosphatase, (2) mixed function oxidation results in inactivation of the 6-phosphofructo 2-kinase but no affect on the fructose 2,6-bisphosphatase, (3) N-ethylmaleimide treatment also inactivates the kinase but does not affect the bisphosphatase, and (4) p-chloromercuribenzoate immediately inactivates the fructose 2,6-bisphosphatase but not the 6-phosphofructo 2-kinase. Our findings indicate that the bifunctional enzyme is a rather complicated enzyme; a dimer, probably with two catalytic sites reacting with sugar phosphate, and with an unknown number of regulatory sites for most of its substrates and products. Three enzymes from Escherichia coli, isocitric dehydrogenase kinase/phosphatase, glutamine-synthetase adenylyltransferase, and the uridylyltransferase for the regulatory protein P_{II} in the glutamine synthetase

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cascade system also catalyze opposing reactions probably at two discrete sites. All four enzymes are important in the regulation of metabolism and may represent a distinct class of regulatory enzymes.

Key words: Fructose 2,6-bisphosphate, 6-phosphofructo 2-kinase/fructose-2,6-bisphosphatase

Fru-2,6-P_2, which was discovered in 1980, is a unique sugar diphosphate that plays a significant role in the regulation of glycolysis and gluconeogenesis in the liver [1–6]. In the last two years it has become clear that both the synthesis and degradation of Fru-2,6-P_2 are catalyzed by a unique bifunctional enzyme that is itself subject to regulation by covalent modification as well as by a number of low molecular weight effectors [1,2,5,6]. It is the object of this report first to review briefly the discovery, structure, and effects of Fru-2,6-P_2 and then to summarize what is known about the properties of the bifunctional enzyme responsible for determining the steady-state concentration of Fru-2,6-P_2. The emphasis will be on the relationship between the 6-phosphofructo 2-kinase and fructose 2,6-bisphosphatase activities of the enzyme, and evidence will be presented for two discrete catalytic sites. In addition, we will demonstrate that there are essential sulfhydryl groups that play an important role in the sugar phosphate affinity of the 6-phosphofructo 2-kinase reaction, raising the possibility that the enzyme activity in intact cells may be regulated by such a mechanism in addition to covalent modification. Finally, we will put forth the hypothesis that this bifunctional enzyme belongs to a distinct class of regulatory bifunctional enzymes that catalyze their opposing reactions at discrete active sites.

THE ROLE OF FRUCTOSE 2,6-BISPHOSPHATE IN THE CONTROL OF HEPATIC CARBOHYDRATE METABOLISM

Fru-2,6-P_2 whose structure is shown in Figure 1, is a unique sugar diphosphate that is an important regulator of hepatic carbohydrate metabolism (for review, see [1]). Fru-2,6-P_2 was discovered in the course of studies on the hormonal regulation of hepatic 6-phosphofructo 1-kinase. We first noted in 1979 that addition of glucagon to hepatocytes resulted in inhibition of 6-phosphofructo 1-kinase when measured in crude cell extracts [7]. A number of groups subsequently reported the existence in hepatocyte extracts of an unknown activator of 6-phosphofructo 1-kinase whose level was dramatically decreased by the addition of glucagon [8–11]. Van Schaftingen and Hers first suggested that the activator was Fru-2,6-P_2 [12] and the definitive structure was established after chemical synthesis of the compound and ^{13}C-nuclear magnetic resonance (NMR) analysis by our group [13] as well as subsequently by others [14,15]. The NMR analysis also revealed that Fru-2,6-P_2 exists as the β-anomer (Fig. 1).

Work from a number of laboratories has established that this compound is a potent activator of 6-phosphofructo 1-kinase [16–18] and a potent inhibitor of fructose 1,6-bisphosphatase [19,20]. It is synergistic with AMP in both of these actions [16–20]. A scheme summarizing regulation of the Fru-6-P/Fru-1,6-P_2 substrate cycle in liver brought about the interaction of Fru-2,6-P_2 and AMP with both enzymes is shown in Figure 2. Fru-2,6-P_2 and AMP cooperate in activating 6-phosphofructo 1-kinase and tend to overcome inhibition by ATP and citrate. Thus, in situations where Fru-2,6-P_2 levels are high in the liver cell one would expect substantial flux through 6-phosphofructo 1-kinase and high rates of glycolysis. In general, this has been the finding in a number of cell types and metabolic situations [1–6]. It is

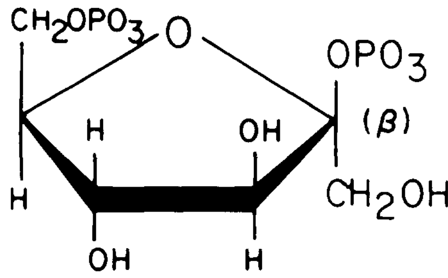


Fig. 1. Structure of fructose 2,6-P_2.

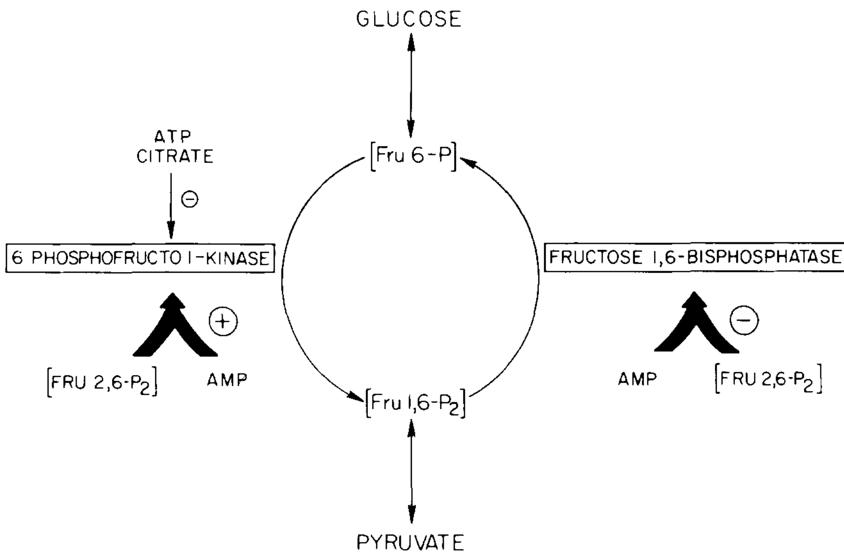


Fig. 2. Scheme for the regulation of the enzymes of the Fru 6-P/Fru 1,6-P_2 substrate cycle by Fru 2,6-P_2 and AMP.

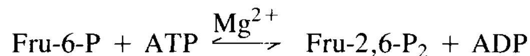
generally agreed that in liver Fru-2,6-P_2 is the most significant physiologic effector of the enzyme in the intact cell. Fructose 2,6-P_2 also acts synergistically with AMP to inhibit fructose 1,6-bisphosphatase, the enzyme opposing 6-phosphofructo 1-kinase in this substrate cycle, and we have argued that this synergism is of major importance in regulating the activity of this key gluconeogenic enzyme [21]. A number of groups have shown that in situations where Fru-2,6-P_2 levels are diminished—for example, in diabetes, starvation, or after glucagon treatment flux thru fructose 1,6-bisphosphatase is accelerated and flux thru 6-phosphofructo 1-kinase is diminished with concomitant increased gluconeogenesis (for review, see [1–6]).

The hepatic concentration of Fru-2,6-P_2 is in the micromolar range and is subject to both dietary and acute hormonal regulation [1]. Glucagon and β-adrenergic agents act to lower its level via elevations in the level of cyclic AMP; and insulin, by virtue of its ability to lower cyclic AMP, opposes the action of these agents [22]. The level of the compound is also decreased in diabetes and starvation [23]. In general the level of Fru-2,6-P_2 is elevated in situations where glycolytic flux is high and decreased in states where gluconeogenesis is elevated [1–6].

The compound has also been found in all mammalian tissues examined [3,4,24], as well as in yeast [25] and plants [2,26]. While regulation of its level in liver by hormones and the relationship of this regulation to glycolysis and gluconeogenesis has been well established, the role of Fru-2,6-P_2 in regulation of carbohydrate metabolism in other tissues such as muscle and adipose tissue is as yet unclear. Further work in these areas is clearly indicated. The results of the above studies and many others [1–6] suggest that the sugar diphosphate is widely distributed in nature and probably plays a pivotal role in the regulation of 6-phosphofructo 1-kinase and hence glycolysis in many cell types and that it is also significant in the regulation of gluconeogenesis in cells having that capability [1].

CHARACTERIZATION OF 6-PHOSPHOFRUCTO 2-KINASE/FRUCTOSE 2,6-BISPHOSPHATASE

The existence of Fru-2,6-P_2 in liver and other tissues and the observations that the level of this ester can be altered indicates that the enzyme activities responsible for its synthesis and degradation are regulated by hormones and substrates. An enzymatic activity responsible for the synthesis of Fru-2,6-P_2 in rat liver was discovered independently in three laboratories [27–29] and shown to catalyze the reaction shown below



$$\text{Fru-6-P} + \text{ATP} \xrightarrow{Mg^{2+}} \text{Fru-2,6-P}_2 + \text{ADP}$$

The enzyme transfers the γ-phosphate of ATP to the C2 position of Fru-6-P and can therefore be designated a 6-phosphofructo 2-kinase (ATP:D-fructose 6 phosphate 2-phosphotransferase). The enzyme appears to be specific for Fru-6-P, but extensive substrate specificity studies have not yet been completed. This enzyme is distinct from the classical 6-phosphofructo 1-kinase with regard to many properties including molecular weight and kinetic properties [1,5,6]. An enzymatic activity responsible for the degradation of Fru-2,6-P_2 was also discovered independently in three laboratories [30–32] and shown to catalyze the following reaction

$$\text{Fru-2,6-P}_2 + H_2O \rightarrow \text{Fru-6-P} + P_i$$

This enzyme is a fructose 2,6-bisphosphatase, which is completely distinct from the classical fructose 1,6-bisphosphatase with regard to molecular weight and kinetic properties [1,5,6]. It is specific for Fru-2,6-P_2 and does not hydrolyze Fru-1,6-P_2.

Recently, we have shown that a single protein catalyzes both these reactions; ie, the enzyme is bifunctional [30]. This conclusion was based primarily on copurification of both activities to homogeneity. Both activities coeluted from a phosphocel-

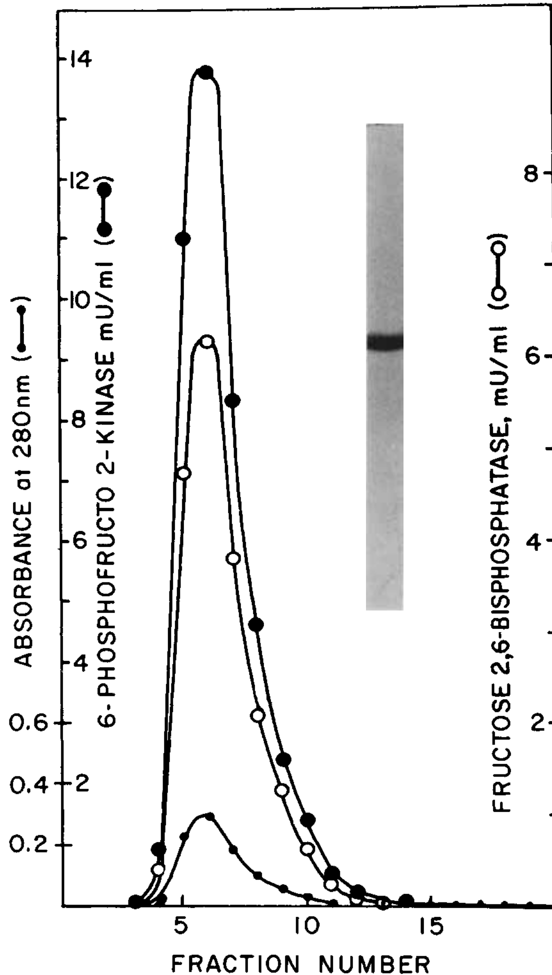


Fig. 3. Copurification of 6-phosphofructo 2-kinase and fructose 2,6-bisphosphatase from phosphocellulose after elution with Fru-6-P. Fractions containing both enzyme activities were applied to a phosphocellulose column and eluted with 2 mM Fru-6-P as described in [25,30,38]. The purified enzyme was subjected to SDS-disc gel electrophoresis. It had a subunit molecular weight of 50,000.

lulose column by Fru-6-P in the last step of the purification scheme, and this enzyme preparation contained a single peptide band (MW 50,000) when analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). Further attempts to separate the two activities on Sephadex G-100 or by sucrose gradient density centrifugation resulted in their coelution with an apparent molecular weight of about 100,000, suggesting that the enzyme is a dimer of two 50,000-dalton subunits. We have designated this enzyme as 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase.

As shown in (Fig. 4A) incubation of the purified enzyme with the catalytic subunit of the cyclic-AMP-dependent protein kinase and [γ-^{32}P]ATP resulted in phosphorylation of the enzyme with about one mol of phosphate incorporated per mol of enzyme subunit [27,30,33]. Figure 4B shows that associated with this phosphoryla tion was an inhibition of the kinase activity and activation of the bisphosphatase

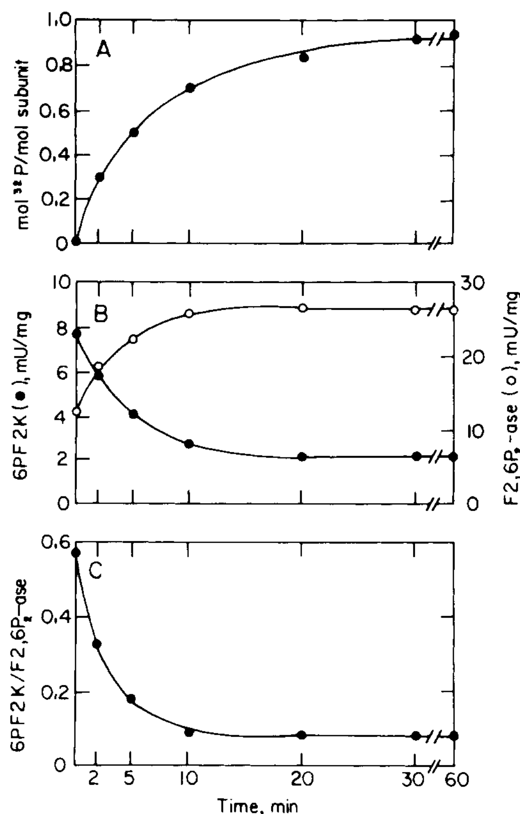


Fig. 4. Cyclic AMP-dependent protein kinase catalyzed phosphorylation of 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase. A) Time course of ^{32}P incorporation from [γ-^{32}P]ATP into the enzyme. B) Time course of the effect of phosphorylation on the activities of the enzyme. C) Time course of the effect of phosphorylation on the ratio of 6-phosphofructo 2-kinase to fructose 2,6-bisphosphatase activity.

activity. The bottom panel (Fig. 4C) shows the effect of this phosphorylation on the ratio of kinase to bisphosphatase activities, indicating a substantial decrease in this ratio after only about 0.3 mol of P_i was incorporated per mol of subunit. This suggests that small increases in protein kinase activity leading to small increases in the phosphate content of the enzyme can result in substantial changes in the balance of the synthetic and degradative activities for Fru-2,6-P_2, and we have demonstrated that this does occur in intact cells [22]. Table I shows the amino acid sequence around the site phosphorylated by protein kinase compared to phosphorylation site sequences of a number of other substrates for the cAMP-dependent protein kinase. The phosphorylated residue is a serine, and like other substrates for the cAMP-protein kinase it contains basic residues N-terminal to the phosphorylated residue. In this case, rather than the usual two or one such residues, the sequence contains three consecutive arginines. This may account for the finding that the K_m for the bifunctional enzyme for protein kinase is lower than that for pyruvate kinase, fructose 1,6-bisphosphatase or 6-phosphofructo 1-kinase from rat liver.

Figure 5 summarizes how this enzyme's activities are regulated by phosphorylation and by various low molelcular weight effectors [1,5,6]. Inorganic phosphate, at

TABLE I. Amino Acid Sequences at the Phosphorylation Sites of Various Gluconeogenic Enzymes and Their Km for Protein Kinase*

Substrate	Sequence	K_m
6-Phosphofructo 2-kinase/ Fru-2,6-P_2ase (Rat liver)	Val-Leu-Gln-*Arg*-*Arg*-*Arg*-Gly-Ser(P)-Ser-Ile-Pro-Gln	10 μM
Pyruvate kinase (Rat liver)	*Arg*-*Arg*-Ala-Ser(P)Val-Ala-Glu-Leu	39 μM
Fructose 1,6-bisphosphatase (Rat liver)	*Arg*/*Lys* -Ser-*Arg*-Pro-Ser(P)-Leu-Pro-Leu-Pro	222 μM
6-Phosphofructo 1-kinase (Skeletal muscle) (Rat liver)	His-Ile-Ser-*Arg*-*Lys*-*Arg*-Ser(P)-Gly-Glu-Ala	230 μM 600 μM

*Data taken from [33].

ALLOSTERIC AND COVALENT REGULATION OF 6-PHOSPHO-FRUCTO 2-KINASE/FRUCTOSE 2,6-BISPHOSPHATASE

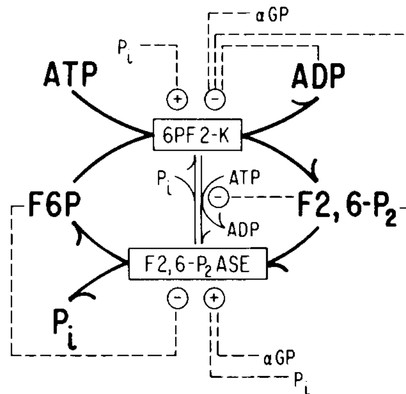
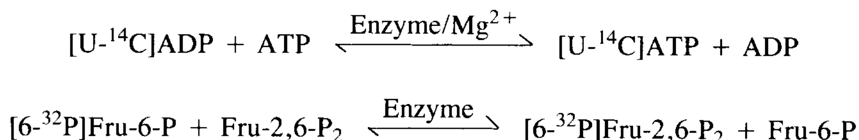


Fig. 5. Regulation of 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase by covalent modification and ligands.

concentrations which exist in the liver cell, activates both the kinase and bisphosphatase. Fru-6-P is a potent product inhibitor of the bisphosphatase, while ADP and Fru-2,6-P_2 are product inhibitors of the kinase activity. α-Glycerol phosphate is an activator of the bisphosphatase but an inhibitor of the kinase and probably acts by competing for Fru-6-P binding sites since it represents the C4-C6 part of the Fru-6-P molecule. Since Fru-2,6-P_2 can only be recycled back to Fru-6-P, regulation of the level of Fru-2,6-P_2 by covalent modification of a single protein represents a very sensitive and efficient mechanism for regulating the level of this compound.

Recent studies on the mechanism of catalysis of the enzyme's reactions suggest that catalysis may involve a two step transfer mechanism that includes a phosphoenzyme intermediate. Support for the existence of a phosphoryl enzyme intermediate

was obtained when we discovered that the enzyme catalyzed the exchange reactions, shown below between ADP/ATP and Fru-6-P/Fru-2,6-P_2 [5,34].



$$[U\text{-}^{14}C]ADP + ATP \xrightleftharpoons{Enzyme/Mg^{2+}} [U\text{-}^{14}C]ATP + ADP$$

$$[6\text{-}^{32}P]Fru\text{-}6\text{-}P + Fru\text{-}2,6\text{-}P_2 \xrightleftharpoons{Enzyme} [6\text{-}^{32}P]Fru\text{-}2,6\text{-}P_2 + Fru\text{-}6\text{-}P$$

The finding of these exchange reactions strongly suggested that a phospho-enzyme intermediate or intermediates are involved in one or both reactions. The existence of such an intermediate was directly demonstrated by the isolation of a ^{32}P-labeled enzyme after incubation with [2-^{32}P]-Fru-2,6-P_2 [5,34,35]. We were able to show that the phospho-enzyme intermediate was acid labile and base stable and, that the labeled amino acid residue was 3-phosphohistidine [35]. We can speculate that the fructose 2,6-bisphosphatase undergoes reversible phosphorylation at N3 of a histidine residue at the active site forming an E-P and Fru-6-P. In the second step, hydrolysis regenerates the free histidine residue forming inorganic phosphate. In addition to providing evidence for a two-step transfer mechanism in the reactions of the enzyme, the finding of a sugar phosphate and adenine nucleotide exchange reaction allows one to distinguish the sugar phosphate and ATP site by following these exchange reactions.

EVIDENCE FOR TWO DISCRETE CATALYTIC SITES ON THE ENZYME

The results of these studies are consistent with one or both of the catalytic mechanisms shown in Figure 6. Either both reactions are catalyzed at a single site (A) or they proceed at two discrete sites (B). We have been fascinated for some time with the question of whether the one- or two-site model is correct. We originally believed that the one-site model was consistent with most of the data and it was a simple and attractive system for explaining our observations. In the last year we have accumulated a large amount of evidence that favors the two-site model. If both reactions occurred at a common site, we should be able to demonstrate E-P formation from Fru-2,6-P_2 and from ATP. However, we have not succeeded in demonstrating E-P formation from ATP [34]. Furthermore, in the case of the one-site model, one would expect that adenine nucleotides such as ATP and ADP would affect the fructose 2,6-bisphosphatase reaction by virtue of their ability to donate or accept phosphate from the common E-P. However, we have not been able to demonstrate any ATP or ADP inhibition of the bisphosphatase reaction [5,34].

Effect of Limited Proteolysis on Enzyme Activities

There are many examples of bifunctional enzymes in the literature, particularly in bacteria, and in general these enzymes have arisen as a result of gene fusion [36,37]. In these cases the two activities can be separated by limited proteolysis. Figure 7 shows the effect of limited proteolysis of the enzyme with thermolysin as a runction of time. Figure 7A is a Coomassie blue-stained SDS gel, while Figure 7B represents an autoradiogram of (A). In this experiment enzyme was either labeled at the phosphorylation site as shown in lane 1 or incubated with [2-^{32}P]Fru-2,6-P_2 to label the bisphosphatase active site and then subjected to SDS-slab gel electrophoresis. The native enzyme phosphorylated in lane 1 by protein kinase migrated in the same

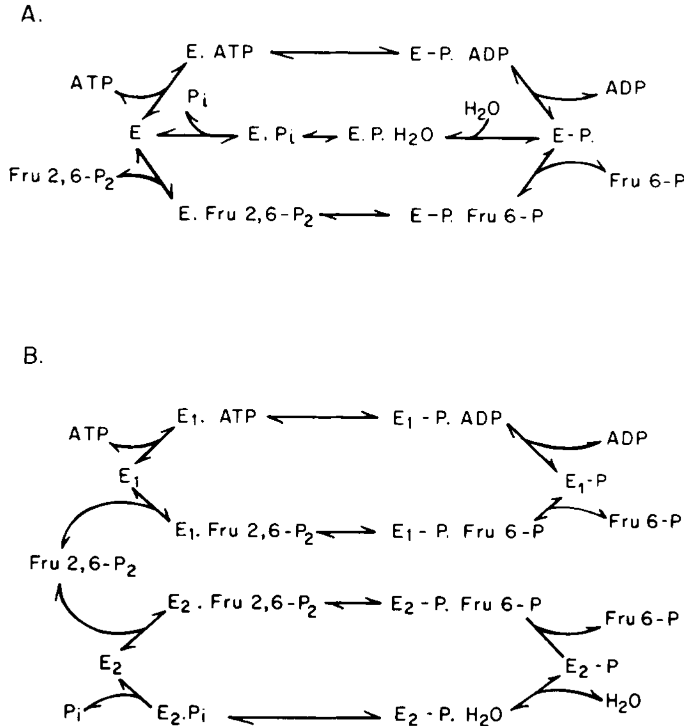


Fig. 6. A one-site (A) or two-site (B) model for catalysis of the 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase reaction.

position as the phosphoryl-enzyme, was coincident with enzyme protein and had a MW of 50,000 (standards in lane 2). Enzyme treated with thermolysin for 2, 5, and 15 min accumulated apppreciable amounts of a 48,000-dalton peptide. Longer digestion resulted in a mixture of ^{32}P-phosphopeptides of molecular weights ranging from 35,000 to 38,000. Labeled active site of the enzyme was seen in all these fragments. These results suggest that the active site remained in the large enzyme core. Figure 8 shows the effect of thermolysin on the two activities as a function of time of digestion. There was a rapid inactivation of the kinase and surprisingly an almost twofold activation of the bisphosphatase seen after 30 min. These findings suggest that the kinase domain has been removed or modified while the bisphosphatase domain remains intact. Inactivation of the Fru-6-P/Fru-2,6-P_2 exchange was similar to that of the kinase shown in Figure 8 (data not shown), suggesting that most of the sugar phosphate exchange occurs at the kinase site [38]. The ATP/ADP exchange was unaffected indicating that only the kinase sugar-phosphate site was removed by thermolysin treatment [38].

Effect of 5′-p-Fluorosulfonylbenzoyl Adenosine

The results of the thermolysin experiments are consistent with the two-site model, with two separate or overlapping domains for the two activities. If the reactions are catalyzed at separate sites it should be possible to inhibit the kinase reaction by interfering with adenine nucleotide binding but to leave the bisphosphatase

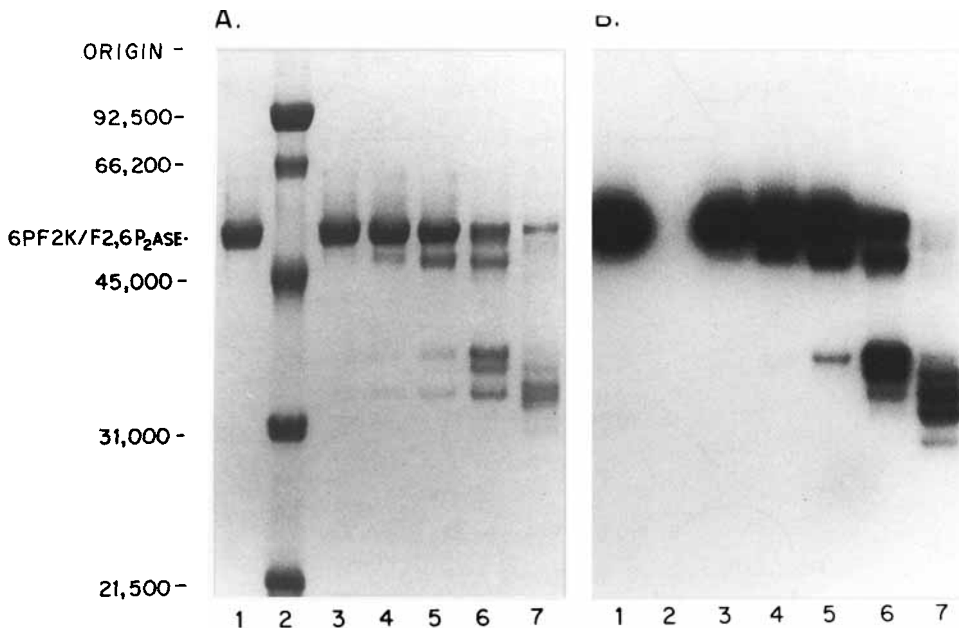


Fig. 7. SDS-polyacrylamide gel electrophoresis after digestion with thermolysin of 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase. The bifunctional enzyme was labeled either by incubation with [2-^{32}P]Fru 2,6-P_2 to label the active site (lanes 3–7) or with protein kinase and [γ-^{32}P]ATP to label the phosphorylation site (lane 1). The enzyme was then digested with thermolysin and aliquots removed at 0,2,5,15, and 30 min of incubation and subjected to electrophoresis (lanes 3–7). A mixture of molecular weight standards were run in lane 2 (phosphorylase B, 92,800; bovine serum albumin, 66,200; carbonic anhydrase, 31,000; and soybean trypsin inhibitor, 21,000). A) Coomassie blue-stained electrophoretogram. B) Autoradiogram of A.

activity unaffected. 5′p-Fluorosulfonylbenzoyl adenosine has been shown to react at nucleotide binding sites and inactivate a number of enzymes [39–41]. The effect of this reagent was tested on the bisphosphatase and kinase activities. The results shown in Table II indicated that only the kinase activity was susceptible to inactivation by the nucleotide analogue.

Effect of Ascorbate/Fe^{3+} in 6-Phosphofructo 2-Kinase/Fructose 2,6-Bisphosphatase

A number of key metabolic enzymes from various sources, many of which contain readily oxidizable groups such as histidine at their catalytic centers, have been reported to be inactivated by mixed-function oxidation [42]. The suggested mechanism of inactivation has been reported to be a site-specific, transition-metal ion-dependent generation of H_2O_2 under aerobic conditions that produce free radicals which in turn attack a histidine or other oxidizable amino acids at the catalytic site [42,43]. Cysteine, methionine, tryptophan, and tyrosine are among the other likely candidates [42]. Since we have shown that 3-phosphohistidine is an intermediate in the reaction mechanism of the fructose 2,6-bisphosphatase reaction the effects of a model system for mixed-function oxidation consisting of ascorbate and Fe^{3+} on the activities of the enzyme were tested [38]. Figure 9 shows the results of a time-course

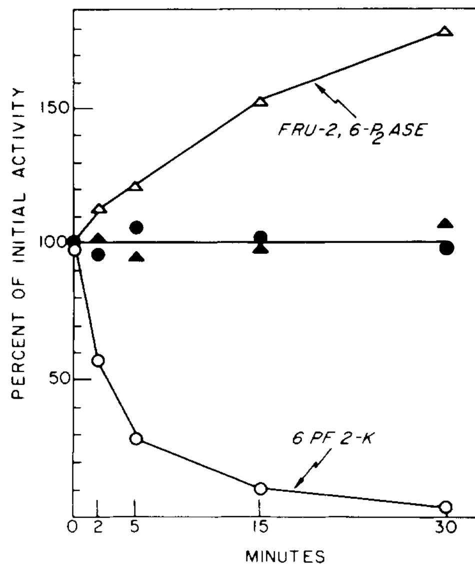


Fig. 8. Time course of the effect of thermolysin on 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase. Conditions were the same as in Figure 7, and aliquots were assayed for 6-phosphofructo 2-kinase and fructose 2,6-bisphosphatase activities.

TABLE II. Effect of 5′-p-Fluorosulfonylbenzoyl Adenosine on 6-Phosphofructo 2-kinase and Fructose 2,6-Bisphosphatase Activities*

	% Inhibition	
Addition	Kinase	Bisphosphatase
DMF	0	0
pFSBA		
0.1 mM	0.2	0
0.2 mM	5.0	0
0.5 mM	48.0	0
1.0 mM	71.0	0
2.0 mM	86.0	0

*Enzyme was incubated for 15 min at 30°C with 5′-p-fluorosulfonylbenzoyl adenosine (pFSBA) or dimethylformamide (DMF) and the enzyme activities assayed as described in the Appendix.

of ascorbate/Fe^{3+} action on kinase activity. There was a rapid inactivation of the kinase with no change in the bisphosphatase activity (data not shown). The presence of ATP, caused a 25% activation of the kinase and completely prevented its inactivation when added before ascorbate/Fe^{3+} or stopped further inactivation when added at 30 min. Also shown are the effects of dithiothreitol on mixed-function oxidation of the kinase. Like ATP, dithiothreitol activated the kinase by 35% and prevented its inactivation by ascorbate/Fe^{3+} but, in contrast to ATP, when added after 30 min it restored the kinase activity to a slightly higher level than that of the native enzyme. The reactivation of the kinase by dithiothreitol clearly suggests that a reversible

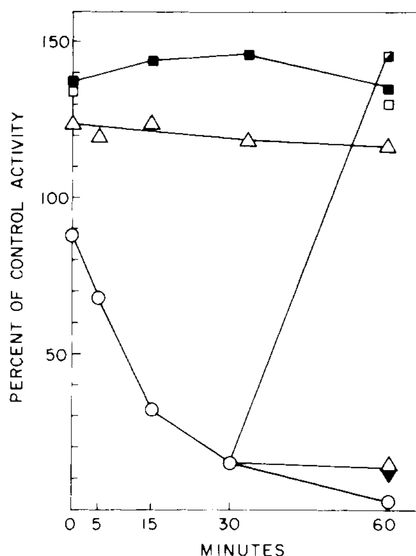


Fig. 9. Effect of ascorbate/Fe^{3+} on 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase activities. Enzyme was incubated with 0.1 mM $FeCl_3$ and 2 mM ascorbate at 30°C with no additions (○), with 5 mM ATP-Mg at zero time or at 30 min (△), or with 5 mM dithiothreitol at zero time (■) or at 30 min (◪). The reaction was terminated by the addition of catalase. 6-Phosphofructo 2-kinase was assayed using the radioactive assay as described in the Appendix. There was no effect of Fe^{3+}/ascorbate on the fructose 2,6-bisphosphatase.

modification of thiol groups was probably taking place at or near the active site. The modification may involve formation of an enzyme disulfide or of sulfenic acid [38]. The inability of ascorbate/Fe^{3+} to affect bisphosphatase activity is an indication that the catalytic histidine residue(s) was not susceptible to ascorbate/Fe^{3+} oxidation, in addition to providing further evidence for a separate catalytic site for the kinase activity.

As with other enzyme systems, the inactivation of 6-phosphofructo 2-kinase by ascorbate and iron probably involves the production of H_2O_2, since the addition of catalase completely inhibited the inactivation [38]. Furthermore, incubation of the enzyme with H_2O_2 was found to inhibit the kinase in a manner similar to that seen with ascorbate/Fe^{3+}, and the activity could also be protected from, and restored after, oxidation by dithiothreitol [38].

Ascorbate/Fe^{3+} treatment also inhibited the Fru-6-P/Fru-2,6-P_2 exchange of the bifunctional enzyme in the absence but not in the presence of dithiothreitol [38]. The inactivation of the sugar-phosphate exchange by ascorbate/Fe^{3+} oxidation correlated well with the inactivation of the kinase (Fig. 9) and was restored after oxidation by dithiothreitol. Thus, the mixed-function oxidation result reinforces our conclusion that sugar-phosphate exchange occurs at the kinase site. Ascorbate/Fe^{3+} had no effect on the ADP/ATP exchange [38].

Ascorbate/Fe^{3+} oxidation was similar to thermolysin treatment in that the inactivation of the kinase and Fru-6-P/Fru-2,6-P_2 exchange may have been due to a large decrease in the affinity of a Fru-6-P site on the enzyme. The reversibility of

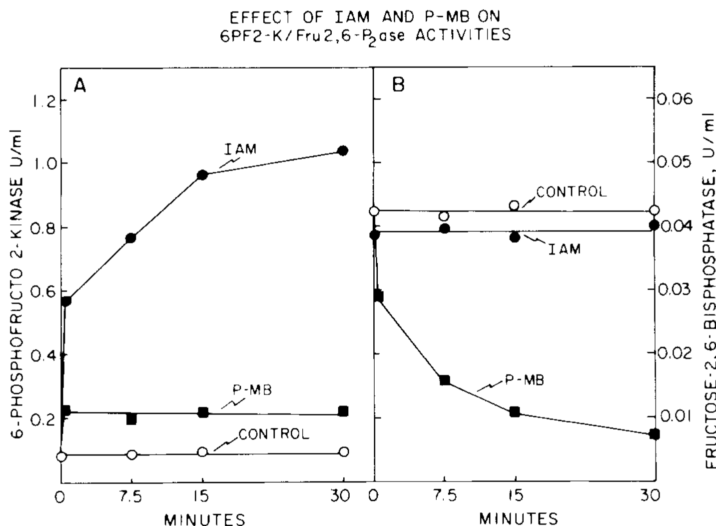


Fig. 10. Effect of sulfhydryl agents on the 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase activities. The bifunctional enzyme was incubated with increasing concentrations of iodoacetamide (IAM) and p-meribenozoate (P-MB) and the kinase (A) and bisphosphatase (B) activities measured as described in the Appendix. Both activities were measured with saturating substrate concentrations.

oxidative-inhibition suggests a role of cysteine in that affinity. The results in both instances are consistent with a two-site model.

Effect of Sulfhydryl Modifying Agents

The results with ascorbate/Fe^{3+} suggest an essential role for sulfhydryl groups in the kinase reaction of the bifunctional enzyme. If this is true, it would be expected that some of the more traditional sulfhydryl modifying agents would have differential effects on the two activities of the enzyme. N-ethylmaleimide treatment inactivated the kinase but had no effect on the fructose 2,6-bisphosphatase [44]. Figure 10 shows the effect of iodoacetamide and p-mercuribenzoate on the kinase and bisphosphatase activity. Iodoacetamide caused a tenfold activation of the V_{max} of the kinase but had no effect on the bisphosphatase activity. In contrast, p-mercuribenzoate caused a rapid inhibition of the bisphosphatase while slightly enhancing the kinase. In the case of iodoacetamide we were able to identify the residues modified as cysteines since only carboxymethylcysteines appeared in protein hydrolyzates with no alterations in lysine,

TABLE III. Some Examples of Bifunctional Enzymes*

1) Pathways for the synthesis of aromatic amino acids in E coli [37] and Neurospora crassa [52]
2) Histidinol dehydrogenase/histidinol-phosphate aminotransferase, histidine operon in Salmonella typhimurium [36] experimentally induced
3) Asparate kinase I/homoserine dehydrogenase I—E coli [53]
4) Glucose 6-Phosphatase—Rat liver and brain [50]
5) Glutamine-synthetase adenylyltransferase—E coli [45]
6) Regulatory protein P_{II} uridylyltransferase of the E coli glutamine-synthetase cascade system [46]
7) Isocitric dehydrogenase kinase/phosphatase—E coli [47]
8) 6-Phosphofructo 2-kinase/fructose 2,6-bisphosphatase—rat liver [27,30,38]

*No attempt has been made to be all-inclusive.

histidine, or methionine content [44]. The affinity of the carboxyaminomethylated enzyme for Fru-6-P was greatly decreased and the reaction became almost entirely dependent on inorganic phosphate [44]. Preliminary results indicate that the native enzyme is labeled with [1-^{14}C]-iodoacetamide to the extent of 2 mol per mol of subunit, and that alkylation of these freely accessible thiol groups, critical for Fru-6-P binding or transfer, is involved. Apparently, alkylation of these groups results in a conformational change that actually enhances the reaction rate, suggesting that there is a negative regulatory influence on the kinase reaction. Whether this group is the same as that modified by the mixed function oxidation system, is under investigation. The striking effects of both mixed function oxidation and iodoacetamide on kinase activity strongly suggest that the affected group(s) are associated with the active site. The iodoacetamide effect is one of the few instances of activation of enzyme reaction rate by a sulfhydryl-reagent.

CONCLUSIONS

The differential effects of various protein modification agents and limited proteolysis are most consistent with a two-site model for catalysis of the two reactions and this is currently our working hypothesis. The most striking finding of these studies was the apparent essential role of sulfhydryl groups in the sugar phosphate affinity in the kinase reaction. It is possible that the kinase activity in intact cells is regulated in part by sulfhydryl modification, perhaps via the oxidized/reduced glutathione couple, methylation, or a cellular mixed function oxidation system. However, there is at present no evidence for such regulation, though it remains an intriguing possibility. It is more likely that the effects of mixed-function oxidation and iodoacetamide will turn out to be useful in further defining structure/function relationships in the enzyme, particularly once the primary structure is known and various active and regulatory sites can be placed in this sequence. In addition, the tenfold activation of the V_{max} of the kinase will be very useful in studies on the stereochemistry of this reaction which will require the production of large amounts of product.

While the data presented here are consistent with the two site model, it is not possible to rule out completely the one-site model, which predicts a single phosphocarrier, capable of transferring phosphate from ATP or Fru-2,6-P_2 to Fru-6-P or H_2O, in the kinase or bisphosphatase reactions, respectively [34]. In this case two Fru-6-P binding sites would be necessary: one, the kinase and sugar phosphate exchange acceptor, which is lost during proteolysis and oxidation; and the other, the bisphosphatase inhibitory site, which is not. Alternatively, a single Fru-6-P site may be altered after proteolysis or oxidation such that it no longer accepts phosphate in the kinase reaction but can still inhibit the bisphosphatase [38]. This question can only be resolved when the number of Fru-6-P binding sites are known as the active site(s) can be identified and placed in the primary structure.

Although the bifunctionality of 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase is unusual, it is by no means unique, as shown in Table III. Other examples include the activities responsible for adenylation and deadenylation of glutamine synthase in E coli [45], the enzyme catalyzing the uridylylation and deuridylylation of the regulatory protein P_{II} in the E coli glutamine synthase cascade [46], and the enzyme catalyzing phosphorylation and dephosphorylation of E coli isocitric dehydrogenase [47]. All four of these enzymes play an important role in the control of

metabolism. Many other bifunctional or multifunctional proteins have been reported [48,49], particularly with regard to amino acid catabolism in bacteria [37], but these enzymes do not catalyze opposing reactions, with the exception of glucose 6-phosphatase [50], and/or are not regulatory in nature. Glucose 6-phosphatase is also a multifunctional catalyst which has both phosphotransferase and phosphohydrolase activity [50], though the physiological significance of the former activity is still uncertain. The reaction mechanism involves the formation of a phosphoryl enzyme intermediate, which has been shown to be 3-phosphohistidine [51]. It is possible that 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase belongs to a distinct but small class of enzymes—those that catalyze opposing physiologically relevant, regulatory reactions at discrete catalytic sites. This group would include 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase, isocitric dehydrogenase kinase/phosphatase, and adenylyl transferase and perhaps the uridylyltransferase of the E coli glutamine synthase cascade. While the uridylyltransferase has been purified to homogeneity and shown to be bifunctional, the presence of two active sites has not yet been demonstrated [46]. A number of other bifunctional enzymes can probably be placed in this class, and it is probable that others will be discovered in the future.

Nordlie [48] has recently defined a class of multifunctional enzymes involving branched reaction pathways. These are group-transfer enzymes of ping-pong mechanism with covalently bound enzyme-group intermediates capable of donating the group to more than one acceptor. To the extent that 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase has an ATPase activity in the absence of added Fru 6-P it may fit the Nordlie definition. If 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase had only one catalytic site, it would clearly conform to a multifunctional catalyst as defined by Nordlie. However, the presence of two sites with opposing functions does not fit easily into the Nordlie definition which is based primarily on kinetic considerations. The advantage of having two opposing activities physically associated is uncertain but with regard to 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase it is an efficient mechanism for regulating both activities by phosphorylation and ligand interaction. It seems reasonable to propose that the bisphosphatase site arose from the kinase site by gene duplication which left a fused product. Use of the modern methods of molecular genetics should allow us to ascertain the significance of this bifunctional structure and to define the relationship between the kinase and bisphophatase domains. Determination of the primary sequence by direct amino acid sequencing methods and indirect cloning methods is in progress.

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APPENDIX: ENZYME PURIFICATION AND ASSAYS

Purification of rat liver 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase to homogeneity was accomplished as previously described [27,30,38].

In the course of this study two methods have been used to measure the activity of 6-phosphofructo 2-kinase activity. In the first method, the enzyme was incubated with Fru-6-P and ATP and the reaction was terminated by the addition of 0.25 N NaOH followed by heating at 90°C for 30 min. The pH was readjusted to 7 to 8 with 1 N acetic acid, and the amount of Fru-2,6-P_2 formed was determined by the 6-phosphofructo 1-kinase activation assay [1,11]. The maximal activity of the enzyme was measured with 5 mM Fru-6-P and 2 mM ATP. In the second method, fructose 6-phosphate was incubated with Mg-[γ-^{32}P]ATP and 6-phosphofructo 2-kinase in order to generate [2-^{32}P]Fru-6-P. The sample was then made 0.25 N in NaOH and heated at 90°C for 30 min. Alternatively, the excess [γ-^{32}P]ATP was removed by charcoal treatment [5], and the ^{32}P-radioactivity in Fru-2,6-P_2 was counted. The sample was then diluted to 10 ml with 20 mM Triethylamine (TEA)-HCO_3, pH 8.2, and applied to a diethylaminoethyl (DEAE)-Sephadex column (0.7 × 2 cm) equilibrated in the same buffer. Degradation products of Fru-6-P were removed by washing the column with 265 mM TEA-HCO_3, pH 8.2. The [2-^{32}P]fructose 2,6-bisphosphate was then the eluted with 500 mM TEA-HCO_3, pH 8.2, and aliquots of eluate counted. Blanks without enzyme were run in parallel with the samples.

Two methods were used to measure fructose 2,6-bisphosphatase activity. In the first method, fructose 2,6-bisphosphatase was incubated with 20 mM TES (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, and 10 μM fructose 2,6-bisphosphate. Aliquots of the incubation were removed at 0, 5, 15, 30, and 60 min, made 0.25 N in NaOH, and heated at 90°C for 30 min. The samples were then neutralized to pH 7–8 with acetic acid and assayed for Fru-2,6-P_2 with the 6-phosphofructo 1-kinase activation assay.

In the second method, the enzyme was incubated with various concentrations of [2-^{32}P]Fru-2,6-P_2 [800 cpm/pmol] in the presence of 20 mM TES (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM dithiothreitol for varying periods of time. The reaction was stopped by adding NaOH to a final concentration of 0.25 N, and the reaction mixture diluted tenfold with 20 mM TEA-HCO_3, pH 8.2. The mixture was then immediately applied to a DEAE-Sephadex column (0.7 × 5 cm) and washed with 2 column volumes of 200 mM TEA-HCO_3, pH 8.2, to elute the labeled inorganic phosphate which was collected directly into scintillation vials and counted as Cerenkov radiation. The remaining, unhydrolyzed fructose 2,6-[2-^{32}P]bisphosphate could be eluted from the column with 600 mM TEA-HCO_3, pH 8.2.

Labelled fructose 2,6-bisphosphate was prepared by first converting carrier-free ^{32}P-inorganic phosphate (10 mCi) to [γ-^{32}P]ATP enzymatically. The labeled substrate was then prepared by incubating 5 mM fructose 6-phosphate and 0.1 mM [γ-^{32}P]ATP (5,00–10,000 cmp/pmol) in the presence of 20 mM TES (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.2 mU of a homogeneous preparation of rat liver 6-phosphofructo 2-kinase for 120 min. The reaction mixture was made 0.25 N with NaOH and heated at 90°C for 30 min, then cooled, and nucleotides removed by charcoal treatment, diluted tenfold in cold 20 mM TEA-HCO_3, pH 8.2, and applied to a DEAE-Sephadex (1 × 10 cm) column equilibrated in the same buffer. The material was eluted from the column with a linear gradient of 20–600 mM TEA-HCO_3 and labelled fructose 2,6-bisphosphate eluted at 375 mM TEA-HCO_3.