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FRUCTOSE-2,6-BISPHOSPHATE

Simon J. Pilkis

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Fructose-2,6-Bisphosphate

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INTRODUCTION

Fructose-2,6-bisphosphate was discovered in the course of studies on the hormonal regulation of rat hepatic 6-phosphofructo-1-kinase by the laboratory groups of Hers, Pilkis, and Uyeda. In the first chapter Hers describes the exciting discovery of the compound in his laboratory. Subsequent to its discovery, the properties and regulatory effects of fructose-2,6-bisphosphate on 6-phosphofructo-1-kinase and fructose-1,6-bisphosphatase and the hormonal regulation of fructose-2,6-bisphosphate levels in liver were characterized in great detail. The chapters by Kemp and Marcus and by Liu and Fromm discuss the mechanism whereby fructose-2,6-bisphosphate exerts its effects on these enzymes. The question of whether fructose-2,6-bisphosphate binds to the substrate site or to a separate allosteric site on fructose-1,6-bisphosphatase has been controversial and is discussed in detail in these chapters. The chapter contributed by Dr. Claus and colleagues discusses those structural properties of fructose-2,6-bisphosphate which confer on it its potency as an activator of 6-phosphofructo-1-kinase and as an inhibitor of fructose-1,6-bisphosphatase. Most of the structural analogs and their relative activities as activators and/or inhibitors have not been heretofore reported.

Second in importance only to the discovery of fructose-2,6-bisphosphate itself was the finding that the enzyme responsible for determining its steady state level in liver was bifunctional, and that the kinase and bisphosphatase reactions were regulated in a reciprocal manner by cyclic-AMP-dependent phosphorylation. This unique protein has been extensively studied; its structure, regulation, and the molecular basis for its catalysis have now been delineated. The current state of knowledge about this enzyme is summarized by El-Maghrabi and co-workers. Great strides have been made in defining the relationship between the hepatic kinase and bisphosphatase domains of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. The kinase occupies the amino terminal half of the enzyme subunit, while the bisphosphatase occupies the carboxyl terminal half. The reaction mechanism of the bisphosphatase involves the formation of a phosphoenzyme intermediate which has been shown to be phosphohistidine. Sequencing of the bifunctional enzyme also revealed that the bisphosphatase domain was evolutionarily related to yeast phosphoglycerate mutase, while the 6-phosphofructo-2-kinase domain may be analogous to *E. Coli* 6-phosphofructo-1-kinase. These findings suggest the bifunctional enzyme arose as a result of a gene fusion event. The relationship between the bisphosphatase and mutases and between the hepatic 6-phosphofructo-2-kinase and *E. coli* 6-phosphofructo-1-kinase and some further speculation on evolution of these proteins are found in the chapter by Bazan and Fletterick. It is possible that the hepatic bifunctional enzyme belongs to a distinct but small class of enzymes — those that catalyze opposing, physiologically relevant, regulatory reactions at discrete catalytic sites. The significance of enzyme bifunctionality is discussed by La Porte. In ad-

dition, Bazan and Fletterick point out a very interesting structural and functional similarity of a general class of acid phosphatases with the mutases and fructose-2,6-bisphosphatase.

During the time of the discovery of fructose-2,6-bisphosphate, a number of groups had shown that hormones that acted on hepatic glycolysis and gluconeogenesis via changes in cAMP affected substrate cycling at the fructose 6-phosphate/fructose 1,6-bisphosphate level. The discovery of the compound, characterization of its effects on both enzymes of this cycle, and elucidation of the acute regulation of this cycle by hormones completed the picture with regard to regulation of glycolysis/gluconeogenesis in liver. Prior to this, the pyruvate/phosphoenolpyruvate substrate cycle was believed to be the major locus of hormone action. The site of regulation at this level was pyruvate kinase, the activity of which was shown to be modulated by cAMP-dependent protein kinase-catalyzed phosphorylation. The demonstration that the enzyme activities involved in the synthesis and degradation of fructose-2,6-bisphosphate were also modulated by cAMP-dependent protein kinase-catalyzed phosphorylation established two enzyme systems, one at each hormonally regulated substrate cycle, which acted in a concerted fashion to provide a coordinated control system. It is now firmly established that fructose-2,6-bisphosphate plays a key role in the regulation of carbohydrate metabolism in liver, and this is the subject of the chapter by Van Schaftingen.

While a great deal is known about fructose-2,6-bisphosphate metabolism and the bifunctional enzyme in liver, much less is known on extrahepatic tissues where bidirectional flux does not occur. As reviewed by Hue and coworkers, fructose-2,6-bisphosphate has effects on glycolysis in a number of extrahepatic tissues and in cell culture systems. Although our knowledge of the significance of the role of fructose-2,6-bisphosphate in the regulation of extrahepatic carbohydrate metabolism is still fragmentary, it is clear from their review that several forms of fructose-2,6-bisphosphate metabolizing enzymes occur in these tissues, and that the forms are distinctly different from the hepatic enzyme, particularly with regard to their regulation.

Fructose-2,6-bisphosphate plays a role in carbohydrate metabolism of nonmammalian systems as well. Macdonald and Buchanan review its role in plants, including its effects on sucrose synthesis, carbon partitioning, and respiration. Holzer reviews its role in yeast and Van Schaftingen and colleagues do the same in even more primitive systems. In all these systems, there are again a number of different forms of fructose-2,6-bisphosphate-metabolizing enzymes, but, as reviewed by Huber for plants, their relationship to each other and to the liver enzyme is currently unknown.

This monograph was intended to present the current state of our knowledge about fructose-2,6-bisphosphate, its metabolism, and its effects. This appears to be a particularly propitious time for such a volume since we now understand the role of this metabolite in the regulation of hepatic metabolism, and the structure and regulation of the unique bifunctional enzyme responsible for its

turnover has been elucidated. At this point in time, research in this area appears poised for another major advance, and Pilkis summarizes briefly in the last chapter the future avenues for research in fructose-2,6-bisphosphate as well as metabolic regulation in general. It is hoped that this book will inspire others to continue to expand our knowledge in these areas in the future, using new techniques on both old and new systems.

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THE EDITOR

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Dr. Pilkis received his Ph.D. in Physiology from the University of Chicago, Illinois, in 1969. In 1971 he obtained his M.D. degree, also from the University of Chicago. After a postdoctoral fellowship with Dr. Asher Korner at the University of Sussex in England he spent a year as a Visiting Investigator of the Howard Hughes Medical Institute, in the Department of Physiology at Vanderbilt University School of Medicine, Nashville, Tennessee. From 1972 to 1986 he served on the faculty of the Department of Molecular Physiology and Biophysics and was a Howard Hughes Investigator at Vanderbilt University from 1972 to 1982. He joined the faculty of SUNY at Stony Brook as Chairman of the Department of Physiology and Biophysics in the School of Medicine, in 1986.

Dr. Pilkis is a member of the American Society for Biochemistry and Molecular Biology, the American Chemical Society, the New York Academy of Sciences, American Association for the Advancement of Science, and the American Diabetes Association. He has been the recipient of numerous grants from the U.S. Public Health Service.

Dr. Pilkis has been an invited lecturer at both National and International Meetings as well as presenting numerous guest lectures at Universities and Institutes. He has authored or co-authored more than 130 scientific publications. His major research interests include hormonal regulation of hepatic gluconeogenesis/glycolysis and the molecular mechanisms governing the catalysis and regulation of 6-phosphofructo-2-kinase, fructose-2,6-bisphosphatase, fructose-1,6-bisphosphatase, and 6-phosphofructo-1-kinase.

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Chapter 1

HISTORICAL PERSPECTIVE AND DISCOVERY OF FRUCTOSE-2,6-BISPHOSPHATE

Henri-Géry Hers

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I. THE CONTROL OF GLYCOLYSIS AND GLUCONEOGENESIS

Fructose-2,6-bisphosphate (Fru-2,6-P_2) was identified in 1980[1-3] as the positive effector of phosphofructokinase that is normally present in the liver of a fed animal, but disappears during fasting and under the action of glucagon, allowing gluconeogenesis to take over. Its discovery was, therefore, the outcome of a long series of investigations devoted to the clarification of the mechanism by which glucagon inhibits glycolysis and stimulates gluconeogenesis in the liver. Since Fru-2,6-P_2 is not only a potent stimulator of phosphofructokinase but also an inhibitor of fructose-1,6-bisphosphatase (Fru-1,6-P_2ase),[4,5] its most obvious action is to favor the formation of Fru-1,6-P_2 from fructose-6-phosphate (Fru-6-P) and to prevent the reverse conversion.

The effect of glucagon to stimulate gluconeogenesis was first reported in 1963 by Schimassek and Mitzkat,[6] who precisely described a rise in the concentration of glucose-6-phosphate (Glu-6-P) and Fru-6-P and a remarkable fall in that of Fru-1,6-P_2 in the perfused livers from fed rats. If, as critically stressed by Ross et al.,[7] the rise in hexose-6-phosphate could be explained by an increased rate of glycogenolysis, this was not the case for the decrease in Fru-1,6-P_2. A similar drop in Fru-1,6-P_2 was repeatedly confirmed, thereafter (reviewed in Reference 8).

It is clear, however, that, if the disappearance of Fru-2,6-P_2 has to explain the effect of glucagon to stimulate gluconeogenesis, this can only be true for conditions under which it was initially present in the liver. This is the case in the livers of well-fed animals or, after fasting, in liver preparations incubated in the presence of substrates, like glucose, fructose (below but not above 5 m*M*), or dihydroxyacetone, which favor its formation.[9,10] By contrast, Fru-2,6-P_2 disappears from the liver in the presence of glycerol[9,11] or fructose at concentration above 5 to 10 m*M*,[9,10] explaining the absence of effect of glucagon on gluconeogenesis from these substrates. Indeed, the failure to demonstrate an effect of glucagon on gluconeogenesis from 20 m*M* fructose or 10 m*M* glycerol[7,12] retarded the recognition of the Fru-6-P/Fru-1,6-P_2 interconversion as a site of action for glucagon. It was only in 1971 that Veneziale[13,14] clearly established that glucagon actually stimulates gluconeogenesis from fructose (2 m*M*) and trioses in the perfused liver. Like glucagon but more slowly, ethanol causes the disappearance of Fru-2,6-P_2[11,15] and can, therefore, mimick some effects of the hormone.[16]

II. THE MODE OF ACTION OF GLUCAGON

It has been recognized for a long time that glucagon exerts its action via the formation of cyclic adenosine monophosphate (cAMP) and the activation of (cAMP)-dependent protein kinase, and this rule applies to the control of gluconeogenesis in the liver.[17] Changes in metabolite concentration revealed

that there are most likely two points of impact of the hormone (reviewed in References 8, and 18a). One of them is at the level of the pyruvate/P-enolpyruvate cycle and the other at the level of the Fru-6-P/Fru-1,6-P_2 interconversion. The mechanism of the first of these effects received an adequate explanation when Engström discovered in 1974 (reviewed in Reference 19) that liver pyruvate kinase is a substrate for cAMP-dependent protein kinase and that its phosphorylation causes its inactivation. One effect of glucagon is, therefore, to arrest glycolysis at the level of P-enolpyruvate of which it favors the conversion to glucose. The clarification of the second crossover, initially observed by Schimassek and Mitzkat,[6] has been more laborious. Its solution came from studies of the Fru-6-P/Fru-1,6-P_2 futile cycle and of the kinetic properties of its constitutive enzymes.

III. THE FRUCTOSE-6-PHOSPHATE/FRUCTOSE-1,6-BISPHOSPHATE FUTILE CYCLE

The presence in the liver of both 6-phosphofructo-1-kinase (6PF-1-K) and Fru-1,6-P_2ase implies that either the activity of these two enzymes is controlled by an on/off mechanism which renders one of them inactive when the other is active, or that they are both simulaneously active, causing a futile recycling of metabolites.

Various isotopic procedures have been used to measure the Fru-6-P/Fru-1,6-P_2 recycling. Several of them were based on the formation of $[^3H]H_2O$ from glucose labeled with ^3H at various positions. These methods were either grossly in error or not sufficiently precise to allow reliable conclusions to be drawn (reviewed in Reference 20). Another method, developed by Rognstad and Katz,[16] and based on the randomization of carbons in glucose formed from [1-^{14}C]galactose, gave reliable semiquantitative information. It clearly indicated that recycling between Fru-6-P and Fru-1,6-P_2 occurs in the fed but not in the fasted animal.[16,21] Since gluconeogenesis is known to operate under both conditions, this observation allowed one to reach the important conclusion that phosphofructokinase is inactive in the liver during fasting.[21] The futile recycling occurring in the fed animal, despite the presence of Fru-2,6-P_2, is now understood by the fact that the inhibition of Fru-1,6-P_2ase by Fru-2,6-P_2, which is competitive, is incomplete in the presence of a high concentration of substrate; furthermore, the sensitivity of phosphofructokinase towards Fru-2,6-P_2 is greater than that of Fru-1,6-P_2ase. The control at this level is, therefore, an incomplete on/off mechanism. The biological significance of this futile cycle has been discussed elsewhere.[18,18a]

IV. THE CONTROL OF PHOSPHOFRUCTOKINASE ACTIVITY BY SUBSTRATES AND METABOLITES

There is a general agreement in the literature that a major point of control

of glycolysis is at the level of phosphofructokinase. The activity of this enzyme is regulated by the concentration of its two substrates and of numerous metabolites, which act as positive or negative effectors.[22] One substrate, adenosine triphosphate (ATP), acts as a negative allosteric effector, which induces a marked cooperativity for the second substrate, Fru-6-P. The latter acts as a positive effector, which relieves inhibition by ATP. Other important positive effectors are adenosine monophosphate (AMP), glucose-1,6-bisphosphate (Glu-1,6-P_2), Fru-1,6-P_2, NH_4^+, and P_i, whereas citrate and H^+ act negatively. The concentration of AMP is expected to rise and that of ATP to decrease during anoxia and to be important factors in the Pasteur effect. Citrate concentration is high when fats are oxidized, explaining that their oxidation is preferential to that of glucose in many tissues.

It is remarkable that, soon before the discovery of Fru-2,6-P_2, Reinhart and Lardy[23] had reached the conclusion that, at the concentration of substrates and effectors known to be present in the liver, phosphofructokinase would be completely inactive, even in the fed state. It is also of interest to recall that, as early as in 1974, Katyare and Howland[24] had observed an altered allosteric control of phosphofructokinase in the livers of genetically obese mice, the enzyme being less inhibited by excess ATP than normally. This situation was later explained by a greater than normal concentration of Fru-2,6-P_2 in these livers.[25,26]

V. A PHOSPHOFRUCTOKINASE STABILIZING FACTOR

Dunaway and co-workers[27-30] have concluded that the decrease in liver phosphofructokinase-L_2 (the major isoenzyme of phosphofructokinase in the liver) in fasting and diabetes is the result of an increased degradation rate, with little or no change in synthetic rate. They also reported that the livers of fed rats contain a phosphofructokinase-stabilizing factor, believed to be a polypeptide for which a molecular weight of 3800 was determined by gel filtration. This factor protected phosphofructokinase-L_2 against thermal or enzymic inactivation and was also a stimulator of the enzyme, since it increased its affinity for Fru-6-P. It was unstable at slightly acid pH, and it was also an inhibitor of Fru-1,6-P_2ase.[29] Its concentration in the liver was inversely proportional to the rate of phosphofructokinase degradation. It was therefore proposed that insulin regulates the rate of degradation of phosphofructokinase-L_2 by controlling the level of the stabilizing factor.[30]

Fru-2,6-P_2 is not only a potent stimulator of liver phosphofructokinase, but has also a remarkable ability to protect phosphofructokinase against thermal or enzymic inactivation[11,31,32] and, like the stabilizing factor, is an inhibitor of Fru-1,6-P_2ase.[4-5] Furthermore, it appears to have an abnormal behavior upon gel filtration when performed at low ionic strength (the procedure used for the determination of the molecular weight of the stabilizing peptide), being then eluted in the molecular weight range of 3000 to 4000.[33] From the work

of Van Schaftingen and Hers,[34] it appears highly probable that the phosphofructokinase stabilizing factor was actually Fru-2,6-P_2.

VI. THE CONTROL OF PHOSPHOFRUCTOKINASE ACTIVITY BY GLUCAGON

As early as in 1972, Taunton et al.[35,36] reported that the i.v. administration of glucagon to rats caused a remarkable activation of Fru-1,6-P_2ase and an inactivation of both phosphofructokinase and pyruvate kinase, whereas insulin had the opposite effect. These enzymatic changes were observed not only in the liver but also in the renal cortex, skeletal muscle, and epididymal fat. Although the effect of glucagon on the liver enzymes is in general agreement with what is now well established, the data of Taunton et al. were never confirmed, and it is doubtful that they could easily be reproduced. Indeed, the changes in enzymatic activities due to glucagon can only be demonstrated under suboptimal assay conditions, an important point that was not specified by the authors. Furthermore, the reported short-term insulin effect is rather unique in the literature and its uncritical report greatly decreased the credibility of the work. The paper therefore had little impact and is only indicative of how difficult the *in vivo* approach of biochemical regulation can be. In 1976, Veneziale[37] also briefly mentioned an inactivation of phosphofructokinase by glucagon in isolated hepatocytes, but no data were presented.

In 1979, four groups of investigators[38-41] reported that the activity of liver phosphofructokinase could be greatly decreased by treatment of hepatocytes with glucagon; the hormonal effect was similar to that of a negative effector, i.e., an decrease in affinity for Fru-6-P and an increased inhibition by ATP with no change in V_{max}. Castaño et al.[38] as well as Kagimoto and Uyeda[39] reported that these modifications persisted after purification of the enzyme, indicating a stable change due to a covalent modification of the protein. This view was reinforced by further work of Kagimoto and Uyeda[42] who concluded that phosphorylation of phosphofructokinase by cAMP-dependent protein kinase was responsible for its inactivation.

In 1980, it became apparent, from the work of several groups, that the effect of glucagon on phosphofructokinase was not stable, but could actually be mimicked by gel filtration or partial purification of the enzyme. Van Schaftingen et al.[1] isolated a low-molecular-weight positive effector of phosphofructokinase by ultrafiltration or gel filtration of a liver extract obtained from a fed animal. The concentration of this effector could be accurately measured by its stimulatory effect on phosphofructokinase when measured at low Fru-6-P concentration. This effector had a molecular weight, as determined by filtration on Biogel P-2 comprised between 400 and 600, and was destroyed within a few minutes in the presence of 0.3 *M* trichloroacetic acid, even at 0°C; it was also destroyed upon incubation in the presence of purified alkaline phosphatase, indicating that it was an extremely acid labile phosphate