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Review

Allosteric regulatory properties of muscle phosphofructokinase

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Summary

We have reviewed the allosteric regulatory properties of skeletal muscle phosphofructokinase and recent results on the phosphorylation of this enzyme. The number and affinities of various ligand binding sites are described, and a simple three state model is presented to explain the kinetic and ligand-binding properties of the enzyme. Data describing a lack of fit to a concerted transition model are presented. The widespread occurrence of partial phosphorylation of phosphofructokinase at a specific site near the carboxyl terminus is documented, as well as the lack of significant kinetic consequences of such phosphorylation.

Introduction

Phosphofructokinase is an enzyme of central importance to the regulation of carbohydrate metabolism and has been studied extensively over the last 15 years. It is subject to regulation by a considerable number of allosteric regulators. Three isozymic protomer forms of phosphofructokinase in mammals have been described along with various hybrids of these three forms. Although phosphofructokinase isozymes differ in their sensitivity to various allosteric effectors, the basic patterns of response are similar. Mammalian muscle phosphofructokinases have a protomer molecular weight in the range of 80–85 000; the smallest active form is a tetramer. Most of these properties have been reviewed in recent years; Uyeda has reviewed both procaryote and eucaryote phosphofructokinase (1), and Goldhammer and Paradies have reviewed phosphofructokinase structural properties (2).

The present review will principally cover skeletal muscle phosphofructokinase, with emphasis on recent developments, and will focus, for the most part, on work from our own laboratory. We will describe: 1) recent results on the interaction of effectors with the enzyme, and 2) data relating to the phosphorylation of phosphofructokinase.

Effector binding sites and the regulation of activity

At pH 8.0 the enzyme displays typical Michaelis-Menten kinetics with respect to both its substrates, ATP and fructose-6-P. Below pH 7.5, however, the enzyme shows a strong sigmoid dependence on fructose-6-P concentration and inhibition by ATP at high concentrations. Fructosebisphosphates, AMP, ADP, cyclic-AMP, and P_i antagonize inhibition by ATP, whereas citrate, phosphenolypyruvate and 3-phosphoglycerate are inhibitors that act synergistically with ATP (1). The activators K^+ and NH_4^+ increase V_{max} without greatly affecting inhibition by ATP.

Table I summarizes results from a number of studies (3-6, unpublished work) on effector binding by phosphofructokinase. The numbers for Kd's are those that were obtained under rather restrictive conditions; that is, usually at protein concentrations far exceeding that seen in kinetic assays and usually an order of magnitude higher than that found in skeletal muscle. In most cases they were performed in rather non-physiological buffers. The results then are qualitative and only roughly quantitative. It is not likely that additional sites go undetected or that the relative order of affinities varies from that shown, only that the *in vivo* affinities may

Table 1. Ligand binding sites of phosphofructokinase.

Name (function) of site	Order of binding affinities[a]	Kd (μM) for highest affinity ligand[b]
ATP (catalytic)	MgATP > MgITP > MgGTP	5-15[c]
ATP (inhibitory)	MgATP > MgADP, ATP	5-15[c]
Adenine nucleotide (activating)	ADP = cAMP > AMP > MgADP > ADPR > ATP	0.5
Citrate (inhibitory)	Citrate > 3-P-glycerate > PEP	20[d]
Fructose 6-P (catalytic)	Fru 6-P > Sedoheptulose 7-P >> Fru 1-P, Glu 1-P	17
Fructose bisphosphate	Fru 2,6-P_2 > Fru 1,6-P_2 > Glu 1,6-P_2	0.02

[a] Determined from binding data (Refs. 3–6, and unpublished) or inferred from kinetic data (1). Positive or negative cooperativity can be observed with some ligands under certain conditions and Kd's are approximated.
[b] Data from (3, 5) and unpublished experiments.
[c] Affinities are for free nucleotide (3); the magnesium complex binds more tightly (4, 5). Data did not distinguish between these two sites.
[d] In presence of 20 μM MgATP.

differ significantly from those given in the table.

From kinetic studies (7) and conformational studies based on thiol reactivity (8), it can be concluded that the catalytic site will accept a number of nucleoside triphosphates in addition to ATP, whereas the inhibitory site is rather specific for MgATP. The Kd's given for ATP binding are those of the free nucleotide. Other studies (3,8–10) suggest that MgATP complex binds tightly to the catalytic and inhibitor sites but binds weakly to the activating site (called the AMP site). It is not certain whether MgATP binds to the inhibitor site more tightly than free ATP. Although free ATP appears to inhibit the enzyme more strongly than MgATP, as can be shown by carrying out kinetic experiments with limiting Mg^{2+} concentrations, it has not been shown that this 'excess' inhibition is allosteric rather than competitive. Excess free ATP would also chelate free Mg^{2+}, which may be required for catalytic activity (Mathias and Kemp; unpublished). From conformational studies based on thiol reactivity (4, 8), it appears that MgATP is much more effective than free ATP in bringing about the inhibited conformation. On the other hand, free ATP was reported to bind to the inhibitor site more tightly than MgATP, based on relative quenching of intrinsic protein fluorescence (10). It is obvious that more work is required to resolve this question.

The activation site or AMP site binds AMP, cyclic AMP, and ADP, resulting in antagonism of ATP inhibition by decreasing the affinity of ATP binding by the inhibitor site. We (3) have seen that in the absence of Mg^{2+}, ADP binds more tightly than other nucleotides to the activating site; but that in the presence of Mg^{2+}, the affinity for ADP is considerably reduced. The activating site is very specific for the adenine ring (3) but will bind a variety of phosphorylated derivatives of adenosine besides AMP, cyclic AMP, and ADP, including ADPribose and NADH (6).

The enzyme binds citrate with high affinity only in the presence of MgATP. The nucleotide site whose occupancy is responsible for the enhanced citrate affinity is apparently the inhibitory site, on the basis of the weak effect of MgITP. The citrate site will also accommodate the inhibitors 3-P-glycerate and phosphoenolpyruvate (5). Based both on the affinity of the enzyme for these three compounds and on their relative *in vivo* concentrations, one must conclude that only citrate of the three can represent an important physiological regulator. Structurally, these three inhibitors share in common three negative charges at physiological pH and the binding site must have a cluster of positive charges. We (11) have modified a lysine residue at this site by reaction with pyridoxal phosphate followed by reduction with sodium borohydride. The enzyme so modified was not capable of binding citrate and was exquisitely sensitive to ATP inhibition.

As stated above, most of the data of Table 1 were obtained from binding studies employing the high protein concentrations that are required for the Hummel-Dreyer method (12) or the fast-flow equil-

ibrium technique (13). Under those conditions, binding of most ligands followed a hyperbolic isotherm with no apparent cooperativity (3, 5, 6). Despite this lack of cooperativity, positive heterotropic effects could be seen in cyclic AMP binding due to the presence of fructose-6-P or vice versa (3). Using different buffer conditions at several hydrogen ion concentrations, and employing lower protein concentrations, Hill and Hammes (14) could show both positive and negative cooperativity in binding studies of fructose-6-P and fructose-1,6-bisphosphate. They interpreted the results on cooperative binding as reflecting changes in the aggregation state of the enzyme.

Frieden and co-workers (15–19), on the basis of pH and cold lability studies and of measurements of intrinsic fluorescence in the presence of ligands, concluded that ligands affect the enzyme by preferentially binding to either protonated or unprotonated forms of the enzyme. They concluded that all of the regulatory properties are displayed by the tetramer and that dissociation is too slow to be the mechanism of ligand induced inhibition. Earlier studies from our laboratory on the use of a reactive thiol group to monitor the active–inactive transition (8) also suggested that the allosteric transitions were readily measured at protein concentrations where the enzyme existed as tetramer or higher polymer, and that dissociation to dimers was not required for the inhibited conformation.

Shown in Fig. 1 is a model for allosteric conformational states of phosphofructokinase. This model is the same as that previously described by this laboratory (20) and incorporates the binding and conformational properties described above. This for the most part is a concerted transition model and is an extension of the Monod-Wyman-Changeaux model for allosteric proteins (21), which suggests that site occupancy should displace the equilibrium to the form with the highest affinity for the ligand. However, there are some problems with this interpretation. Site occupancy does not necessarily produce a conformational change. ADP-ribose and nicotinamide dinucleotides occupy the AMP binding site as shown by fast-flow equilibrium binding and by reversal of the AMP activating effect (6). Although the dinucleotides occupy the site, they clearly do not promote the active conformation, a fact confirmed by thiol reactivity conformation studies (6). Perhaps the extended structure of the dinucleotides sterically prevents the full conformational change to the active form. This, however, suggests an induced change and not the displacement of a pre-existing equilibrium of conformers. Alternatively, the dinucleotides could bind equally well to both the protonated, inhibited and the unprotonated, active conformations of the enzyme, and thus have no influence on the equilibrium.

That a full conversion to the active form is not always achieved can be shown by the actions of the

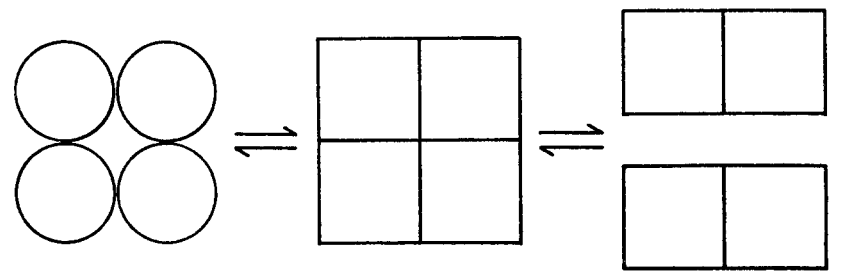

Fig. 1. Proposed model for allosteric transitions of phosphofructokinase.

sugar bisphosphates on phosphofructokinase. On the basis of equilibrium binding studies, fructose 2,6-P_2 and glucose-1,6-P_2 compete for a single site per protomer and the affinity for the fructose derivative is about two orders of magnitude higher than for the glucose bisphosphate (unpublished data). Figure 2 shows the ability of the sugar bisphosphates to reverse citrate inhibition of phosphofructokinase. Not only is the concentration of glucose-1,6-P_2 required to half-activate about two orders of magnitude higher than that of fructose-2,6-P_2, but the total extent of activation is considerably lower. This would not be predicted by the simple model of Fig. 1. Obviously, the real situation must be one somewhat more complex than that model, and one cannot on the basis of current information provide for a model that fits all observations. One can presume either additional conformation states that may represent subgroups of the indicated form, or one can assume induced states which do not represent full interconversion from one form to the other.

Phosphorylation of phosphofructokinase

Interest in the potential regulation of muscle phosphofructokinase by phosphorylation dates back to the 1960s. Ling et al. (22) showed that the presence of fluoride in the extraction medium permitted a higher yield of enzyme and that addition of ATP to extracts made without fluoride increased the activity of phosphofructokinase. Parmegianni et al. (23) confirmed these results and attempted unsuccessfully to phosphorylate phosphofructokinase in extracts by the addition of [γ-^{32}P]-ATP or by the addition of phosphorylase kinase and labeled ATP to purified phosphofructokinase. Subsequently, several laboratories, including our own, tried without success to phosphorylate phosphofructokinase. Interest in phosphorylation was rekindled in 1975 with the report by Brand and Soling (24) that phosphofructokinase in a low pH precipitate fraction of liver homogenate could be phosphorylated by added [γ-^{32}P]-ATP. Shortly thereafter, Hofer and Furst (25) reported the isolation of [^{32}P]phosphofructokinase from muscle tissue of mice previously injected with [^{32}P]phosphate. They calculated a content of about 0.7 moles phosphate per mole protomer assuming equilibration with the tissue phosphate pool. Hussey et al. (26), Uyeda's laboratory (27) and our own laboratory (28) reported covalently bound phosphate in rabbit muscle at levels closer to 0.2 moles per mole, and we found (29) that the phosphate content of mouse muscle phosphofructokinase as determined by incorporation of injected [^{32}P]phosphate was 0.12 to 0.15 moles per mole protomer when the incorporation was calculated on the basis of the specific activity of the γ-phosphate of tissue ATP.

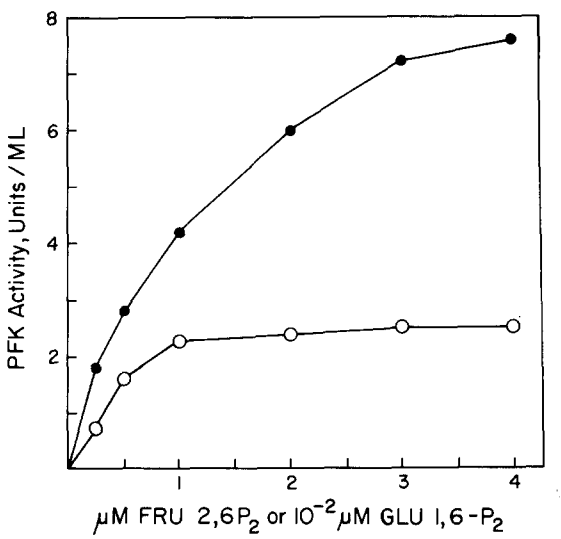

Fig. 2. Activation of phosphofructokinase by sugar bisphosphates in the presence of an inhibitory concentration of citrate. Phosphofructokinase was assayed at pH 7.0 as described by Foe and Kemp (39) with 1.0 mM fructose 6-P, 0.5 mM ATP, and 1.0 mM citrate. ●, plus fructose 2,6-P_2 as indicated; ○, plus glucose 1,6-P_2 as indicated.

Hofer's group recently reported that phosphofructokinase from *Ascaris suum* muscle contained up to 2 phosphates per subunit, and that fully phosphorylated enzyme was more active than partially phosphorylated enzyme (30, 31). Two phosphofructokinase kinases, one cyclic AMP dependent and one cyclic AMP independent, have been reported to exist in *Ascaris suum* extracts (32).

Rabbit muscle phosphofructokinase was shown to be phosphorylated by ATP in the presence of the catalytic subunit of cyclic AMP-dependent protein kinase (28). That this was not detected in earlier experiments is probably attributable to the high K_m for the substrate (33) and to the exquisite sensitivity of the carboxyl terminal site of phosphorylation to the action of proteases (34). We have observed (35) that unless care is taken the isolated enzyme may contain a population of molecules

lacking the carboxyl terminal 'tail' that contains the site of phosphorylation. The kinetics of the phosphorylation event show that the affinity of cyclic AMP-dependent protein kinase for muscle phosphofructokinase is relatively low, with a K_m of 230 μM (33), which is about an order of magnitude higher than the K_m's reported for histone, casein, and a preparation of lysozyme that has been modified to unfold its structure (36). On the other hand, the V_{max} for the phosphorylation of phosphofructokinase approached that observed with histone as a substrate (33). Allosteric activators of phosphofructokinase, AMP and fructose bisphosphates, increase V_{max} but do not affect K_m; whereas inhibitors of phosphofructokinase, citrate and phosphoglycerate, decrease V_{max} (33). The site of phosphorylation of rabbit muscle phosphofructokinase has been determined and is at a serine residue that is the sixth amino acid from the carboxyl terminus (33). This serine is preceded by the sequence Arg-Lys-Arg, which makes it similar to other known substrates for cyclic AMP-dependent protein kinase (37) wherein basic amino acid residues are found several positions toward the NH_2 terminus from the serine undergoing phosphorylation.

More recently we have used this protein kinase to phosphorylate phosphofructokinases isolated by affinity chromatography (38) from beef heart and mouse and rat skeletal muscle, and as was seen with the rabbit muscle enzyme (34), a brief subtilisin digestion of the *in vitro* labeled enzyme rapidly removed the position of phosphorylation. The phosphopeptides were separated from the rest of the enzyme by Sephacryl S-200 chromatography followed by purification on Sephadex G-25 and DEAE-Sephadex (33). The composition of these peptides are shown in Table 2 along with the subtilisin peptide from rabbit skeletal muscle. It would appear that these peptides are very similar in structure, differing slightly in their overall lengths.

Studies (33) with *in vivo* [^{32}P]-labeled phosphofructokinase isolated from tissue of a rabbit injected with [^{32}P]phosphate showed that virtually all of the *in vivo* phosphate was located at the same site as that labeled *in vitro*. A brief tryptic digest of the *in vivo* labeled enzyme produced a phosphopeptide whose position of elution from Sephadex G-25 and position of migration on thin layer cellulose chromatography was identical to that isolated from a digest of enzyme labeled by incubation with cyclic

Table 2. Composition of subtilisin-phosphopeptides from various phosphofructokinases[a].

Amino acid	Source of phosphofructokinase			
	Rabbit muscle	Beef heart	Rat muscle	Mouse muscle
Threonine	1	0	0	0
Serine	2	2	2	2
Glutamic[b]	1	2	2	2
Glycine	1	2	2	2
Alanine	1	1	2	3
Valine	1	0	1	1
Isoleucine	1	1	1	1
Histidine	1	1	1	2
Lysine	1	1	1	1
Arginine	2	2	2	3
Total	12	12	14	17

[a] Hydrolysates of phosphopeptides of *in vitro* labeled phosphofructokinase from beef heart and rat and mouse skeletal muscle were analyzed on a Glenco modular amino acid analyzer. Enzymes were prepared, phosphorylated, and digested as described in the text and in (33). Amino acid compositions were rounded off to nearest whole integer. Data for rabbit muscle enzyme has been published (33).

[b] Amide nitrogen was not determined.

AMP-dependent protein kinase. Furthermore, the kinetics of phosphate removal from *in vivo* and *in vitro* labeled enzyme by alkaline phosphatase were identical (39). Thus all our data on phosphorylation *in vivo* and *in vitro* are consistent with the idea that the phosphate found in muscle phosphofructokinase has been introduced by cyclic AMP-dependent protein kinase. The high K_m (230 μM) with respect to phosphofructokinase coupled with a tissue concentration of about 15 μM dictates a rather low rate of phosphorylation in contrast to other substrates for the protein kinase. It might be argued that yet-to-be-discovered factors interact with phosphofructokinase to make it a better substrate, but known effectors have only a relatively modest effect on V_{max}, not on K_m.

An argument against cyclic AMP-dependent protein kinase as the *in vivo* catalyst of phosphorylation of phosphofructokinase in muscle comes from the recent work of Cohen's laboratory (40) that the phosphate content of phosphofructokinase was not significantly different between propranolol- and epinephrine-treated rabbits under conditions where striking differences were seen in the phosphorylation state of glycogen synthase. This finding may be

a result of the relatively low rate of phosphorylation of phosphofructokinase by cyclic AMP-dependent protein kinase described above. On the other hand, Kahn's laboratory (41) has shown that erythrocyte phosphofructokinase in intact cells preincubated with [^{32}P]phosphate would become phosphorylated upon addition of exogenous cyclic AMP. Also, phosphorylation of phosphofructokinase in liver was increased by glucagon or cyclic AMP perfusion (42).

No kinases other than the cyclic AMP-dependent protein kinase have been found that will introduce phosphate into mammalian phosphofructokinase *in vitro*. We have tried unsuccessfully phosphorylase kinase, myosin light chain kinase and calmodulin provided by Robert Adelstein's laboratory (43), and casein kinase provided by Jolinda Traugh's laboratory. Crude fractions from skeletal muscle extracts have also been tried without success. Because of the high K_m observed when the cyclic AMP-dependent protein kinase is used, the amount of protein kinase added in *in vitro* phosphorylation experiments is very high. It has been privately suggested that these levels of protein kinase will phosphorylate anything. This is not so; comparable experiments with muscle pyruvate kinase and muscle aldolase show no detectable phosphorylation by cyclic AMP-dependent protein kinase.

The functional consequences of the phosphorylation of phosphofructokinase remain obscure. Our laboratory (39) has compared the properties of phosphophosphofructokinase (0.13 moles per mole protomer endogenous phosphate plus 0.62 moles incorporated with protein kinase) and dephosphophosphofructokinase (0.02 moles per mole after treatment with intestinal alkaline phosphatase). No difference in V_{max} was observed between the two forms. The differences in allosteric regulatory properties were very small, but were consistent from preparation to preparation and assay to assay. The highly phosphorylated enzyme is more sensitive to allosteric inhibition by citrate and ATP and requires higher concentrations of AMP, sugar bisphosphates, and inorganic phosphate for activation. Thus, all of these differences are in the direction of the phosphorylated enzyme having less activity than the dephospho-enzyme. There were no differences observed between states of aggregation of the phospho- and dephospho-enzyme, nor were there differences in stability under conditions of low pH inactivation. The low content of organic phosphate in the enzyme as isolated does not measurably affect the kinetics of the enzyme as indicated in Fig. 3A. The small differences become measurable only when the enzyme phosphorylated *in vitro* is compared with enzyme as isolated (native) or *in vitro* dephosphorylated enzyme (Figs. 3B and 3C).

In the light of these small kinetic regulatory differences, it is unlikely that changes in kinetics due to phosphorylation make a significant contribution to

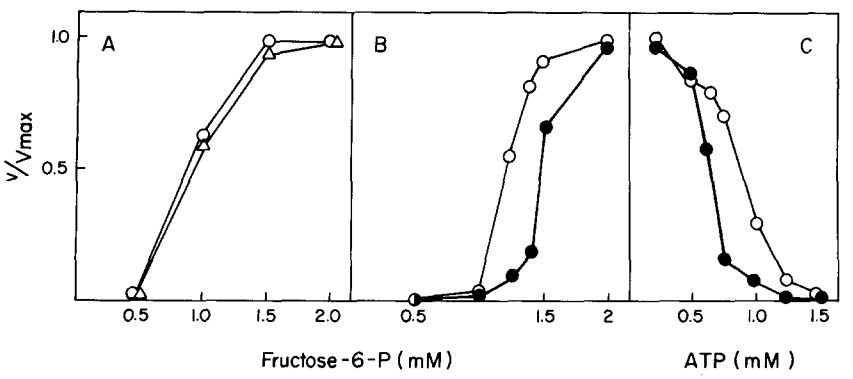

Fig. 3. Comparison of kinetics of phosphofructokinase with different degrees of phosphorylation. Phosphofructokinase was assayed at pH 7.0 as described by Foe and Kemp (39). A: native phosphofructokinase, phosphate content 0.13 moles/mole protomer (△) and *in vitro* dephosphorylated phosphofructokinase, phosphate content <0.02 moles/mole protomer (○). B: *in vitro* phosphorylated phosphofructokinase, phosphate content 0.75 moles/mole protomer (●) and native phosphofructokinase (○). C, *in vitro* phosphorylated phosphofructokinase (●) and *in vitro* dephosphorylated phosphofructokinase (○). In A and B, the ATP concentration was 0.5 mM; in C, the fructose 6-P concentration was 2 mM.

the overall regulation of phosphofructokinase. Furthermore, it would not seem reasonable to have cyclic AMP-dependent protein kinase producing an inhibition of muscle phosphofructokinase, considering the fact that the production of cyclic AMP also leads to the activation of glycogen phosphorylase to produce glucose phosphate for glycolysis. On the other hand, that phosphorylation of phosphofructokinase may vary with physiological state comes from two lines of evidence. Hofer and Sorensen-Ziganke (44, 45) reported that rabbit muscle phosphofructokinase isolated under three different conditions contained very different covalent phosphate contents. Muscle excised and stored on ice contained about 0.5 moles per mole protomer; muscle removed and quickly frozen in liquid nitrogen from deep anesthesia had slightly more than 1 mole per mole protomer; whereas muscle that had been rhythmically contracted for 10 min as a result of nerve stimulation had close to 2 moles per mole protomer. Even the lowest of these contents are higher than anything we (28, 29) or others (26, 27) have reported for phosphate content of muscle phosphofructokinase. Furthermore, the data suggest more than one site of phosphorylation, such as that indicated with rat liver (46) and *Ascaris suum* muscle (30) enzyme. It would further suggest that the additional site may have functional significance and that it is not the site that we have studied in the kinetic studies described above. In view of the potential significance of the work from Hofer's laboratory regarding phosphorylation state, it is very important that this work be confirmed and extended.

Another suggestion that state of phosphorylation is varied by physiological state comes from our recent work (35) with the genetically diabetic mouse (C57BL/KsJ). Organic phosphate content of five control animals (db/m) varied from 0.11 to 0.19 mole/mole protomer with a mean content of 0.14 moles/mole. This agrees with our earlier data obtained with outbred Swiss mice (29). In contrast, the serine phosphate content of diabetic mice (db/db) had a broad range (0.11 to 0.39 of phosphate content with a mean value of 0.24 moles/mole. All of this phosphate was in the terminal region described above as indicated by its facile removal by limited proteolysis. High- and low-phosphate forms of phosphofructokinase from both diabetic and normal mice, resolved by ion exchange chromatography, showed consistent kinetic differences, with the more highly phosphorylated form being more sensitive to ATP inhibition. However, it is unlikely that the slight kinetic differences between low and high phosphate forms contributed to the impairment in glycolysis that is observed in diabetic tissue.

In summary, phosphorylation of phosphofructokinase occurs in all four mammalian species that we have examined and in all three isozymic species, including the type C subunit found in brain (Foe and Kemp, unpublished). Although some evidence suggests more than one site of phosphorylation, the most carefully studied event occurs at a specific sequence near the carboxyl terminus. The extent of phosphorylation appears to vary under differing physiological and pathological states. Yet, no profound kinetic consequences are apparent with regard to the state of phosphorylation of the major site. If an important regulatory function results from the phosphorylation/dephosphorylation cycle at the carboxyl terminal site, the function will undoubtedly not be a direct kinetic effect but some indirect action such as a role in the interactions of the enzyme with cellular organelles. The possibility and significance of a second site of phosphorylation remains to be examined.

Acknowledgements

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