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MODULATION OF PROTEIN FUNCTION

edited by
DANIEL E. ATKINSON
C. FRED FOX

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MODULATION OF PROTEIN FUNCTION

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PREFACE

During the past two decades it has come to be recognized that biological functions of many, if not all, types are controlled to a very large extent through modulation of the functions of individual proteins or of multimolecular protein systems that result from interaction with metabolites or with specialized messenger compounds of low molecular weight. It was the aim of this symposium to bring together workers from several fields, all of which deal with such modulation of protein function. Discussion of representative metabolic control systems, ranging from single-enzyme responses to complex regulatory cascades, and the control of photosynthesis and of protein synthesis and enzyme inactivation dealt with the general topic at perhaps its most fundamental cellular level. Modulations and conformational changes in proteins that underlie higher-level interactions, such as those involved in cyclic nucleotide function, sensing and chemotactic response to foreign materials, and the complement system, were described. Two talks dealt with potential clinical relevance of phenomena of the types described by other participants. The common thread of functionally significant consequences of protein–small-molecule interaction led to extensive interaction among participants who work on widely diverse systems, and the editors hope that common thread will similarly unify this published record of the symposium.

We wish to thank the symposium speakers and poster session contributors for providing the basis of the program. We also wish to acknowledge the continuing support that the Life Sciences Division of ICN Pharmaceuticals, Inc., endows for the general support of this conference series, and, finally, we cite the generous contribution made by The National Foundation in partial support of the present meeting.

Daniel E. Atkinson

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MOLECULAR PROPERTIES OF PHOSPHOFRUCTOKINASE (PFK) RELEVANT TO MODULATION OF ITS FUNCTION[1]

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Studies during the past twenty years on the molecular properties of phosphofructokinase have contributed immensely to our understanding of its role as an important regulatory enzyme in glycolysis. Both covalent and non-covalent changes in enzyme structure have been reported. Evidence has been accumulating showing variation in phosphofructokinase activity in connection with different physiological conditions. In many cases the changes in enzyme activity is implied from indirect evidence and on the basis of what we already know of the properties of the enzyme. We wish to summarize briefly our current knowledge of some of the most important molecular properties of the enzyme. We will then report on some recent experiments on its allosteric sites and the nature of inhibition by vanadate. Finally, we will discuss briefly the relationship between these properties and the regulatory function of phosphofructokinase.

Molecular structure. Information based on data from our own laboratory on heart phosphofructokinase as well as laboratories of Lardy (1,2) and others indicates that the smallest fully active phosphofructokinase is a tetramer with a molecular weight of 360,000 and an $S_{20,w}$ value of 13. High enzyme concentration or the presence of fructose-1,6-P_2 or fructose-6-P favor the formation of high aggregates of the enzyme with an $S_{20,w}$ value as high as 54, while the presence of ATP or low enzyme concentration favor the low molecular form. The tetrameric form of the enzyme can be dissociated to dimers which are inactive. Enzyme protomers can be obtained in the presence of 4mM of SDS. Each protomer can be dissociated to 4 subunits with a molecular weight of 24,000 in the presence of 5M guanidine HCl.

Kinetics. Studies on the kinetics of phosphofructokinase have indicated that pH determines the nature of these kinetics (1). At pH 8.2, which is the optimal pH for enzyme activity, it exhibited Michaelis-Menten type of kinetics. At pH 6.9 typical allosteric kinetics are seen. The curve for ATP is hyperbolic until the activity

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TABLE 1

SOME OF THE IMPORTANT EFFECTORS OF PHOSPHOFRUCTOKINASE

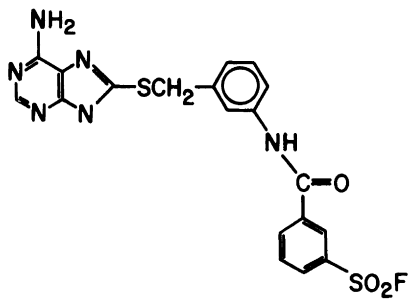
Inhibitors	Deinhibitors of ATP or Activators
ATP	3',5'-cyclic AMP
Citrate	5'-AMP
P-creatine	ADP
3-P-glycerate	Fructose 6-P
2-P-glycerate	Fructose-1,6-P_2
2,3-P_2-glycerate	Glucose-1,6-P_2
P-enolpyruvate	NH_4^+ , Pi

is maximal, followed by a steep inhibition curve as the ATP concentration is increased. In the presence of an activator the catalytic part of the curve is not changed while the inhibitory curve reaches almost a plateau after maximal activity. Thus activators exert their effect by relieving ATP inhibition, i.e. by "de-inhibition". The saturation curve for fructose-6-P at pH 6.9 is sigmoidal. Inhibitors will increase the sigmoidicity while activators will convert the sigmoidal kinetics to hyperbolic kinetics.

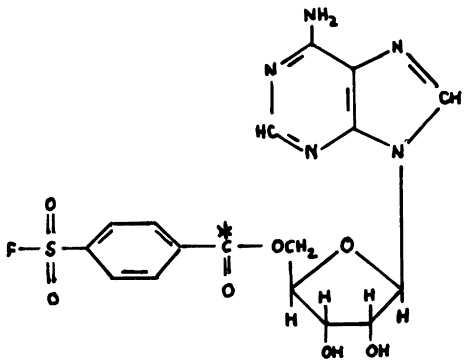
The list of allosteric effectors of mammalian phosphofructokinase (Table 1) is long and more agents are being added to it. Among the activators listed, AMP and Pi are of special interest since their levels are increased after anoxia. Furthermore, cyclic 3',5'-AMP, whose level is increased following administration of several hormones, also is one of the activators.

Nature of Allosteric Sites. Our approach to study the molecular properties of allosteric sites of PFK is largely through chemical modification and through identification of the structures that have been modified. Previously we have used photo-oxidation (3,4) and ethoxyformic anhydride (4) to modify sheep heart PFK. The modified enzyme became less sensitive to ATP inhibition in connection with the loss of ATP inhibitory binding sites; its sigmoidal kinetics for fructose-6-P was also abolished, while the catalytic effect was only slightly decreased. Reaction of ethoxyformic anhydride with PFK specifically modified four histidine residues per protomer; thus those histidine residues presumably served as cationic binding sites for inhibitory ATP.

The use of affinity label reagents is a more effective way of selectively binding to the site in the enzyme prior to its covalent reaction. Recently we have used two reagents that react covalently



8-[m-(m-FLUOROSULFONYLBENZAMIDO)BENZYLTHIO]ADENINE
(FSB-ADENINE)



5'-p-FLUOROSULFONYLBENZOYL ADENOSINE
(FSB-ADENOSINE)

FIGURE 1.

with sheep heart phosphofructokinase making it much less sensitive to inhibition by ATP (5,6). Modified enzyme becomes insensitive to activation by AMP, ADP and cyclic AMP.

Fig 1 shows the structure of these two compounds. The first compound used is 8-[m-(m-fluorosulfonylbenzamido)benzylthio] adenine which will be referred to as FSB-adenine (7). The other reagent is an adenosine derivative, 5'-p-fluorosulfonylbenzoyl adenosine which will be referred to as FSB-adenosine (8). Both affinity label reagents have been used to label several other enzymes. For example, FSB-adenine was first used by Graves to label the AMP site on glycogen phosphorylase (7). The adenosine reagent was used as an affinity label of the inhibitory DPNH site of bovine liver glutamate dehydrogenase by Colman (8). The catalytic sites of rabbit muscle pyruvate kinase (9) and mitochondrial ATPase (10) were also labelled with the same reagent following inactivation of the enzymes.

Our recent studies show that the adenosine reagent is a specific reagent for the AMP-ADP sites of phosphofructokinase and protects against ATP inhibition better than the adenine derivatives (Figure 2). This may be due to the presence of the ribose moiety which is important for the right orientation of the reagent molecule. Conditions were first established to abolish allosteric kinetics without affecting maximal enzyme activity at pH 8.2. This was achieved when the enzyme covalently binds approximately 1 mole of the reagent per protomer. The modified enzyme completely lost its sensitivity to inhibition by ATP at moderately low levels. Inhibition can only be produced at concentrations as high as 700μM.

The results summarized in Fig. 3 show the sensitivity of the enzyme to AMP activation when inhibited by ATP at a concentration that causes 60% inhibition. The results show that while the native enzyme is sensitive to activation by AMP at concentration as low as

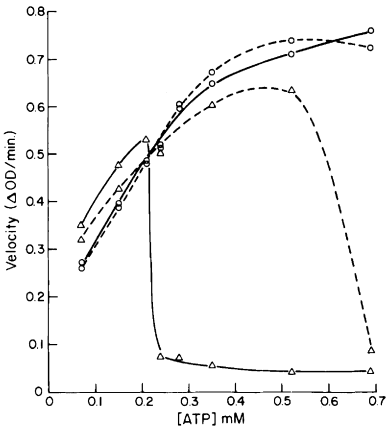


Fig. 2

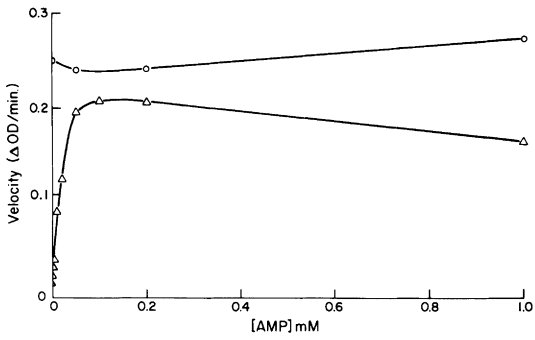


Fig. 3

Fig. 2. Sensitivity of native (▲) and modified (●) phosphofructokinase to inhibition by ATP. Enzyme modified by 5-$FSBO_2B_Z$-adenosine and native enzyme were prepared as reported. Initial velocity of phosphofructokinase was measured at different ATP concentrations in the regular reaction mixture at pH 6.9 without AMP (straight line) or with 1 mM AMP (dashed lines). The concentration of fructose-6-P was fixed at 0.5 mM and the $MgCl_2$ at 1 mM.

Fig. 3. Sensitivity of native (▲) and modified (●) phosphofructokinase to activation by AMP. Enzyme activity was measured at pH 6.9 in the presence of 0.25 mM ATP, 0.5 mM fructose -6-P and 1 mM $MgCl_2$.

10μM, the modified enzyme is completely insensitive to AMP activation. Titration curves for the second substrate fructose 6-P showed no sigmoidal kinetics for the modified enzyme. Kinetics of the enzyme at pH 8.2, on the other hand, was not significantly influenced.

The effect of enzyme modification on nucleotide binding to PFK showed that the binding of AMP, cAMP and ADP was abolished following enzyme modification. In contrast, the modified and native enzyme did not differ significantly to the maximal amount of binding of the ATP imidoanalog, App(NH)p. Affinity of the modified enzyme to App(NH)p was reduced.

This reagent therefore appears to be affecting the allosteric sites specifically and does not appear to involve the catalytic sites of PFK. The fact that this reagent abolished the binding of the activators AMP, cAMP and ADP but only interfered with the affinity for the ATP analog App(NH)p indicates that occupation of the AMP site does not eliminate ATP binding. Thus, the desensitization to ATP inhibition by FSB-adenosine modification must result from a change in the interaction of PFK with ATP subsequent to ATP

binding.

Our success in labelling the allosteric site with a specific affinity label prompted us to investigate the chemical nature of the AMP-ADP site. Following modification of PFK with [^{14}C]-FSB-adenosine, we attempted to isolate the labelled peptide and to identify the residue which is modified. For these experiments we used the [^{14}C] reagent that is labelled at the carbonyl moiety instead of the adenosine (10); since the ester bond between the benzoyl and adenosine is hydrolyzed slowly under slightly alkaline conditions. Following modification with the reagent, PFK was subjected to reduction and carboxymethylation. This was followed by citraconylation in order to solubilize the modified enzyme and to prevent cleavage at the lysine sites . The enzyme was then treated with trypsin and chromatographed on Sephadex G-50 column. The major radioactive peptide was identified in one peak. We are currently isolating the labelled peptide for sequence analysis.

We have also attempted to identify the amino acid residue that is modified by the adenosine reagent. The side chains of serine, tyrosine, lysine and histidine residues in protein are capable of reacting with sulfonyl halides. Since sulfonylated serine and histidine are base-labile (10) they cannot be expected to isolate under such an isolation step; thus the sulfonylated derivatives of lysine and tyrosine are the reasonable candidates. Carboxybenzensulfonyl (CBS) derivatives of lysine and tyrosine (CBS-Lys and CBS-Tyr) can be synthesized (10) as standards for the identification. A sample of the radioactive fraction was treated with alkali and hydrolyzed with 6NHCl for 22 hrs. at 110°C. The acid hydrolysate was subjected to high voltage paper electrophoresis (HVPE) at pH 3.5 along with samples of CBS-Lys and CBS-Tyr, which had been treated under similar conditions. The spot with the major radioactivity moved at the same velocity as CBS-lysine. The results therefore identify lysine as the amino acid involved in enzyme modification. It is possible that this lysine residue serves as a cationic binding site for AMP, ADP and cAMP.

Vanadate as a Potent PFK Inhibitor. Ortho vanadate has been reported to inhibit several enzymes, including alkaline phosphatase (11), Na, K-ATPase (12), and dynein ATPase (13,14). Some of the chemical properties of vanadate are summarized in Figure 4. Since the tetrahedral structure of vanadate is so similar to that of phosphate, vanadate may inhibit enzymes by competing at phosphate-binding sites. Other studies have shown that vanadate can form a trigonal bipyramidal structure in solution and may inhibit as a transition-state analog. Enzymes catalyzing phosphate-transfer reactions have been proposed to go through this transition state. Vanadate can also exist in several polymeric states. The degree of polymerization is dependent upon the pH and concentration of vanadate in solution. At a basic pH, monomeric vanadate is

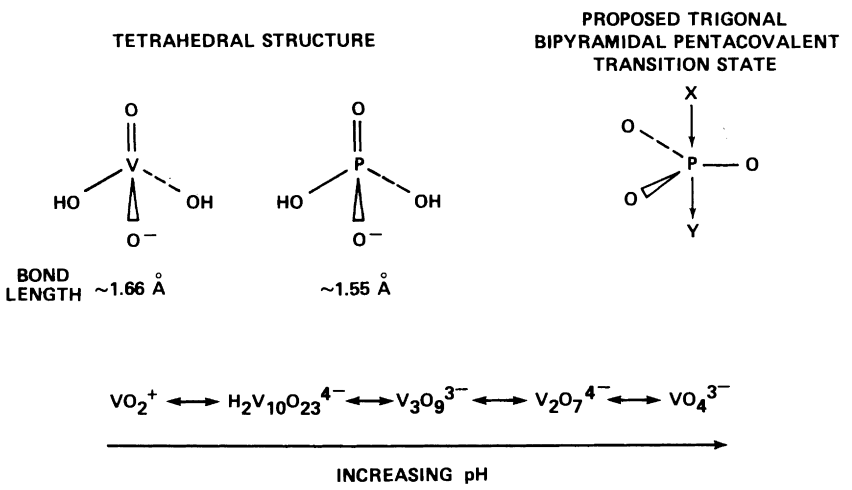


Fig. 4

predominant, and at an acidic pH, dimeric, trimeric and decameric species are found. Vanadate was chosen for this study as a potential inhibitor of PFK, since PFK is a phospho-transfering enzyme, binds several phosphate-containing ligands, and is inhibited by several polyanionic effectors.

Preliminary experiments showed that vanadate, when prepared under conditions that favored decavanadate, was a potent inhibitor of PFK (15). The enzyme was not inhibited by monomeric vanadate. When inhibitory curves for different samples of vanadate were normalized on the basis of decavanadate concentration, there was a good correlation between decavanadate concentration and the inhibitory effect on the enzyme.

Unlike the known inhibitors of PFK, vanadate was shown to inhibit both at pH 8.2 and at pH 6.9. Vanadate also can inhibit in the presence of 50mM of phosphate. Thus, the inhibitory effect of vanadate is not because of its structural similarity to phosphate but because of the effect of decavanadate as an allosteric modifier of the enzyme.

Further kinetic data showed that vanadate was a more potent inhibitor at pH 6.9 than at pH 8.2. The concentration of vanadate to inhibit PFK to the extent of 50% of its activity (I_{50} value) at pH 6.9 was 0.45 µM and at pH 8.2 was 5.5 µM. The usual allosteric activators of PFK such as cyclic AMP and glucose-1,6-P_2 were also activators of the vanadate inhibited enzyme (Table 2). In many ways, vanadate inhibition behaves like other inhibitors that act synergistically with ATP. Citrate is a good representative of this group.

TABLE 2

INHIBITION OF PFK BY VANADATE AND
DE-INHIBITION BY ALLOSTERIC EFFECTORS

PFK was assayed at pH 6.9 in 50mM morpholinopropane sulfonic acid buffer or at pH 8.2 in 50mM glycylglycine buffer. The reaction mix also contained 20mM KCl, 3mM $MgCl_2$, 1mM DTT, 0.1mg/ml BSA, 0.2mg/ml NADH, aldolase, triose-P-isomerase, α-glycero-P-dehydrogenase, and where indicated, vanadate, glucose-1,6-diphosphate, and cyclic AMP. FSB-adenosine modified PFK was prepared as described previously (6). The concentrations of vanadate reported below represent the concentration required for maximal inhibition at each condition in the absence of positive effectors.

Preparation	pH	[Vanadate] (μM)	Additions	Activity (% control)
Native	6.9	1.5	none	2
		1.5	5μM Glu-1,6-P_2	41
		1.5	20μM cAMP	38
	8.2	15	None	1
		15	20μM Glu-1,6-P_2	57
		15	50μM cAMP	41
Modified	6.9	13	None	6
		13	20μM Glu-1,6-P_2	44
	8.2	125	None	10
		125	500μM Glu-1,6-P_2	38

Vanadate, like citrate, acts synergistically with ATP; however, unlike other inhibitors, vanadate remains effective at pH 8.2. As well as inhibiting synergistically, vanadate affects the response of PFK to fructose-6-P. At pH 8.2, vanadate converts the response of PFK with respect to fructose-6-P from hyperbolic to sigmoidal kinetics.

The question arose whether PFK modified with FSB-adenosine reagent changes the sensitivity of the enzyme to vanadate inhibition. The results indicate that the modified PFK, although completely desensitized to ATP inhibition, was still sensitive to vanadate inhibition. Modified enzyme however was less sensitive to the inhibition than the native enzyme (Table 2), yielding I_{50} values over six times

greater than that observed for the native enzyme. Vanadate inhibition was still sensitive to deinhibition by glucose-1,6-P_2, but was uneffected by cAMP, consistent with the observation that the modification blocks the AMP site.

The results obtained with the vanadate studies have shown that PFK possesses a polyanionic site for a potent synergistic inhibitor. Since decavanadate depolymerizes rapidly in solution and has not been detected in tissues, it is probably not an inhibitor of PFK in vivo. The physiological inhibitor may be a polyphosphate or a polyanion yet to be determined. At pH 6.9, poly $(P_i)_{14}$ was found to inhibit PFK in vitro at concentrations less than 1μM and was deinhibited by glucose 1,6-P_2 and cAMP.

The vanadate inhibition studies have also increased our understanding of allosterism in PFK. Some workers have proposed that allosteric kinetics is coupled to the protonation of active tetrameric PFK and eventual dissociation to inactive dimers. This proposal is not supported by the present studies showing allosterism at pH 8.2, where PFK is unlikely to dissociate. Inactivation studies provided further evidence against allosterism coupling to dissociation. At pH 6.5, dilute concentrations of PFK inactivate rapidly due to dissociation. Rather than enhancing dissociation, decavanadate actually was found to protect PFK against inactivation. PFK can no longer be defined as an allosteric enzyme below pH 7.5 and as a Michaelis-Menten enzyme above it and is certainly not regulated by a simple two-state concerted process. Rather, the activity and allosteric sensitivity of PFK varies over a wide range and reflects numerous conformations elicited by substrates and effectors.

Activation of PFK by hormones and by changes in physiological condition of the cell. A change in enzyme activity is often assumed on the basis of a decrease or an increase of enzyme modifiers. In other cases a change in enzyme activity is based on more direct evidence; that is, an increase or a decrease in assayable enzyme activity in tissue extracts. A summary of these changes in enzyme activity is included in Table 3.

Activation of PFK in aerobic cells as a result of anoxia is assumed on the basis of an increase in the levels of activators such as AMP, ADP and Pi (21). An increase in enzyme activity in this case is kinetic since the agents act as allosteric deinhibitors.

In the case of serotonin activation of PFK in the liver fluke, the evidence seems to indicate that the effect is more direct. Enzyme activation is more stable and there is an increase in the specific activity of the enzyme. This means that when the enzyme is diluted for the assay and any effector ligand present is also diluted, there is still an increase in enzyme activity. We previously showed that cyclic AMP is necessary for such an effect. We ascribe such an activation to either covalent modification or tightly bound activators.