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Heart Phosphofructokinase: Allosteric Kinetics with Fructose 6-Sulfate†

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ABSTRACT: The allosteric regulation of heart phosphofructokinase was studied at pH 6.9 with an alternative substrate, fructose 6-sulfate. The alternative substrate allowed kinetic studies to be carried out at high enzyme concentrations (0.1 mg/ml) where the effect of allosteric ligands on enzyme physical structure has been studied. A K_m for ATP binding (8-10 μM) in the presence of saturating AMP concentrations was found which agreed well with the value obtained at pH 8.2. ATP inhibitory effects closely followed saturation of its substrate site. Hill plots for ATP inhibition gave an interaction coefficient of 3.5, indicating cooperativity between at least four enzyme subunits. Neither AMP nor fructose 6-sulfate affected the cooperativity between the ATP inhibitory sites but only increased the inhibitory threshold. As the ATP concentration was increased from suboptimal to inhibitory levels, interaction coefficients for AMP and fructose 6-sulfate changed from 1 to 2. Increasing citrate concentration resulted in an increase in the interaction coefficient for fructose 6-sulfate to a value of 1.9. Citrate inhibition was synergistic with ATP inhibition with an interaction coefficient of 2. The data indicate that allosteric kinetics of the enzyme can be shown at high enzyme concentrations with the alternative substrate. ATP inhibition appears to involve interaction between at least four subunits, while citrate, AMP, and fructose 6-sulfate interact minimally with two subunits.

The important role played by phosphofructokinase in regulating glycolysis is reflected in its complex allosteric regulation (cf. reviews by Mansour (1972) and by Lardy and Bloxham (1973)). The enzyme is inhibited by increasing concentrations of ATP, the phosphoryl donor substrate. The kinetic effect of ATP inhibition is manifested by a lowered affinity and an increased cooperative response to the second substrate, fructose 6-phosphate. The allosteric inhibitor, citrate, displays a kinetic effect similar to that of ATP, and its inhibitory action is dependent on the concentration of ATP. Allosteric inhibition is counteracted by the products of the phosphofructokinase reaction, ADP and fructose-1,6-P_2,[1] as well as by the allosteric effectors AMP and cyclic AMP. The kinetic effect of these activators is opposite to that of ATP and citrate. The enzyme is also activated by divalent anions HPO_4^{2-} and SO_4^{2-} as well as by monovalent cations K^+ and NH_4^+.

Physical studies of phosphofructokinase have demonstrated that the enzyme exists in a variety of polymeric forms which undergo transformations in response to enzyme concentration, metabolic effectors, temperature alteration, and pH changes. Information on enzyme structure and ligand binding properties carried out at high enzyme concentration (mg/ml) is often correlated with kinetic studies utilizing enzyme concentrations at μg/ml levels. Since the degree of polymerization of certain regulatory enzymes affects their kinetic behavior and is dependent on their concentration, it is possible that the kinetic data obtained on highly diluted enzyme may not apply to the enzyme in concentrated form.

We reported that fructose 6-sulfate can be used as an alternative substrate for the enzyme (Martensen and Mansour, 1976b). The use of this substrate for studies on phosphofructokinase allosteric regulation seemed to offer several advantages. The reduced catalytic efficiency of greater than 30-fold and the decreased binding affinity by 100-fold allowed activity measurements at enzyme concentrations two orders of magnitude greater than that possible with the native substrate. The effect of enzyme concentration on allosteric regulation could therefore be investigated.

Experimental Procedure

Materials. Fructose 6-sulfate was synthesized by sulfurylation of fructose. The 6-sulfuryl ester was isolated and purified by a procedure which was reported before (Martensen and Mansour, 1976b). Nucleotides, fructose-6-P, fructose-1,6-P_2, and dithiothreitol were purchased from Sigma Chemical Co. Pyruvic kinase, lactic dehydrogenase, both from rabbit skeletal muscle, and phosphoenolpyruvate were purchased from Boehringer Mannheim Corporation.

Methods: Preparation and Assay of Phosphofructokinase. Sheep heart enzyme was purified and crystallized by the procedure described previously (Mansour et al., 1966; Lorenson and Mansour, 1969). The low catalytic activity of phosphofructokinase with the alternative substrate allowed the use of high concentrations of the enzyme to study its allosteric properties at pH 6.9. All assays described herein contained

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[1] Abbreviations used: fructose-6-P, fructose 6-phosphate; fructose-1,6-P_2, fructose 1,6-diphosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NADH, reduced nicotinamide adenine dinucleotide.

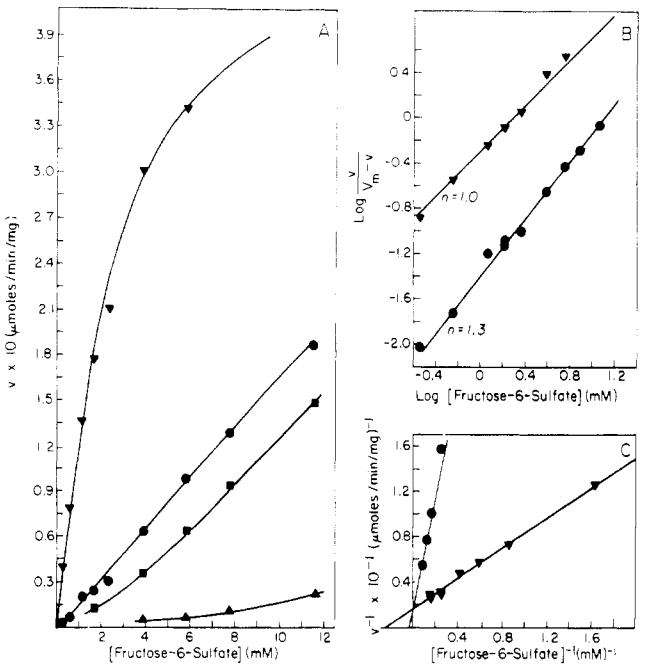

FIGURE 1: Effect of AMP and ATP on the response of phosphofructokinase activity to fructose 6-sulfate. (A) Plot of initial velocity vs. fructose 6-sulfate concentration with 10 μM ATP (●), 10 μM ATP and 500 μM AMP (▼), 20 μM ATP (■), and 60 μM ATP (▲). (B) Hill plot of the data of A. (C) Lineweaver–Burk plot with 10 μM ATP (●) as well as 10 μM ATP and 500 μM AMP (▼).

phosphofructokinase at a concentration of 0.1 ± 0.01 mg/ml. Crystalline enzyme was solubilized at approximately 5 mg/ml in 10 mM Tris-Cl (pH 7.5), 100 mM KCl, 1 mM K_2SO_4, 10 μM fructose-1,6-P_2, 1 mM EDTA, and 5 mM dithiothreitol. The dissolved enzyme (0.5 ml) was layered onto a Sephadex G-25-80 column (10 ml) and eluted with buffer containing 10 mM Tris-Cl, pH 7.5, 100 mM KCl, 1 mM EDTA, and 5 mM dithiothreitol. Fractions containing protein were shown to separate from fractions containing ammonium sulfate and other low-molecular-weight compounds. The optical densities of the protein fractions were determined at 260 and 280 nm. All samples had a 280/260 ratio of at least 1.4. Pooled protein fractions were diluted to a concentration of 1.0 ± 0.01 mg/ml and kept on ice. ATP and K_2SO_4 were added to concentrations of 10 μM and 1.0 mM, respectively, to the pooled fraction. In certain experiments where ATP kinetics were investigated this nucleotide was not added. Unless the enzyme was kept in the presence of KCl, dithiothreitol, K_2SO_4, and ATP, aggregation of the protein occurred within an hour. Addition of the above compounds maintained the nonaggregated state for several hours. In the presence of the above compounds, the enzyme aggregated when left overnight on ice; yet, little or no loss of specific activity occurred.

The phosphofructokinase reaction was followed spectrophotometrically at 340 nm using a Cary Model 118 recording spectrophotometer. The light beam was masked both horizontally and vertically to avoid stray light effects. Full-scale range of 0.1 absorbance was used in the spectrophotometer. Kinetic experiments were carried out using a 100-μl reaction mixture in 10-mm, dark-walled, semimicrocuvettes at room temperature. No significant change in enzymatic specific activity was observed during the experimental period.

The forward reaction was measured by coupling phosphofructokinase to the pyruvate kinase–lactic dehydrogenase coupled reaction (ADP determination system). Reactions were initiated by the addition of phosphofructokinase which was diluted tenfold in the assay. No change in the kinetic behavior was observed if the enzyme was added to the assay mixture prior to the addition of the alternative substrate. No apparent effects were observed if the enzyme concentration was decreased by one-half or increased twofold. The assay contained 50 mM imidazole chloride, pH 6.9, 100 mM KCl, 1 mM K_2SO_4, 5 mM dithiothreitol, 0.1 mM EDTA, 0.1 mg/ml bovine serum albumin, 0.8 mM $MgCl_2$, 0.15 mM NADH, 0.4 mM phosphoenolpyruvate, and 5 units/ml each of pyruvate kinase and lactate dehydrogenase. Assays which displayed high initial velocity rates were retested with 12 units/ml each of coupling enzymes. The coupling enzymes were desalted on a G-25-80 Sephadex column.

Results

Assay of Phosphofructokinase Activity with Fructose 6-Sulfate at pH 6.9. Enzymatic assay of phosphofructokinase at pH 6.9 with the alternative substrate was carried out by coupling the enzyme with pyruvate kinase and lactic dehydrogenase to measure ADP production. Use of the aldolase–α-glycerophosphate dehydrogenase coupled assay was not practical because the second product of the reaction, fructose 1-phosphate 6-sulfate, was shown to be a poor substrate for aldolase (Martensen and Mansour, 1976a). The use of high concentration of aldolase could not be adopted as part of the procedure because it has been found to inhibit phosphofructokinase (El-Badry et al., 1973). Furthermore, as will be indicated below, the ADP-coupled assay was necessary to maintain a fixed level of ATP concentration. This was particularly important at low concentrations of ATP. The alternative product of the reaction using fructose 6-sulfate as the substrate was shown to be a poor activator of phosphofructokinase [$K_a \simeq 1$ mM]. Thus, the accumulation of the fructose diester would have little influence on the enzymatic rate since all initial velocity data were obtained prior to 8 μM product formation.

Potassium and sulfate ions are known to stimulate phosphofructokinase activity at pH 6.9. Since the potassium salt of fructose 6-sulfate was varied over a 40-fold range in some of the experiments, the effect of the additional potassium ion was negated by carrying out all assays in the presence of 100 mM KCl. The possibility that sulfate ion produced by hydrolysis or contamination of the alternative substrate would affect the reaction was investigated. Turbidimetric assay for free sulfate ion (Grazi et al., 1973) of the alternative substrate preparation showed less than 0.5%. To minimize any contribution this small amount might have on the initial velocities measurements at different concentrations of the alternative substrate, all assays contained 1.0 mM K_2SO_4. Addition of glucose 6-sulfate to an assay using inhibitory concentrations of ATP did not stimulate the rate, indicating that observed activity changes were not due to increasing concentrations of carbohydrate sulfate ion.

A plot of initial velocity vs. fructose 6-sulfate concentration is shown in Figure 1A. In these experiments fructose 6-sulfate was varied in the presence of the optimal as well as inhibitory levels of ATP. The effect of AMP, the allosteric activator, on the response of enzyme to fructose 6-sulfate at optimal concentration of ATP was also studied. (See below). In the presence of the optimal ATP concentration (10 μM), the curve for velocity vs. substrate concentration was slightly sigmoidal. Increasing the concentration of ATP above 10 μM resulted in a sharp decrease in enzyme activity. A single V_{max} was obtained from reciprocal plots of the data for enzyme activity

with 10 μM of ATP in the presence as well as in the absence of 500 μM of AMP (Figure 1C). A Hill plot of the data (Figure 1B) using the V_{max} extrapolated from the reciprocal plots gave interaction coefficients of 1.0 and 1.3 in the presence and absence of AMP, respectively. A V_{max} could not be experimentally obtained from the data with 20 and 60 μM ATP for calculating Hill plot interaction coefficients. The data were analyzed by an Eadie-Scatchard plot (Segel, 1975). An interaction coefficient can be approximated by plotting $1/V$ vs. $1/S^n$. The value of n was determined from the exponent that gave the best straight line. The n value obtained with 20 μM ATP was 2.2 ($r^2 = 0.999$). This analysis of the data with 60 μM ATP could not differentiate values of n from 1.3 to 2.2. The above results suggest that the interaction coefficient increases as a result of ATP binding to the enzyme. The presence of 0.5 mM AMP at the optimal level of ATP (10 μM) caused a marked increase in enzyme activity and a hyperbolic response to fructose 6-sulfate. Activation by AMP was due to a greater than sixfold increase in the apparent binding affinity to fructose 6-sulfate and a reduction of its interaction coefficient to 1.0. The apparent K_m for fructose 6-sulfate with AMP (4 mM) agrees well with the value of 3.3 mM obtained at pH 8.2 (Martensen and Mansour, 1976b).

Effect of ATP and GTP on Phosphofructokinase Activity at pH 6.9. At pH 8.2 when fructose 6-sulfate was used as the phosphoryl acceptor (Martensen and Mansour, 1976b), the K_d for ATP binding (6 μM) was found to be in good agreement with the K_d obtained with the natural substrate, fructose-6-P. Kinetic determination of the K_m for ATP at pH 6.9 using the natural substrate is complicated by the interrelationship between the cooperative response to fructose 6-phosphate and the inhibitory response to ATP. As the ATP concentration was increased above a certain value, inhibition of enzymatic activity occurred. This threshold was affected by the concentration of fructose 6-phosphate in the assay. The effect of ATP on enzymatic activity with the alternative substrate was investigated to determine if the binding affinity of the enzyme at 0.1 mg/ml for ATP was affected by the change in pH from 8.2 to 6.9 and if the use of the alternative substrate affected the response of enzymatic activity to ATP. The effect of ATP and GTP concentrations on the initial velocity of the enzyme is shown in Figure 2. The concentration of the triphosphate nucleosides ranged from 0 to 20 μM. The enzymatic activity was sensitive to concentrations of ATP less than 10 μM. This would be expected if the equilibrium between enzyme and the two substrates is attained rapidly relative to the breakdown of the ternary complex as was the case at pH 8.2 where the reaction mechanism was described by the ordered equilibrium case. The slight activity which existed in the absence of added nucleotide in the experiment depicted in Figure 2 may be attributed to ATP bound to the enzyme. This amount of ATP was estimated to be 0.2 μM from the difference in the initial velocities in the absence and presence of added ATP concentrations which gave a linear response. In the absence of the alternative substrate no significant enzyme activity was observed. The initial velocity response to increasing concentrations of ATP appeared to plateau at 10 μM and then decreased rapidly at higher concentrations. Enzyme activity at low ATP concentrations was greater than activity obtained with equivalent concentrations of GTP. However, with GTP the activity continues to increase and appears to plateau at a concentration of 20 μM. Concentrations above 20 μM of GTP inhibited enzymatic activity. Double-reciprocal plots (not shown) of the data of Figure 2 from 2 to 10 μM of GTP gave a straight line with an apparent K_m for GTP binding of 10 μM. If this K_m value approximates

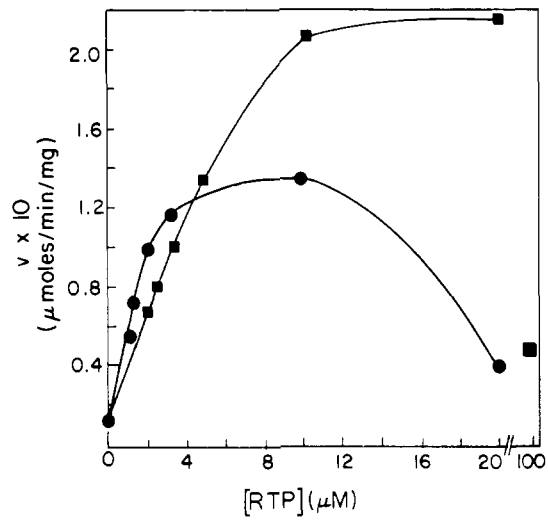

FIGURE 2: Effect of nucleoside triphosphates on phosphofructokinase activity at pH 6.9. Fructose 6-sulfate concentration was 3.9 mM. Plots of initial velocity vs. ATP (●) or GTP (■) concentration are shown.

the K_d, no correction of the data is required for GTP bound to the enzyme since it would not amount to more than 8.5% of the lowest GTP concentration (2 μM). This would be in good agreement with the value of 9 μM obtained at pH 8 with muscle phosphofructokinase (Bar-Tana and Cleland, 1974). A double-reciprocal plot obtained using ATP as the variable substrate was nonlinear. Since several ATP concentrations were so low as to be affected by the concentration of the enzyme, an apparent K_m could not be calculated.

Effect of ATP on the Response of Phosphofructokinase to AMP. AMP affects phosphofructokinase activity by abolishing the cooperative interactions with fructose 6-sulfate. Experiments were carried out to study the relationship between ATP inhibition and AMP activation. Therefore, the effect of AMP was tested at a nonsaturating concentration of fructose 6-sulfate in the presence of suboptimal (3.3 μM), optimal (10 μM), and inhibitory (20 μM) concentrations of ATP (Figure 3A). Both the extent and rate of activation of phosphofructokinase activity by increasing concentrations of AMP appear to be dependent on the concentration of ATP. The extent of the activation appears to be a saturable function of the ATP concentration. The change in rate is illustrated by a double-reciprocal plot (Figure 3B) of $(\Delta v)^{-1}$ vs. $(AMP)^{-1}$, where Δv is the difference between a velocity obtained at a particular concentration of AMP and a velocity obtained in its absence. At the suboptimal concentration of ATP, the response to AMP is nearly linear, analogous to that of a simple activator which increases the binding affinity of fructose 6-sulfate to the enzyme. As the ATP concentration is increased to optimal and inhibitory levels, an increase in the parabolic nature of the reciprocal plots also occurs. This is illustrated by Hill plots of the data shown in the outset of Figure 3B. Interaction coefficients of 1.1, 1.3, and 1.7 are obtained at ATP concentrations of 3.3, 10, and 20 μM, respectively. The value of the interaction coefficient for AMP at 20 μM ATP is not changed by using observed v vs. Δv for the Hill plot. Under this condition AMP acts analogously to an essential activator. The increase of the AMP interaction coefficient with increasing ATP concentrations indicates that allosteric inhibition by ATP changes the enzymatic activation by AMP from Michaelian to cooperative kinetics. Extrapolation of the reciprocal-plot data of Figure 3B appears to give a common intercept value of 6 μM for the binding constant of AMP. This would be in good agreement

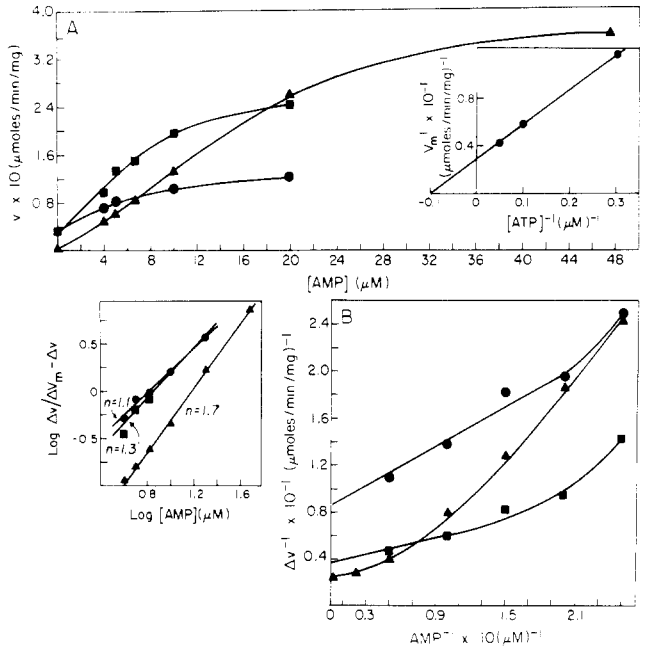

FIGURE 3: Effect of ATP on the response of phosphofructokinase activity to AMP at pH 6.9. Fructose 6-sulfate concentration was 3.9 mM. (A) Plot of initial velocity vs. AMP concentration with 3.3 μM ATP (●), 10 μM ATP (■), and 20 μM ATP (▲). Inset: Lineweaver–Burk plot of apparent V_{max} vs. ATP concentration. (B) Lineweaver–Burk plot of activation vs. AMP concentration. Outset: Hill plot of the data used for B.

with the K_d of 5 μM obtained from equilibrium dialysis binding studies (Setlow and Mansour, 1972). If a replot of apparent ΔV_{max} is plotted against the concentrations of ATP at 3.3 and 10 μM (data not shown), the line passes through the origin. If the reciprocal of apparent V_{max} obtained from a double-reciprocal plot of the data of Figure 3A is plotted against the reciprocal of the ATP concentration (inset Figure 3A), a straight line is observed. An apparent K_m for ATP of 10 μM was obtained. The amount of ATP bound to the enzyme at the lowest ATP concentration would be 8% of the total if the K_m approximates the K_d. This percentage is balanced by the estimated amount of ATP carried over with the enzyme into the assay which was estimated to be approximately 0.2 μM (see above). Thus no corrections were made on the kinetic plots. These data indicate that the stimulation of phosphofructokinase activity by AMP occurs concomitantly with the binding of ATP. The optimal concentration of ATP (10 μM) in the absence of AMP should not be considered as a saturation value but as a threshold value for the ATP inhibitory response.

Effect of AMP on ATP Inhibition. Since increasing ATP concentrations resulted in a change from Michaelian to sigmoidal kinetics for AMP activation, the effect of AMP on ATP inhibition was studied. The response of enzymatic velocity as a function of ATP concentrations in the presence of 0, 10, 20, and 48 μM AMP is shown in Figure 4A. When no AMP is present, the threshold for ATP inhibition occurs at less than 10 μM ATP. As AMP concentration was increased, the inhibitory threshold occurred at higher concentrations of ATP. The influence of AMP on the inhibitory response was studied by using the Hill equation for an allosteric inhibitor (Pogell and Taketa, 1965; Cooperman and Buc, 1972). The allosteric inhibitor (I = ATP) would combine with the enzyme (E) (or an enzyme-substrate complex) to form an inactive complex (EI_n) with n sites in the following fashion:

$$E + nI = EI_n$$

$$K = [EI_n]/([E][I]^n)$$

If EI_n is catalytically inactive, then $V_m - v$ can be used as EI_n, where V_m is the optimal activity observed with ATP; v would equal (E), the noninhibited enzyme, to yield:

$$\log\left(\frac{V_m - v}{v}\right) = -\log K + n \log [I]$$

A plot of log $[(V_m - v)/v]$ vs. log [ATP] (Figure 4B) was drawn from the data using the optimal activity observed as V_m. The slopes of the Hill plots gave an interaction coefficient of 3.5 for the data in the absence as well as in the presence of the three concentrations of AMP. When values for ATP concentrations which gave 50% inhibition of the optimal velocity were obtained from the graph and plotted against the concentration of AMP, a linear relationship was seen (data not shown). Although AMP did not affect the interaction coefficient for ATP inhibition, it altered the threshold at which inhibition appeared. This result is analogous to the effect of AMP on the response of yeast phosphofructokinase to fructose-6-P in the presence

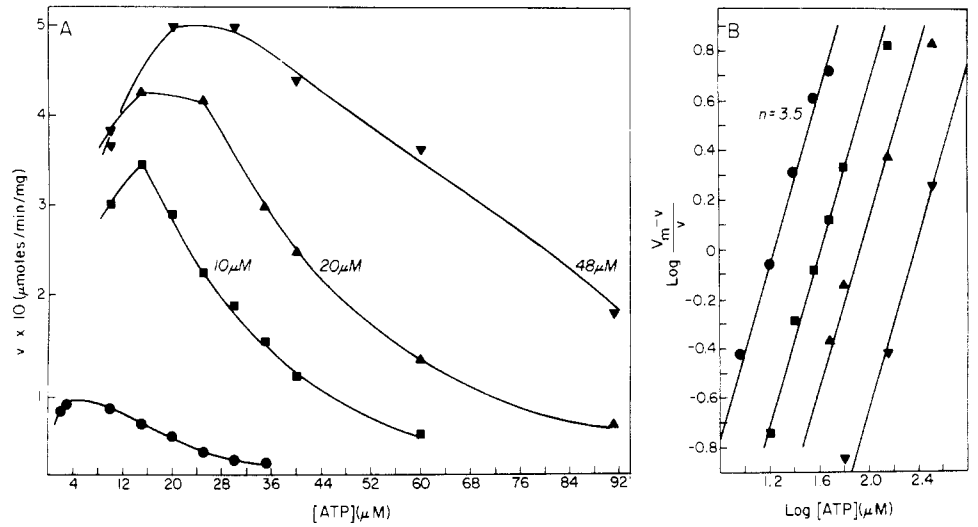

FIGURE 4: Effect of AMP on the response of phosphofructokinase activity to ATP. Fructose 6-sulfate concentration was 3.9 mM. (A) Plot of initial velocity vs. ATP concentration with no AMP (●), 10 μM AMP (■), 20 μM AMP (▲), and 48 mM AMP (▼). (B) Hill plot for allosteric inhibition by ATP for the data of A.

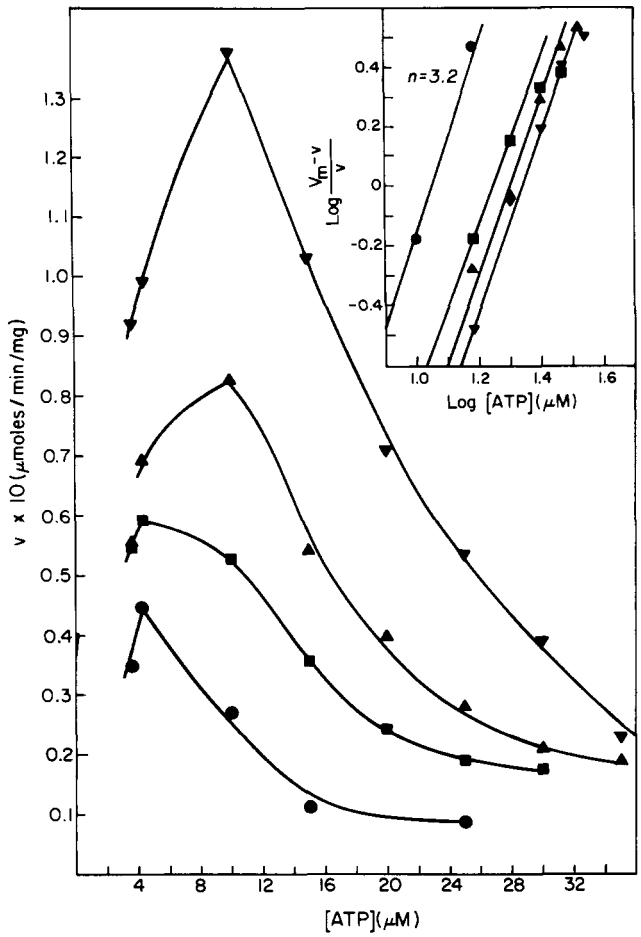

FIGURE 5: Effect of fructose 6-sulfate on inhibition of phosphofructokinase activity by ATP. Fructose 6-sulfate concentration was 3.9 (●), 5.8 (■), 7.7 (▲), and 11.6 mM (▼). Inset: Hill plot for allosteric inhibition by ATP.

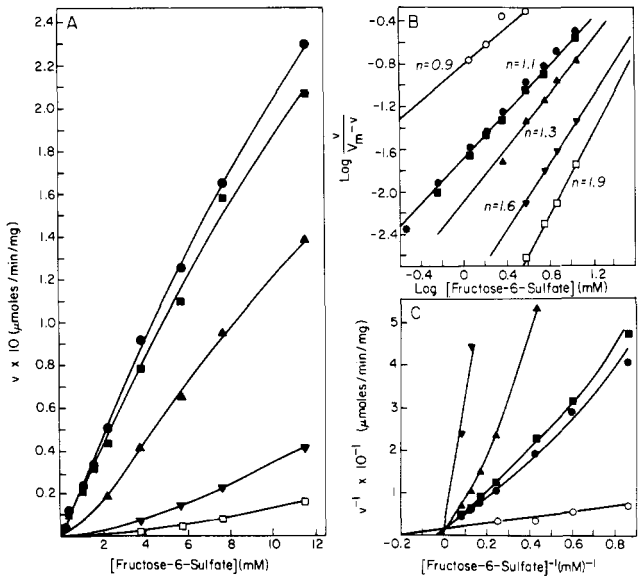

FIGURE 6: Effect of citrate on the response of phosphofructokinase activity to fructose 6-sulfate. ATP concentration was 10 μM. (A) Plot of initial velocity vs. fructose 6-sulfate concentration with no citrate (●), 5 μM citrate (■), 25 μM citrate (▲), 75 μM citrate (▼), and 250 μM citrate (□). (B) Hill plot of the data of A including data with 500 μM of AMP (not shown in Figure 6A). (C) Lineweaver–Burk plot of the data of A with three concentrations of citrate and with 500 μM AMP (○).

of ATP or ITP (Atkinson et al., 1965). The molecular significance of the high interaction coefficient for ATP binding is not clear, but it might suggest that ATP interacts with at least four sites to produce inhibition.

Effect of Fructose 6-Sulfate on ATP Inhibition. The relationship between fructose 6-sulfate concentration and ATP inhibition was investigated to see if the kinetic order of the inhibition by ATP was affected by the concentration of the alternative substrate. The response of enzymatic activity to increasing ATP concentrations at 3.87, 5.8, 7.8, and 11.6 mM fructose 6-sulfate is shown in Figure 5. The effect of higher concentrations of the alternative substrate on the ATP inhibition profile is qualitatively like that observed when adding higher concentrations of AMP; the inhibitory threshold for ATP concentrations is increased. A Hill plot of the inhibition data (inset Figure 5) shows that the interaction coefficient for ATP inhibition (3.2) was not altered by changing the concentration of fructose 6-sulfate over the range of 3.9 to 12 mM. The ATP concentration which gave 50% inhibition of the optimal velocity appears to vary linearly with the concentration of the alternative substrate (plot not shown).

Effect of Citrate on the Response of Phosphofructokinase Activity to Fructose 6-Sulfate. The activity of phosphofructokinase from a variety of sources is known to be inhibited by citrate. The effect has been attributed to the dissociation of active tetramers to inactive dimers (Lad et al., 1973). In the experiments presented here the assay concentration of enzyme was approximately two orders of magnitude higher than that used in other studies with the natural substrate. It was of interest to determine the effect of citrate at high enzyme concentrations which might favor aggregated forms. The effect of citrate concentrations from 5 to 250 μM on the response of phosphofructokinase activity to increasing concentrations of the alternative substrate at an ATP concentration of 10 μM is shown in Figure 6A. A citrate concentration of 5 μM, while inhibitory, does not result in a significant change of the interaction coefficient for fructose 6-sulfate (Figure 6B). At citrate concentrations of 25, 50, and 250 μM interaction coefficients were obtained for the alternative substrate of 1.3, 1.6, and 1.9, respectively, indicating that increasing citrate concentrations cause an increase in the cooperative response to the alternative substrate. The kinetic effect of citrate on enzymatic activity with fructose 6-sulfate may be seen from a double-reciprocal plot of the data (Figure 6C). Included in the figure for the reciprocal data is a plot for enzyme activity in the presence of 500 μM AMP. The data show that the plots of enzyme activity with citrate, AMP, or without additions have a common ordinate intercept. Citrate inhibition results from a severalfold increase in the apparent binding constant for the alternative substrate. A plot of v/v_0 against citrate concentration (data not shown), where v_0 is the velocity in the absence of citrate and v is the velocity in the presence of the inhibitor, was made for the four highest concentrations of fructose 6-sulfate. It was found that the decrease in v/v_0 with citrate was inversely related to the concentration of the alternative substrate. Thus, the inhibition of phosphofructokinase activity by citrate decreases as the concentration of the alternative substrate increases. The inhibitory interaction coefficient for citrate (data not shown) was found to be 1.7 and 2.0 at 3.9 and 12 mM of fructose 6-sulfate, respectively.

Effect of Citrate on the Response of Phosphofructokinase Activity to ATP. The relationship between ATP and citrate inhibition appears to be complex. It has been reported that the inhibitory action of both ligands is synergistic (cf. review by

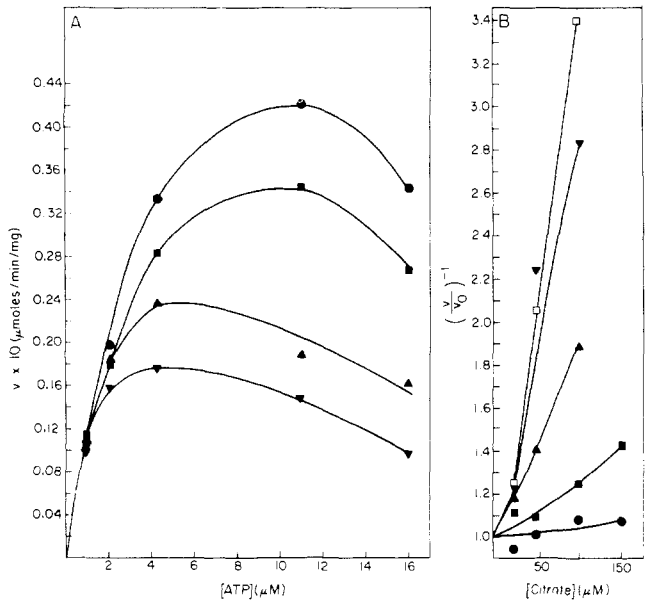

FIGURE 7: Effect of citrate on the response of phosphofructokinase activity to ATP. Fructose 6-sulfate concentration was 2.3 mM. (A) Plot of initial velocity vs. ATP concentration with no citrate (●), 25 μM citrate (■), 50 μM citrate (▲), and 100 μM citrate (▼). (B) Plot of v_0/v vs. citrate concentration with 1 μM ATP (●), 2.1 μM ATP (■), 4.3 μM ATP (▲), 11 μM ATP (▼), and 16 μM ATP (□).

Lardy and Bloxham, 1973). To minimize the chelation effect of citrate on metal ion concentration, the Mg^{2+} concentration was raised to 4 mM in the assay. Concentrations above this level were inhibitory. The effect of citrate on enzymatic activity at low concentrations of ATP is shown in Figure 7A. The response of enzymatic activity to ATP when citrate was excluded was hyperbolic for the lowest three concentrations of ATP and gave an apparent K_m of 10 μM. Addition of 25 μM citrate to the assay resulted in a proportional decrease of activity at each level of ATP. The inhibition then could not be due to citrate competition for ATP at the active site. Citrate concentrations of 50 and 100 μM caused not only a decrease in velocity but a shift in the optimal ATP concentrations to a value less than half that observed when citrate was absent. Thus inhibition may be due to a decreased ability of the enzyme to bind fructose 6-sulfate. This could be caused by citrate binding at an allosteric site as well as an increased ability for ATP to bind at an inhibitory allosteric site. The synergistic effect of the two inhibitors is shown in Figure 7B.

Discussion

Replacement of the phosphoryl moiety at the sixth position of fructose-6-P with a monoanionic sulfuryl group yields a useful substrate analogue for the study of allosteric kinetics of phosphofructokinase. The reduced catalytic efficiency enables kinetic assays to be carried out at enzyme concentration of 100 μg/ml which is within the enzyme concentration range that exists in heart muscle (Mansour et al., 1966). Investigations on the allosteric kinetics of phosphofructokinase using highly diluted enzyme with the natural substrate could be complicated by artifacts that arise from the instability of the enzyme. It has been suggested that increasing concentrations of the natural substrate, fructose-6-P, could result in an increase in the stabilization of the enzyme and give an apparent cooperative response (Lardy and Bloxham, 1973). Such a possibility may indeed account for the high interaction coefficient values that have been reported in the literature as a measure of cooperativity between the binding sites for fructose-6-P (Kemp, 1969) in which values from two to eight were obtained.

Studies on the allosteric kinetics of phosphofructokinase are further complicated by the finding that the response to many effectors is influenced by the concentration of the enzyme in the assay mixture (Karadsheh et al., 1974; Kemp, 1971). For example, the inhibitory action of ATP was reported to be decreased as the concentration of the enzyme was increased (Kemp, 1971). Other factors which complicate investigations on the allosteric kinetics of phosphofructokinase are that the enzyme is activated by the products of the reaction, ADP and fructose 1,6-P_2, and is inhibited by aldolase used in the aldolase-α-glycerophosphate dehydrogenase coupled assay system (El-Badry et al., 1973). The results reported above with the alternative substrate, fructose 6-sulfate, overcome many of these difficulties. For example, under the present assay system using phosphofructokinase at a concentration of 100 μg/ml, the enzyme is known to be stable (Mansour and Ahlfors, 1968) and, therefore, no change in the specific activity would be expected when the enzyme was diluted to this concentration. Furthermore, the velocities obtained by starting the reaction with the alternative substrate were not different from those obtained by starting the reaction with the enzyme. This was not the case when the enzyme was highly diluted (Kemp, 1971). Effects of enzyme concentration on the response to different modifiers were excluded since the enzyme concentration used through the entire investigation was fixed at 100 μg per ml. Neither the products of the phosphofructokinase reaction nor those of the coupling enzymes at the levels that are produced in the assay system had any influence on the kinetics of the enzyme. Thus, the effects observed in our studies appear to be due to the interaction of the alternative substrate and other metabolic effectors at specific sites on stable enzyme species.

The allosteric kinetics of phosphofructokinase using the alternative substrate, fructose 6-sulfate, are qualitatively similar to those reported before, using the natural substrate, fructose-6-P. The results reported above clearly show that ATP is the predominant effector of the enzyme at pH 6.9. At suboptimal concentrations of ATP, the response of enzyme activity to increasing concentrations of the alternative substrate is only slightly cooperative, as indicated by the low interaction coefficient (α = 1.3). Cooperative kinetics were shown to be dependent on ATP concentration. As the concentration of ATP in the assay was increased above the optimal level, enzyme activity at a fixed concentration of the alternative substrate decreased and cooperativity increased. Thus, ATP shows its heterotropic inhibitory effect in the presence of the alternative substrate, fructose 6-sulfate. When GTP was used as the phosphoryl donor, the inhibition was less pronounced than with ATP. Presumably in the presence of GTP greater saturation of the substrate site occurred before an inhibitory response was observed. This could be due to a higher affinity of the inhibitory site for ATP than for GTP, or that the interactions of GTP at the inhibitory sites do not give the equivalent inhibitory response as with ATP. It has previously been reported that ATP inhibition occurs only after saturation of the substrate site (Lardy and Bloxham, 1973). Our data do not support this concept. Inhibition by ATP appears to either coincide or occur prior to saturation of the substrate site with ATP. The significance of this is important but not easily explained. It should not be concluded that saturation of the catalytic ATP site per se results in inhibition since binding studies have shown that more than one site for ATP per protomer exists. Values of three to four sites have been reported (Lorenson and Mansour, 1969;

Kemp and Krebs, 1967). Binding studies in this laboratory (unpublished observations) with an ATP analogue adenylyl imidodiphosphate show two sites per protomer with differing affinities. AMP and fructose-6-P abolish binding at the site with lower affinity. Cooperative interactions between ATP inhibitory sites were suggested from allosteric studies using the natural substrate (Mansour and Ahlfors, 1968). Atkinson (1970) has reported that inactivation of the yeast phosphofructokinase is approximately negative first order. Our data with the heart enzyme suggest that the order is either 3 or 4. Neither the alternative substrate nor the allosteric activator, AMP, changes the order of the inhibition. If one assumes the active form of phosphofructokinase is a tetramer, then it could be postulated that the interaction coefficient of four describes the effect of ATP on four protomers of the tetramer.

Allosteric inhibition by ATP appears to decrease the binding of fructose 6-sulfate prior to saturation of the catalytic site for ATP. This suggests that saturation of the catalytic site with ATP is closely coupled with the onset of a highly cooperative ATP inhibitory response possibly from an inhibitor site which affects the binding of the alternative substrate. This explains why the activation by AMP closely follows saturation of the ATP substrate site. Increasing the concentrations of fructose 6-sulfate and AMP allows increased saturation of the ATP substrate site prior to the onset of inhibition. The increase in ATP concentration likewise causes the cooperative response to AMP and the alternative substrate to increase to a limiting value of two.

Kinetic effects of citrate are similar to those of ATP. Cooperative interactions with fructose 6-sulfate in the presence of citrate are evident from an increase in the interaction coefficient to approximately two. Yet, an interaction coefficient with citrate appears to be two rather than four as seen with ATP. The concentrations of citrate necessary to observe inhibition are similar to those found in our studies using the natural substrate with a highly diluted enzyme. Thus, increasing the protein concentration two orders of magnitude has no effect on the inhibitory activity of citrate. The effect of citrate should not be considered as dependent on ATP concentration but instead as synergistic with ATP inhibition. Binding of both ligands results in inhibition and their combined effects are more than additive. This behavior is kinetically explained by different inhibitory binding sites for citrate and ATP with synergy of binding. Since the inhibition by both compounds is kinetically competitive with the alternative substrate, the effects of both inhibitors are analogous to the case of two competitive inhibitors binding at different sites as described by Yonetani and Theorell (1964). However, this does not suggest that citrate and ATP bind at the fructose-6-P site.

The data reported above clearly showed different degrees of cooperative kinetics with different ligands. For example, cooperativity between the sites for fructose 6-sulfate, AMP, and citrate gave a maximum interaction coefficient of approximately 1.8, while the value for the inhibitory kinetics for ATP was about 3.5. It is realized that the presentation of a model to explain these kinetic effects may be an oversimplification of a very complex system which involves changes in the affinity for different ligands (Hill and Hammes, 1975) as well as the degree of polymerization. Phosphofructokinase was reported to exhibit different degrees of polymerization that are dependent on pH, enzyme, and ligand concentration (Mansour, 1972; Mansour and Ahlfors, 1968; Ling et al., 1965; Lad et al., 1973). Mg-ATP, ATP, AMP and fructose-6-P have been reported to stabilize the fully active tetrameric form of phosphofructokinase at a concentration of 0.15 mg/ml and pH 7.0 while millimolar concentrations of citrate stabilized the inactive dimers (Lad et al., 1973). Accordingly, inhibition by citrate could be due to stabilization of the inactive dimeric form of the enzyme as well as decreasing the affinity for the hexose ester as a result of conformational changes. Inhibition by ATP appears to be due to weakening of the binding for fructose-6-P because of cooperative conformational changes between the subunits. When the ATP concentration is low, the kinetics of fructose 6-sulfate are hyperbolic with no homotropic interactions. Increasing ATP concentration results in highly cooperative conformational change which decreases the affinity for fructose 6-sulfate but does not appear to alter the polymeric state of the enzyme (Lad et al., 1973). The Hill coefficient for ATP binding to the inhibitory sites was 3.5, suggesting occupation of four sites in the tetramer. In the case of fructose 6-sulfate and AMP, the Hill coefficient was not greater than 1.8, which indicates that minimally two sites are involved in the homotropic interactions of the enzyme. Whether these are the true number of sites in the tetrameric inhibited form of the enzyme or simply those that are involved in the cooperative interaction will have to be ascertained by further physical measurements. The availability of an alternate substrate that allows kinetics to be studied with enzyme concentrations that are within the same range of those used for physical studies should enable direct information on the relationship between structure and activity of the enzyme.

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Mechanism of Pancreatic Lipase Action. 1. Interfacial Activation of Pancreatic Lipase†

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ABSTRACT: Hydrolysis of dissolved *p*-nitrophenyl acetate by pancreatic lipase follows the classical acyl enzyme pathway already proposed for other esterases. Kinetic parameters of the hydrolysis have been determined. The turnover rate of the reaction is many orders of magnitude slower than that for the natural emulsified substrates. Nevertheless, several arguments are in favor of the specificity of this hydrolysis: (1) triacetin, which resembles the usual substrates for the enzyme, is also hydrolyzed very slowly in solution; (2) dissolved triacetin and tripropionin are competitive inhibitors for the *p*-nitrophenyl acetate hydrolysis; (3) the same chemical structural features which are required in the case of emulsified substrates are also necessary to promote hydrolysis of dissolved *p*-nitrophenyl esters. This suggests that the same active site (or a part of the same active site) is responsible for hydrolysis of both *p*-nitrophenyl acetate and specific emulsified substrates. Since deacylation is the rate-limiting step in the catalysis of *p*-nitrophenyl acetate, the intermediate acetyl enzyme can be isolated by trapping it at pH 5.0. Kinetic competence of this intermediate has been demonstrated. Hydrolysis by pancreatic lipase of dissolved monomeric *p*-nitrophenyl acetate and triacetin is considerably enhanced (100- to 500-fold) by various interfaces. This suggests that at least the deacylation step, which is rate limiting in absence of interface, is accelerated by the presence of inert interfaces. Siliconized glass beads were directly shown to accelerate the deacylation of isolated [^{3}H]acetyl lipase by at least a hundred times. This step does not directly involve the ester substrate. Thus, it is suggested that a part of the activation of lipase at interfaces may be due to a conformational change resulting from adsorption.

Enzymes acting on insoluble lipids are of special interest for their substrates and are not molecularly dispersed in water, but form aggregates separated from water by an interface. Therefore, these enzymes provide typical examples of heterogeneous catalysis (Sémériva and Desnuelle, 1976) and they offer simple models of lipid–protein associations in membranes and other biological systems.

For example, it is noteworthy that the activity of the two best known lipolytic enzymes, pancreatic lipase (EC 3.1.1.3) and phospholipase A_2 (EC 3.1.1.4), is not impaired but, on the contrary, considerably enhanced by the insolubility of their substrates (Sarda and Desnuelle, 1958; Pieterson et al., 1974).

Several hypotheses have been proposed to explain the necessity of an interface for the full expression of lipase activity (Sémériva and Desnuelle, 1976). The simplest one is to assume that lipase preferentially reacts at the interface simply because it has no affinity for soluble molecules.

On the other hand, interfacial activation can also arise either because the substrate acquires new properties at the interface or because the enzyme itself is modified by adsorption. These two hypotheses are not exclusive and it is probably more sensible to think that the activation results from the sum of several factors.

Some of these factors have been invoked to account for the interfacial activation, such as the increase of substrate local concentration at the interface (Brockman et al., 1973), a better orientation of the strategic ester bond (Garner and Smith, 1970; Zografi et al., 1971; Wells, 1974), or the reduction of the water shell normally surrounding ester molecules dissolved in water (Brockerhoff, 1973). The alternative proposal postulating a conformational change of lipase by adsorption (Sarda and Desnuelle, 1958) has been supported by recent data (Esposito et al., 1973; Entressangles and Desnuelle, 1974). However, no conclusion can be unambiguously drawn, since, in all the results presented above, the properties of both substrate and enzyme are affected by the presence of the interface. This activating effect of interfaces will not be understood unless the mechanism of action of the enzymes on dissolved substrates is more precisely known and compared with that on emulsified substrates. Pancreatic lipase has already been shown to act at a quite measurable rate on dissolved tripropionin (Entressangles and Desnuelle, 1974; Brockman et al., 1973) and PNPA[1] (Sémériva et al., 1974) in the presence of low concentrations of water-miscible organic compounds. Data obtained with this latter substrate were consistent with an acyl enzyme mechanism (Sémériva et al., 1974).

The first purpose of the present paper is to report new results confirming that the hydrolysis of dissolved substrates by lipase

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[1] Abbreviations used are: PNPA, *p*-nitrophenyl acetate; NADH, reduced nicotinamide adenine dinucleotide; ADP, ATP, adenosine di- and triphosphates.