Hexose Phosphate Binding Sites of Fructose-6-phosphate,2-kinase:Fructose-2,6-bisphosphatase

INTERACTION WITH N-BROMOACETYLETHANOLAMINE PHOSPHATE AND 3-BROMO-1,4-DIHYDROXY-2-BUTANONE 1,4-BISPHTOSPHATE*

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N-Bromoacetylethanolamine phosphate and 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate have been tested in order to study the hexose phosphate binding sites of a bifunctional enzyme, fructose-6-P,2-kinase:fructose-2,6-bisphosphatase. N-Bromoacetylethanolamine phosphate is a competitive inhibitor with respect to fructose-6-P (K_i = 0.24 mM) and a noncompetitive inhibitor with ATP (K_i = 0.8 mM). The reagent inactivates fructose-6-P,2-kinase but not fructose-2,6-bisphosphatase, and the inactivation is prevented by fructose-6-P. The inactivation reaction follows pseudo first-order kinetics to completion and with increasing concentrations of N-bromoacetylethanolamine phosphate a rate saturation effect is observed. The concentration of the reagent giving the half-maximum inactivation is 2.2 mM and the apparent first order rate constant is 0.0046 s^{-1}. The enzyme alkylated by N-bromoacetylethanolamine-P has lost over 90% of the kinase activity, retains nearly full activity of fructose-2,6-bisphosphatase, and its inhibition by fructose-6-P is not altered.

3-Bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate is also a competitive inhibitor of fructose-6-P,2-kinase with respect to fructose-6-P in the forward reaction and fructose-2,6-P_2 in the reverse direction. This reagent inhibits 93% of fructose-6-P,2-kinase but activates fructose-2,6-bisphosphatase 3.7-fold. 3-Bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphatase alters the fructose-2,6-P_2 saturation kinetic curve from negative cooperativity to normal Michaelis-Menten kinetics with K_{0.5} of 0.8 mM. The reagent, however, has no effect on the fructose-6-P inhibition of the phosphatase.

These results strongly suggest that hexose phosphate binding sites of fructose-6-P,2-kinase and fructose-2,6-bisphosphatase are distinct and located in different regions of this bifunctional enzyme.

The synthesis of fructose-2,6-P_2 is catalyzed by Fru-6-P,2-kinase (Equation 1) (1-3). This enzyme also catalyzes a slow ATP-ADP exchange, ATP hydrolysis, and the reversal of the forward reaction (4). The degradation of fructose-2,6-P_2 is catalyzed by Fru-2,6-bisphosphatase (Equation 2) (5-8).

\[
\text{Fructose-6-P + ATP} \rightleftharpoons \text{fructose-2,6-P_2 + ADP} \tag{1}
\]

\[
\text{Fructose-2,6-P_2 + H}_2\text{O} \rightarrow \text{fructose-6-P + P} \tag{2}
\]

The enzyme is strongly inhibited by fructose-6-P (6, 8, 9). Both Fru-6-P,2-kinase and Fru-2,6-bisphosphatase activities reside in the same protein (6, 8, 9), consequently an interesting question raised is whether the active sites of both enzymes are overlapping or two distinct sites.

We have shown that a limited proteolysis by trypsin results in nearly complete loss of Fru-6-P,2-kinase without significant loss of Fru-2,6-bisphosphatase (10). The former is also inactivated completely by photoaffinity labeling the enzyme with 8-azido-ATP, which serves as a substrate, but the latter enzyme is not affected by the treatment. Moreover, the demonstration that the native enzyme but not the partially digested enzyme can be photoaffinity labeled with 8-azido-ATP led us to conclude that tryptic digestion results in a cleavage of the ATP binding site from the Fru-6-P,2-kinase. These results provide evidence in support of a hypothesis for two distinct sites for the kinase and the phosphatase. However, they do not rule out a possibility that the active sites are indeed overlapping, but only a part of the active site, i.e. the ATP binding site, is cleaved by the proteolysis without affecting the fructose-6-P and fructose-2,6-P_2 binding sites which may be shared by both enzymes. In order to investigate this possibility synthetic substrate analogs, N-bromoacetylethanolamine phosphate (11) and 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate (12), were examined as possible active site and/or inhibitory site-directed reagents in order to explore the fructose-6-P binding sites of Fru-6-P,2-kinase and Fru-2,6-bisphosphatase, and the fructose-2,6-P_2 binding site of the latter enzyme. N-Bromoacetylethanolamine-P is shown to be a specific active site-directed inactivator of fructose bisphosphatase aldolase and alkylates either an essential histidine residue or an essential lysine residue depending on reaction conditions (12). 3-Bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate affinlty labels two essential lysine residues in ribulose-bisphosphate carboxylase (13, 14).
Materials and Methods

BrAcNHEtOP$^1$ and BrDHBP$_2$ were synthesized as before (11, 15). Fructose-2,6-[2$^{32}$P]P$_2$ was prepared as before (7). Fructose-2,6-bisphosphatase was prepared from fresh rat liver as described previously (9). All other reagents were reagent grade and obtained from commercial sources.

Assay for Fru-6-P,2-kinase

Forward Direction—The reaction mixture contained in 0.1 ml, 100 mM Tris/HCl or HEPES, pH 7.5, 5 mM sodium phosphate, 5 mM ATP, 1 mM Fru-6-P, and 10 mM MgCl$_2$. The reaction was initiated with addition of the enzyme and the mixture was incubated at 30°C. At various time intervals (usually 2.5 and 5 min), aliquots (10 µl) were transferred to 90 µl of 20 mM Tris/HCl, pH 9, and the diluted solution was heated for 1 min at 90°C to stop the reaction. Suitable aliquots of the heated reaction mixture were then assayed for Fru-2,6-P$_2$ as described (16). One unit of the enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 nmol of β-Fru-2,6-P$_2$ per min under these conditions.

Reverse Direction—This assay method is the same as described previously with a slight modification. The reaction mixture contained in a final volume of 50 µl, 100 mM Tris/HCl or HEPES, pH 7.5, 5 mM MgCl$_2$, 0.1 mM [1$^4$C]ATP (4 × $10^6$ cpm/nmol), indicated concentrations of Fru-2,6-P$_2$, 50 µM NADP, 0.4 unit of desalted glucose-6-P dehydrogenase, and 1 unit of phosphoglucoisomerase. The reaction was initiated with addition of Fru-2,6-P$_2$-kinase and the mixture was incubated at 30°C. The formation of [1$^4$C]ATP was determined as described previously (9).

Assay for Fructose-2,6-bisphosphatase

The reaction mixture contained in a final volume of 0.1 ml: 100 mM Tris/HCl or HEPES, pH 7.5, 6 mM MgCl$_2$, 50 µM NADP, 0.4 unit of desalted glucose-6-P dehydrogenase, and 1 unit of phosphoglucoisomerase, and 20 µM Fru-2,6-[2$^{32}$P]P$_2$ (8.2 × $10^4$ cpm/nmol). The reaction was initiated with the addition of the enzyme and the reaction mixture was incubated at 30°C. At given time intervals (usually 2, 4, and 8 min), aliquots (10 µl) were removed, transferred into 100 µl of 0.1 N NaOH, and the solution was heated at 100°C for 2 min. H$_2$O (1 ml) and 0.02 N H$_2$O$_2$ (1 ml) was added to the heated reaction mixture and the sample was adsorbed on a Dowex 1-Cl$^-$ column (0.5 cm × 4 cm) which had been equilibrated with 0.02 M H$_2$O$_2$. The column was washed with 1 ml of 0.02 N H$_2$O$_2$ followed with 1 ml of 0.15 M NaCl in 0.02 N H$_2$O$_2$. [32P]Phosphate was then eluted with 5 ml of the same solution, a portion (1 ml) of the eluate diluted in 10 ml of Aquasol (New England Nuclear) and counted in a scintillation counter. One unit of the activity is defined as the amount of the enzyme that catalyzes the formation of 1 µmol of phosphate/min under these conditions.

Results

Since BrAcNHEtOP and BrDHBP$_2$ may react with Tris, both HEPES and Tris/HCl buffers were used. Identical results were obtained when the enzyme was treated with BrDHBP$_2$ in Tris/HCl and HEPES. However, some differences were observed with BrAcNHEtOP, but in most cases, these differences were small, suggesting that Tris, when the reaction mixture was incubated at 30°C. At given time intervals (usually 2, 4, and 8 min), aliquots (10 µl) were removed, transferred into 100 µl of 0.1 N NaOH, and the solution was heated at 100°C for 2 min. H$_2$O (1 ml) and 0.02 N H$_2$O$_2$ (1 ml) was added to the heated reaction mixture and the sample was adsorbed on a Dowex 1-Cl$^-$ column (0.5 cm × 4 cm) which had been equilibrated with 0.02 M H$_2$O$_2$. The column was washed with 1 ml of 0.02 N H$_2$O$_2$ followed with 1 ml of 0.15 M NaCl in 0.02 N H$_2$O$_2$. [32P]Phosphate was then eluted with 5 ml of the same solution, a portion (1 ml) of the eluate diluted in 10 ml of Aquasol (New England Nuclear) and counted in a scintillation counter. One unit of the activity is defined as the amount of the enzyme that catalyzes the formation of 1 µmol of phosphate/min under these conditions.

The abbreviations used are: BrAcNHEtOP, N-bromoacetylethanolamine-P; BrDHBP$_2$, 3-bromo-1,4-dihydroxy-2-butane-1,4-bisphosphate; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Fru-6-P, fructose 6-phosphate.

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FIG. 2. Rate of inactivation of Fru-6-P,2-kinase and Fru-2,6-bisphosphatase by BrAcNHEtOP. The reaction mixture contained in a final volume of 50 μl, 50 mM HEPES, pH 7.5, 5 mM sodium phosphate, 0.1 mM dithiothreitol, and 1 μM of Fru-6-P,2-kinase:Fru-2,6-bisphosphatase. The reaction was initiated with addition of 0.2 mM (O), 0.3 mM (O), 0.5 mM (O), 0.7 mM (O), and 1 mM (O), BrAcNHEtOP and the mixture was incubated at 30°C. BrAcNHEtOP (mM) was incubated with 0.5 mM BrAcNHEtOP in the presence of 4 mM Fru-6-P. At indicated time intervals, aliquots were removed and the activities of Fru-6-P,2-kinase (A), Fru-2,6-bisphosphatase (Δ and Δ) were determined as described under "Experimental Procedures." The controls without BrAcNHEtOP were (●) Fru-6-P,2-kinase and (●) Fru-2,6-bisphosphatase. The inset shows the plot of inactivation half-time (τ) versus the reciprocal of BrAcNHEtOP as described in the text.

Effect on Fru-2,6-bisphosphatase (Fig. 2). Complete inactivation occurred with 1 mM BrAcNHEtOP within 40 min, and the inactivation of Fru-6-P,2-kinase approximates pseudo-first order kinetics.

If the inhibitor reacts noncovalently with the active site of the enzyme to form a complex prior to inactivation, the inactivation rate should approach a maximum value as the inhibitor concentration approaches infinity, as in the case of saturation kinetics with substrate. A linear expression for the rate of the inactivation has been derived (Equation 3) (17, 18) where τ is the observed

\[ \tau = \frac{1}{[I]} (T_K_{inact} + T) \]  

inactivation half-time, [I] is the inhibitor concentration, T is the minimal inactivation half-time, and K_{inact} is the apparent dissociation constant for the enzyme-reagent complex. The replot of τ versus the reciprocal of inhibitor concentration (Fig. 2, inset) clearly shows rate saturation and indicates that the inactivation involves initial reversible formation of the enzyme-inhibitor complex. From the plot the observed minimum inactivation half-life is estimated as 2.5 min and K_{inact} is 2.2 mM. The first order rate constant is also calculated as 0.0046 s⁻¹.

Fru-6-P, the substrate for the kinase, slows the rate of the inactivation of Fru-6-P,2-kinase by BrAcNHEtOP; the inactivation half-time (τ) at 0.5 mM BrAcNHEtOP is 15 min in the absence but 70 min in the presence of 4 mM Fru-6-P (Fig. 2). Fru-6-P at 5 mM protects against the inactivation completely up to 0.4 mM and 50% above 1 mM BrAcNHEtOP (Fig. 3). These results provide additional evidence in support of the contention that BrAcNHEtOP and Fru-6-P compete for the same site on Fru-6-P,2-kinase. Furthermore, the inactivation by the reagent is essentially the same in the presence and the absence of dithiothreitol eliminating a possible side reaction between BrAcNHEtOP and the SH groups.

Effect on Fru-2,6-bisphosphatase—BrAcNHEtOP has negligible effect on Fru-2,6-bisphosphatase activity as discussed above. However, since this enzyme is inhibited by Fru-6-P and its K_i (4-10 μM) is very similar to its K_i (15 μM) for Fru-2,6-P,2-kinase (9), the question arises as to whether this inhibitory site is the same as that of the catalytic site of Fru-6-P,2-kinase. In order to answer this question, the extent of the inhibition by Fru-6-P of Fru-2,6-bisphosphatase which has been preincubated with BrAcNHEtOP was determined. As shown in Fig. 4, the Fru-6-P inhibition of the modified enzyme which has lost over 90% of Fru-6-P,2-kinase activity but retained nearly all Fru-2,6-bisphosphatase activity is only slightly less than that of the untreated enzyme. Similarly, the inhibition curves of a similar sample of modified enzyme which had lost 65% of the kinase activity was identical to the untreated enzyme (data not shown). These results strongly suggest that the Fru-6-P binding site of Fru-6-P,2-kinase and the Fru-6-P inhibitory site of Fru-2,6-bisphosphatase are also distinct.

BrDHBPz—BrDHBPz is a competitive inhibitor of Fru-6-P,2-kinase with respect to Fru-6-P with a K_i value of 20 μM (Fig. 5A). A plot of the activity versus the reciprocal of ATP in the presence of the reagent (Fig. 5B) indicates that BrDHBPz is a noncompetitive inhibitor versus ATP with a K_i value of about 40 μM. The identical inhibition patterns were obtained when the same reactions were carried out in the HEPES buffer, and the K_i values are also very similar, i.e. they are 17 and 35 μM for Fru-6-P and ATP, respectively. BrDHBPz is also a competitive inhibitor with respect to Fru-
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Fig. 4. Fru-6-P inhibition of Fru-2,6-bisphosphatase which has been covalently reacted with BrAcNHEtOP. Fru-2,6-bisphosphatase (1 mM) was treated with 1 mM BrAcNHEtOP as described in the legend to Fig. 2 for 40 min at 30°C, and under these conditions 97% of Fru-6-P,2-kinase was inactivated but retained nearly full Fru-2,6-bisphosphatase activity. The excess reagent was removed by column centrifugation (19) and concentrated with Centricon 30 (Amicon). Fru-2,6-bisphosphatase activity was determined in the presence of 10 mM Fru-2,6-P₂ as described under "Experimental Procedures." When varying concentrations of Fru-6-P were added, the Fru-6-P-depleting system containing NADP, glucose-6-P dehydrogenase, and phosphoglucone isomerase was omitted. ○, untreated; ●, treated enzyme.

2,6-P₂ in the reversal of the Fru-6-P,2-kinase reaction with $K_i = 85 \mu$M (Fig. 6). BrDHBP₂ does not react covalently with the enzyme since the inhibition by the reagent can be reversed by dialysis or column centrifugation (19).

Assay of the enzyme in the presence of increasing concentrations of BrDHBP₂ resulted in 93% inhibition of Fru-6-P,2-kinase ($K_i = 0.28$ mM), but a 3.6-fold activation of Fru-2,6-bisphosphatase when assayed at 3 mM Fru-2,6-P₂ ($K_{activation} = 0.54$ mM) (Fig. 7). This activation of the latter enzyme by the reagent was further investigated. As shown in Fig. 8, in the absence of BrDHBP₂, Fru-2,6-bisphosphatase exhibits a hyperbolic saturation curve in which it approaches $V_{max}$ very slowly above 3 mM Fru-2,6-P₂. In the presence of the reagent, however, it shows normal Michaelis-Menten kinetics with an apparent $K_{M}$ of 0.8 $\mu$M. These results are similar to the differences observed between phospho- and dephospho-Fru-2,6-bisphosphatase (9) in which the latter enzyme shows negative cooperativity while the former shows normal Michaelis-Menten kinetics. Thus, it is of interest to determine if the reagent-induced change is the same as that produced by the phosphorylation. This possibility was examined by determining the effect of the reagent on the activity of the phosphorylated Fru-2,6-bisphosphatase. As shown in Fig. 9, the $V_{max}$ of the phosphorylated Fru-2,6-bisphosphatase is increased at least 2-fold in the presence of BrDHBP₂. These results indicate that the activation of Fru-2,6-bisphosphatase by the reagent is independent of that produced by the phosphorylation. The inhibition of Fru-2,6-bisphosphatase by Fru-6-P is not significantly affected by the presence of 0.1-0.5 mM BrDHBP₂ (data not shown). Thus, this activation by the reagent is not due to the release of the Fru-6-P inhibition of the enzyme. Various inhibition constants of BrAcNHEtOP and BrDHBP₂ are summarized in Table I.

Discussion

Hartman et al. (11) synthesized BrAcNHEtOP and used it as an active site-directed affinity labeling reagent to probe the structural regions of aldolases. Although BrAcNHEtOP is not a particularly close analog of Fru-1,6-P₂, they were able to demonstrate that the reagent is a useful probe for the active sites of class I fructose-bisphosphate aldolases. They showed that the aldolases are inactivated by the reagent irreversibly after the initial reversible interaction and that the inactivation results from the alkylation of an active-site histidine or lysine. Similarly to the aldolases, BrAcNHEtOP irreversibly inactivates Fru-6-P,2-kinase. The evidence for this reagent reacting with the Fru-6-P binding site of the kinase is: (a) competitive inhibition during the short period of the reaction, (b) pseudo first-order inactivation with the rate saturation,
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FIG. 6. Inhibition of Fru-6-P,2-kinase (reverse direction) by BrDHBPz. Fru-6-P,2-kinase (25 µg/ml) in the reverse direction was assayed as described under “Experimental Procedures” except indicated concentrations of BrDHBPz were added, and V is in nanomoles/ml/min.

FIG. 7. Inhibition of Fru-6-P,2-kinase and activation of Fru-2,6-bisphosphatase by BrDHBPz. Fru-6-P,2-kinase (8 µg/ml) and Fru-2,6-bisphosphatase (3 µg/ml) were assayed as described under “Experimental Procedures” in the presence of varying concentrations of BrDHBPz except 3 µM Fru-2,6-P2 was used for the latter enzyme assay.

(c) complete inactivation, and (d) protection against inactivation by Fru-6-P.

Two observations in these experiments which could be inconsistent with alkylation of an active site of Fru-6-P,2-kinase are that the Ki of BrAcNHEtOP (0.24 mM) and the Kii, (2.2 mM) are significantly different and that the high concentrations of Fru-6-P (1-5 mM) are required to provide protection against the inactivation despite its low Km (16 µM (4)). Thus, we cannot rigorously exclude the possible alkylation outside of the active site which induces conformational change resulting in differential effects on the two activities of the enzyme.

The primary objective of this study is to determine whether the hexosephosphate binding sites of Fru-6-P,2-kinase and Fru-2,6-bisphosphatase are localized at the same or distinct sites. These hexose-P binding sites include (a) the catalytic...
sites for Fru-6-P and Fru-2,6-P₂ of Fru-6-P,2-kinase for the forward and the reverse reactions, respectively, and (b) the catalytic site for Fru-2,6-P₂ and the inhibitory site for Fru-6-P of Fru-2,6-bisphosphatase. The results presented here demonstrate that these hexose-P binding sites of Fru-6-P,2-kinase and Fru-2,6-bisphosphatase are probably distinct. The evidence in support of this conclusion is as follows: (a) BrAcNHEtOP inhibits Fru-6-P,2-kinase without affecting Fru-2,6-bisphosphatase. (b) Similarly, BrDHBP₂ is a competitive inhibitor with Fru-6-P in the forward reaction and with Fru-2,6-P₂ in the reverse direction of the kinase while it activates Fru-2,6-bisphosphatase. (c) The activation of the phosphatase by BrDHBP₂ is not due to the release of the inhibition by Fru-6-P suggesting that BrDHBP₂ does not compete for the inhibitory site, but instead it may bind to an allosteric site of this enzyme. Among several sugar phosphates we have examined, 3-phosphoglycerate shows the similar phenomenon, i.e. the activation of Fru-2,6-bisphosphatase and inhibition of Fru-6-P,2-kinase. (d) The Fru-6-P,2-kinase alkylated by BrAcNHEtOP, which is devoid of the kinase activity, still retains full Fru-2,6-bisphosphatase activity and also its sensitivity to Fru-6-P inhibition is unaltered. (e) Fru-6-P inhibition of Fru-2,6-bisphosphatase is not sigmoidal while the interaction of Fru-6-P with Fru-6-P,2-kinase is highly cooperative (9). Thus, these results as well as the previous studies on the adenine nucleotide binding sites (10) strongly suggest that the catalytic sites and the inhibitory sites of Fru-6-P,2-kinase and Fru-2,6-bisphosphatase are located in the different regions of this bifunctional enzyme. A question that remains unanswered is whether both active sites are located in each subunit or in different subunits of this dimeric protein. All the evidence we have obtained thus far regarding the structure of this enzyme indicates that the subunits are identical, thus favoring the former possibility. However, this does not preclude the latter possibility and requires more information on the chemical structure of the enzyme, and the number of binding sites for hexose phosphate, adenine nucleotides, and various effectors in order to answer the question.

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**REFERENCES**


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**TABLE I**

Inhibition constants of BrAcNHEtOP and BrDHBP₂

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2 S. Kitajima, R. Sakakibara, and K. Uyeda, manuscript in preparation.