Inactivation of Phosphofructokinase by 6-Mercapto-9-β-D-ribofuranosylpurine 5′-Triphosphate†

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ABSTRACT: 6-Mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate (sRTP) is a phosphoryl donor for the reactions catalyzed by phosphofructokinase and hexokinase. The affinity of phosphofructokinase was similar for both sRTP and ATP, whereas the affinity of hexokinase for sRTP was much lower than for ATP. At high concentrations of sRTP (>0.4 mM), phosphofructokinase was inhibited by the nucleotide. Hexokinase was unaffected over a similar concentration range. Incubation of phosphofructokinase with sRTP resulted in inactivation of the enzyme. The monophosphate derivative and the oxidized disulfide forms of mercaptopurine nucleotides both inactivated phosphofructokinase; however, they were less potent than sRTP. The rate of inactivation of phosphofructokinase was dependent on the concentration of sRTP and the loss of 80–90% of the enzyme activity obeyed first-order kinetics. Both fructose 6-phosphate and MgATP slowed the rate of inactivation of phosphofructokinase by sRTP. Incubation of sRTP-inactivated phosphofructokinase with reduced thiols resulted in virtually complete recovery of enzyme activity. [β,γ-^{33}P]sRTP was bound to phosphofructokinase in a covalent bond which could not be reversed by extensive dialysis or by precipitation with trichloroacetic acid. When phosphofructokinase labeled with [β,γ-^{33}P]sRTP was reactivated with mercaptoethanol, the bound radioactivity was released on dialysis. Complete inactivation of phosphofructokinase resulted in the binding of 6–8 mol of [β,γ-^{33}P]sRTP per 380,000 g of phosphofructokinase.

The activity of phosphofructokinase (EC 2.7.1.11) is regulated by the concentration of various adenine nucleotides (Atkinson, 1966; Stadtman, 1966). The enzyme requires ATP for catalytic activity but is inhibited by high concentrations of this nucleotide (Lardy and Parks, 1956; Uyeda and Racker, 1965). The inhibition by ATP can be reversed by adenosine 5-monophosphate (Passoneau and Lowry, 1962; Parmeggiani and Bowman, 1963) and adenosine 3′,5′-cyclic monophosphate (Denton and Randle, 1966). Kinetic studies (Lowry and Passoneau, 1966; Garfinkel, 1966), chemical modifications of the enzyme (Chapman *et al.*, 1969a,b; Lorenson and Mansour, 1969; Setlow and Mansour, 1970; Ahlfors and Mansour, 1969), nucleotide specificity (Uyeda and Racker, 1965; Bloxham, 1973), and fluorescence studies (Bloxham, 1973) have all revealed that these properties are related to spatially separated binding sites (catalytic and regulatory) for the various adenine nucleotides. It is possible that suitable affinity labels could be designed that would allow the structure of each of these sites to be investigated independently.

Recently, Brunswick and Cooperman (1971) described the synthesis of a photoaffinity label that could be used for investigating the nucleoside monophosphate regulatory site; however, at present no affinity labels have been described for the catalytic site. The lack of specificity at the catalytic site of phosphofructokinase for the base moiety of nucleoside triphosphates (Ling and Lardy, 1954; Uyeda and Racker, 1965) suggests that suitable modifications within the purine ring could produce an affinity label. Evidence for an essential (Younathan *et al.*, 1968) and highly reactive (Kemp and Forest, 1968; Kemp, 1969a,b; Mathias and Kemp, 1972) thiol at the active site of the enzyme prompted us to investigate sRTP[1] as a potential affinity label of this site. sRTP can react with thiols to form either a disulfide bond (Murphy and Morales, 1970) or by nucleophilic attack to form a thioether (Fox *et al.*, 1958; Hampton, 1963; Brox and Hampton, 1968a,b; Hulla and Fasold, 1972). In both cases, a stable covalent bond should result which affords the possibility of examining the structure of the active site.

In the present work, it is shown that sRTP is capable of acting as a phosphoryl donor in the reaction catalyzed by phosphofructokinase and that under appropriate conditions the nucleotide can inactivate the enzyme. Inactivation is associated with the covalent binding of sRTP to the enzyme. This covalent bond can be cleaved by mercaptoethanol or dithiothreitol to regenerate the active enzyme.

Materials and Methods

Rabbit skeletal muscle phosphofructokinase was prepared by the method of Ling *et al.* (1965) and crystallized by the procedure of Parmeggiani *et al.* (1966). The enzyme had an activity of 100–120 U/mg when assayed at pH 8 in the presence of saturating concentrations of MgATP and fructose 6-phosphate.

sRMP[1] was obtained as the barium salt from P-L Biochemicals, Milwaukee, Wis. It was converted to the free acid with Dowex 50 (H^+). Precautions were taken to wash the Dowex 50 (H^+) thoroughly with water to remove anionic material from the eluate as this material was found to potentiate the observed inactivating properties of sRMP.

The tri-*n*-butylamine salt of sRMP was converted to the triphosphate by condensation with inorganic phosphate in

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[1] Abbreviations used in the text are: sRMP, 6-mercapto-9-β-D-ribofuranosylpurine 5′-monophosphate; sRTP, 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate. NAD^+ and NADH, oxidized and reduced forms of nicotinamide adenine dinucleotide.

anhydrous pyridine using *N',N'*-dicyclohexylcarbodiimide as a water abstracting agent (Murphy *et al.*, 1970). After chromatography on Dowex 1-Cl, precipitation as the lithium salt and chromatography on Sephadex G-15 (Murphy *et al.*, 1970), the product was shown to be chromatographically pure. Ascending chromatography on DEAE-cellulose paper, using 0.7 M ammonium formate, pH 3.1, gave the following R_F values for nucleotides: sRMP, 0.53; AMP, 0.8; sRTP, 0.10; ATP, 0.20. The concentration of sRTP was determined by using the molar absorption coefficient of 23,100 at 322 nm (Hampton and Maguire, 1961). Use was made of the fact that sRTP is a substrate for phosphofructokinase and its concentration was determined enzymatically in the presence of excess enzyme and fructose 6-phosphate. The two methods agreed within 5%.

Preparation of [β,γ-^{33}P]*sRTP.* The free acid forms of sRMP (0.1 mmol) and ^{33}P-labeled inorganic phosphate (1 mCi) were dissolved in water and dried *in vacuo*. After removal of the last traces of water by coevaporation with anhydrous pyridine, the residue was dissolved in 4 ml of anhydrous pyridine containing 1.2 mmol of tri-*n*-butylamine and 0.2 mmol of phosphoric acid. *N',N'*-Dicyclohexylcarbodiimide (4 mmol) was added and the reaction was allowed to proceed for 12 hr. At this time, a further 4 ml of anhydrous pyridine, tri-*n*-butylamine (5 mmol), phosphoric acid (1 mmol), and *N',N'*-dicyclohexylcarbodiimide (4 mmol) were added. After another 36 hr, the product was isolated as previously described (Murphy *et al.*, 1970). A final purification was achieved by passage through a water-equilibrated Sephadex G-25 column (30 × 1 cm) immediately prior to use.

Oxidation of 6-Mercaptopurine Nucleotides. The conditions used were similar to those described by Goody and Eckstein (1971) for the oxidation of thiophosphate nucleotide analogs. Five micromoles of sRMP or sRTP was dissolved in 1 ml of water, treated with 0.1 ml of H_2O_2 (3%, v/v), and immediately evaporated to dryness *in vacuo*. The residue was dissolved in 1 ml of water and reevaporated to dryness. The resultant compounds exhibited maximum absorption at 260 nm. On exposure to mercaptoethanol at pH 9, the 322-nm absorption appeared rapidly. The concentration of the disulfide was determined on the basis of the recovered mercaptopurine nucleotide.

Assay of Phosphofructokinase. Incubations contained: Tris-HCl, pH 8, 50 mM; $MgSO_4$, 1 mM; ATP, 0.2 mM; dithiothreitol, 1 mM; aldolase, 30 μg; triosephosphate isomerase and α-glycerophosphate dehydrogenase, 1.5 μg each; NADH, 0.2 mM; and phosphofructokinase, 0.2–0.5 μg. The reaction at 28° was started by the addition of 0.05 mM fructose 6-phosphate in a final volume of 1 ml and the decrease in absorption was followed at 340 nm using a Gilford 2000 spectrophotometer.

Assay of Hexokinase. Incubations contained: Tris-HCl, pH 8, 50 mM; $MgSO_4$, 4 mM; glucose, 2 mM; $NADP^+$, 0.125 mM; glucose-6-phosphate dehydrogenase, 2.5 μg; and yeast hexokinase, 0.5 μg. The reaction at 28° was started by the addition of appropriate concentrations of nucleoside triphosphate in a final volume of 1 ml.

Results

sRTP as a Phosphoryl Donor. Phosphofructokinase uses a wide variety of nucleoside triphosphates as phosphoryl donors for fructose 6-phosphate (Ling and Lardy, 1954; Uyeda and Racker, 1965). Figure 1 shows that sRTP can be used as a phosphoryl donor for the reactions catalyzed

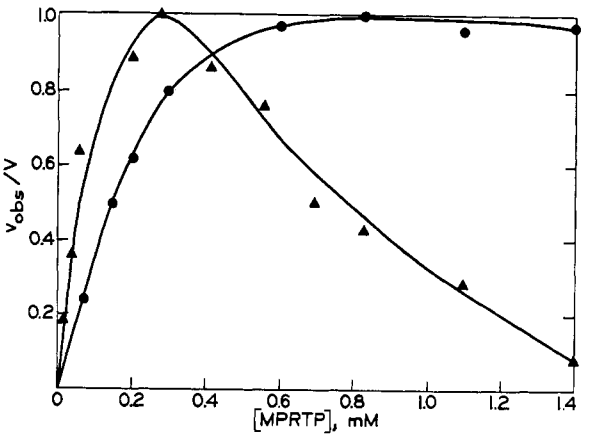

FIGURE 1: Influence of sRTP on the catalytic activity of phosphofructokinase and hexokinase: (▲) phosphofructokinase activity; (●) hexokinase activity. v_{obsd} is the observed reaction rate at each sRTP concentration and V is the maximal catalytic rate observed at the optimal sRTP concentration. V was 25 and 30 μmol per min per mg for phosphofructokinase and hexokinase, respectively.

by both phosphofructokinase and yeast hexokinase. With phosphofructokinase, the apparent K_M for sRTP is 10^{-4} M at 5×10^{-5} M fructose 6-phosphate, which is similar to the apparent K_M for ATP (4×10^{-5} M) determined under the same conditions. In contrast, the affinity of yeast hexokinase for sRTP ($K_{M,app}$ = 2 mM at 2 mM glucose) is much lower than for ATP ($K_{M,app}$ = 0.25 mM at 2 mM glucose). A further difference in the response of the two enzymes is observed at high concentrations of sRTP. In the case of phosphofructokinase the enzyme was inhibited as the concentration of sRTP exceeded the optimal catalytic concentration, whereas, over the same concentration range, yeast hexokinase was not affected. Examination of the reaction rate in the presence of inhibitory concentrations of sRTP revealed that only the initial rate of fructose 1,6-diphosphate production was linear and this rate was used in the calculation of the data shown in Figure 1. After the initial linearity, the rate decreased progressively. Increasing concentrations of sRTP decreased the period during which the reactions were linear. No difference in the inhibitory potency of sRTP was observed when phosphofructokinase was assayed at either pH 7 or 8. This suggests that sRTP inhibits phosphofructokinase by a different mechanism from that involved in the inhibition of enzyme activity by ATP, since inhibition at pH 8 requires >6 mM ATP (Uyeda and Racker, 1965).

Inactivation of Phosphofructokinase by sRTP. When phosphofructokinase was incubated with sRTP for 30 min at 4° and then assayed under conditions of negligible concentration of the nucleotide ($<3.5 \times 10^{-6}$ M), the enzyme was inactivated (Figure 2). At the highest concentration of sRTP employed in the inactivation reaction, almost complete inactivation was achieved. The inactivation was similar when the preliminary incubation was performed at either pH 7 or 8. Inactivation was the result of a decrease in the maximal catalytic capacity of the sRTP-treated enzyme. Incubation of phosphofructokinase with sRMP under similar conditions produced a maximum inactivation of only 18%.

The rate of inactivation of phosphofructokinase by sRTP was concentration dependent (Figure 3). At pH 8, the rate of inactivation obeyed first-order kinetics. At the lower concentrations of sRTP, the inactivation followed first-order kinetics for the loss of only 80–90% of the enzyme activity.

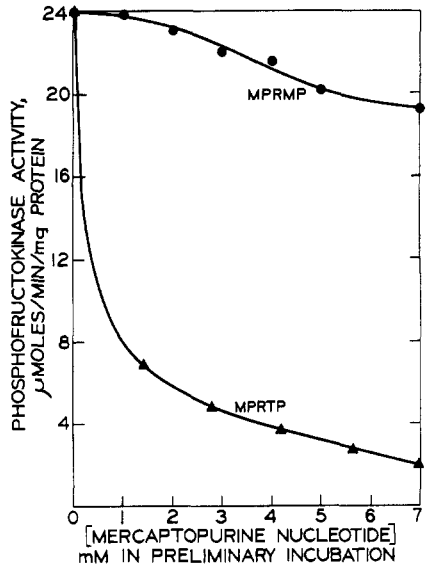

FIGURE 2: Inactivation of phosphofructokinase by mercaptopurine nucleotides. Phosphofructokinase (1 mg/ml) in 50 mM potassium phosphate, pH 8, containing 1 mM EDTA, was incubated with various initial concentrations of mercaptopurine nucleotides for 30 min at 4° when the reaction mixture was diluted 20-fold with buffer. A small aliquot was then removed to measure enzyme activity.

TABLE I: Inactivation of Phosphofructokinase by 6-Mercaptopurine Nucleotides.[a]

Inactivator	Concn (mM)	Inhibition (%)
sRTP (-SH)	0.1	83.4
	0.5	94.5
	1.0	100.0
sRMP (-SH)	0.1	0.0
	0.5	31.2
	1.0	50.0
sRTP (-S-S-)	0.1	27.8
	0.5	68.8
	1.0	95.0
sRMP (-S-S-)	0.1	26.2
	0.5	73.4
	1.0	95.0

[a] Phosphofructokinase (10 μg) was incubated at 4° for 18 hr in 0.1 ml of 50 mM potassium phosphate buffer, pH 8, containing 1 mM EDTA and the appropriate concentration of nucleotide; 5 μl was then removed and assayed for enzyme activity. Control incubations were carried out in the absence of the nucleotide.

Kitz and Wilson (1962) have shown that for an active-site directed reagent (I), the inactive enzyme (E–I) should be formed through a noncovalent intermediate (E·I), *i.e.*

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E\cdot I \xrightarrow{k_3} E\text{-}I$$

K_I, k_3, and k_{app} are related by the equation

$$\frac{1}{k_{app}} = \frac{1}{k_3} + \frac{K_I}{k_3}\frac{1}{[I]} \qquad (1)$$

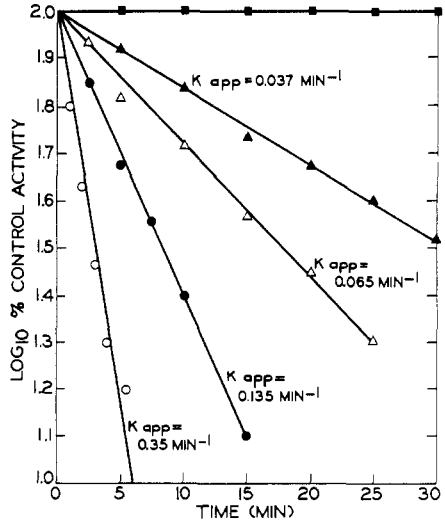

FIGURE 3: The rate of inactivation of phosphofructokinase by sRTP. Phosphofructokinase (1 mg/ml) in 50 mM potassium phosphate buffer, pH 8, containing 1 mM EDTA, was inactivated at 4° with the following concentrations of sRTP: (○) 6 mM; (●) 3 mM; (△) 1.3 mM; (▲) 0.65 mM; (■) none.

A reciprocal plot of the calculated rate constants (k_{app}) and sRTP concentration was found to be linear in accord with eq 1 and K_I and k_3 were calculated to be 6–8 mM and 0.41 min^{-1}, respectively, at pH 8 and 4°.

If the inactivation of phosphofructokinase results from the binding of sRTP to the catalytic site of the enzyme, it would be expected that the normal substrates of phosphofructokinase should protect against inactivation. Figure 4 shows that both fructose 6-phosphate and MgATP markedly decreased the rate of inactivation of phosphofructokinase by a fixed concentration of sRTP. Maximum protection was obtained when the enzyme was incubated with the substrate prior to the addition of sRTP.

Inactivation of Phosphofructokinase by Oxidized 6-Mercaptopurine Nucleotides. Table I shows that the oxidized forms of both sRMP and sRTP inactivated phosphofructokinase In contrast to the observation that sRTP was a more potent inhibitor than sRMP, the oxidized forms of the nucleotides appear to be equally effective. Oxidation of sRTP causes a marked decrease in its inhibitory potency.

Reversal of sRTP Inactivation by Mercaptoethanol. Studies on the use of sRTP as a chromophoric probe of myosin have shown that the covalent bond between the protein and the nucleotide can be broken by exposure to mercaptoethanol and that this resulted in the release of sRTP as detected by the appearance of the characteristic absorption of the 6-mercaptopurine nucleotide at 322 nm (Murphy and Morales, 1970). If sRTP forms a similar covalent bond with phosphofructokinase, the inactivation of the enzyme should be reversed by mercaptoethanol. When phosphofructokinase that had been completely inactivated with sRTP was incubated at 4° with 0.1 M mercaptoethanol in 0.1 M potassium phosphate buffer, pH 8, containing 1 mM EDTA, the activity of the enzyme increased progressively with time. Approximately 90% of the original enzyme activity was restored within 24 hr. Since 0.1 M mercaptoethanol caused a slight inactivation

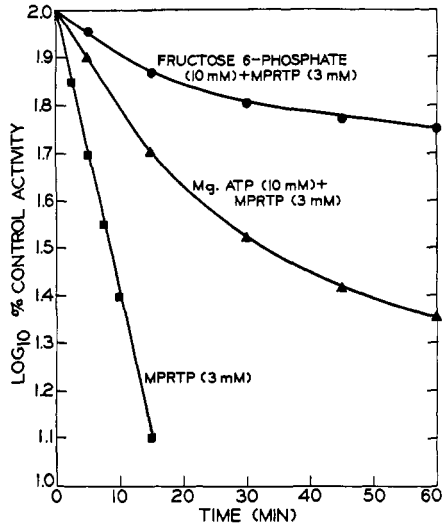

FIGURE 4: Inactivation of phosphofructokinase in the presence of fructose 6-phosphate or MgATP. Phosphofructokinase (1 mg/ml) in 50 mM potassium phosphate buffer, pH 8, containing 1 mM EDTA, was incubated for 5 min at 4° in the presence of MgATP or fructose 6-phosphate. The inactivation reaction was then commenced by the addition of sRTP.

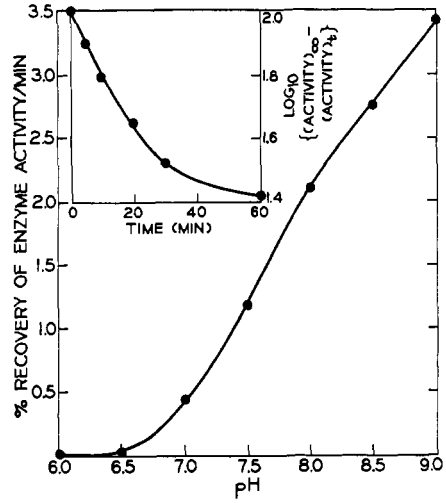

FIGURE 5: Influence of pH on the rate of reactivation of sRTP-inactivated phosphofructokinase by mercaptoethanol. Phosphofructokinase (40 μg), which had been completely inactivated with sRTP, was incubated in 1 ml of 0.1 M potassium phosphate, at the appropriate pH, containing 0.1 M mercaptoethanol. The recovery of enzyme activity was measured at various times. The insert shows a pseudo-first-order rate plot for reactivation at pH 8.

of phosphofructokinase, this recovery of activity represented almost complete reactivation of the enzyme. In the absence of mercaptoethanol, there was no significant recovery of enzyme activity over a period of 3 days.

The rate of reactivation, shown in the insert of Figure 5, revealed that only the initial rate of reactivation was pseudo first order. This initial rate of reactivation was dependent upon the pH of the medium (Figure 5). No reactivation occurred below pH 7, and as the pH became more alkaline the rate of reactivation increased progressively.

Dithiothreitol also reversed the inactivation of phosphofructokinase by sRTP and was ten times more potent than mercaptoethanol on a molar basis. To ensure maximal catalytic capacity of phosphofructokinase, dithiothreitol (1 mM) was always included in the assay mixture. With untreated enzyme under these conditions, a linear reaction rate was usually achieved within 30 sec and only a few minutes were required to complete the assay. Within the usual assay time, at pH 8, 1 mM dithiothreitol produced only a marginal increase in the activity (<1%) of the sRTP-treated phosphofructokinase. However, when the reaction was allowed to proceed over much longer periods, the reaction rate increased significantly. For the purpose of the present work, only the initial rate between 30 and 150 sec was used to calculate enzyme activities.

Binding of [β,γ-^{33}P]sRTP to Phosphofructokinase. The results so far provide indirect evidence for the formation of a covalent bond between phosphofructokinase and sRTP. Proof of this point requires that labeled sRTP is irreversibly bound to the enzyme. When [β,γ-^{33}P]sRTP was allowed to react with phosphofructokinase and the product was subjected to chromatography on Sephadex G-25 (30 × 1 cm column), radioactivity was eluted coincident with the phosphofructokinase protein peak (Figure 6). The radioactivity in the free sRTP fraction was eluted in two overlapping peaks, the base of which is shown in Figure 6. The second peak contained the bulk of the radioactivity (66%) and corresponded to unchanged sRTP, whereas the spectral properties of the leading or higher molecular weight component (beginning at 25 ml) indicated that it was the disulfide. This result was obtained even when the reduced [β,γ-^{33}P]sRTP was subjected to chromatography on Sephadex G-25 immediately prior to incubation with the enzyme to ensure that it eluted as a single peak. From this result, it is concluded that the incubation conditions caused oxidation of the 6-mercaptopurine nucleotide.

In a control experiment, [8-^{14}C]ATP was incubated with phosphofructokinase under identical conditions to those used

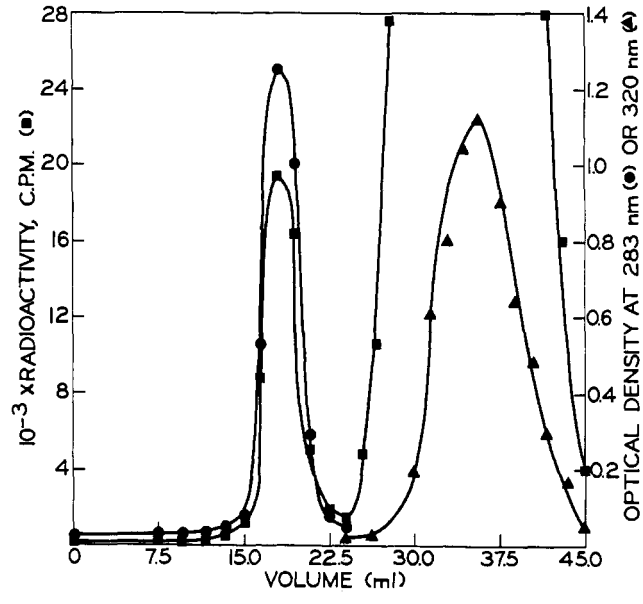

FIGURE 6: Binding of [β,γ-^{33}P]sRTP to phosphofructokinase. Phosphofructokinase (2.5 mg) was incubated for 3 hr at 4° with [β,γ-^{33}P]sRTP (1.5 μmol; 1.6 × 10^6 cpm) in 0.1 M potassium phosphate buffer, pH 8, containing 1 mM EDTA (1.5 ml). The material was chromatographed on a Sephadex G-25 column (30 × 1 cm) using the incubation buffer as a solvent. Fractions of 1.5 ml were collected.

TABLE II: Binding of Labeled Nucleoside Triphosphates to Phosphofructokinase.[a]

Enzyme Treatment	Mol of [8-^{14}C]-ATP/ 380,000 g of Enzyme	Mol of [β,γ-^{33}P]-sRTP/ 380,000 g of Enzyme	Activity of sRTP-Treated Enzyme (%)
Native	0	0	100
Sephadex chromatography	1.19	14.5	5
Dialysis *vs.* buffer (36 hr)[b]	0.07	8.0	5
Dialysis *vs.* buffer + 1 mM ATP (36 hr)	0.07	8.0	5
Trichloroacetic acid[c]	0	4.2	
0.1 M mercaptoethanol (18 hr) prior to dialysis (36 hr)	0	0.43	85

[a] Phosphofructokinase was allowed to react with either ATP or sRTP at the same specific activity and chromatographed on Sephadex G-25 as described in Figure 6. [b] Potassium phosphate buffer (0.1 M), pH 7, containing 1 mM EDTA. [c] The protein was precipitated on a filter paper disk with 10% (w/v) trichloroacetic acid. The disk was washed with 5% (w/v) trichloroacetic acid (×4) and ether (×3), dried, and counted (40% counting efficiency).

for the reaction with [β,γ-^{33}P]sRTP. This resulted in the elution of ^{14}C-labeled phosphofructokinase from Sephadex G-25, demonstrating that phosphofructokinase has a high affinity for nucleoside triphosphates and indicating that the stability of [β,γ-^{33}P]sRTP bound to phosphofructokinase following Sephadex chromatography is not a completely satisfactory criterion of covalent binding. Therefore, it was necessary to evaluate further the nature of the bond between sRTP and the enzyme.

Table II shows the influence of a variety of treatments on the stability of nucleoside triphosphate binding to phosphofructokinase. All of the treatments completely removed [^{14}C]-ATP bound to phosphofructokinase, indicating that binding was the result of a physical interaction between the protein and nucleotide. In contrast, it was not possible to remove all of the [β,γ-^{33}P]sRTP bound to the enzyme. Dialysis did remove about 40% of the radioactivity but the remaining radioactivity was stable even after prolonged dialysis against buffer containing ATP. For a number of preparations of phosphofructokinase, the maximum stable binding of [β,γ-^{33}P]sRTP which resulted in complete inactivation of the enzyme was 6–8 mol of nucleotide per 380,000 g of protein. When [β,γ-^{33}P]sRTP-labeled phosphofructokinase was precipitated with trichloroacetic acid about 50% of the tightly bound radioactivity remained bound to the protein. Since control experiments showed that the terminal phosphate of ATP is susceptible to hydrolysis under these conditions, the retention of this amount of radioactivity provides evidence that the bond between the enzyme and sRTP is covalent.

To determine whether the reactivation of sRTP-treated phosphofructokinase results in the liberation of sRTP, [β,γ-^{33}P]sRTP-labeled phosphofructokinase was treated with 0.1 M mercaptoethanol. Following this procedure, the bulk of the radioactivity was readily removed by dialysis (Table II).

The stoichiometry of the labeling of phosphofructokinase with [β,γ-^{33}P]sRTP was investigated by incubating phosphofructokinase (400 μg) with 0.6 μmol of [β,γ-^{33}P]sRTP (4×10^5 cpm) at 4° in 50 mM potassium phosphate buffer, pH 8, containing 1 mM EDTA (0.4 ml). At appropriate times, from 0 to 120 min, 40-μl aliquots were removed and diluted with 1 ml of 0.1 M potassium phosphate buffer, pH 8, containing 1 mM EDTA, 5 mM fructose 6-phosphate, and 5 mM MgATP. Fructose 6-phosphate and MgATP were included to protect against inactivation. After 36-hr dialysis (3 × 100 ml of buffer) the enzyme activity, protein concentration, and bound radioactivity were determined. There was a linear relationship between the extent of inactivation of the enzyme and the binding of [β,γ-^{33}P]sRTP. Complete inactivation of the enzyme corresponded to the binding of 6.5 mol of sRTP per 380,000 g of protein, which is similar to the values obtained in experiments involving prolonged incubation (Table II).

Discussion

The present work provides evidence that sRTP inactivates phosphofructokinase by reacting with it to form a covalent bond. sRTP has been shown to act as a covalent label for myosin (Murphy and Morales, 1970), for the aspartokinase reaction of threonine-sensitive aspartokinase homoserine dehydrogenase from *Escherichia coli* K12 (Truffa Bachi and d'A Heck, 1971), and rabbit muscle phosphofructokinase. In each of these cases, the interaction of sRTP with the enzyme is similar since all the proteins contain essential thiol groups, use sRTP as a substrate, and are inactivated only by relatively high concentrations of the nucleotide. As in the case of myosin and aspartokinase, sRTP becomes bound to phosphofructokinase by a disulfide bond. Examination of the product formed by treating [β,γ-^{33}P]sRTP-labeled phosphofructokinase with mercaptoethanol yielded sRTP as the only identifiable radioactive product. Had sRTP reacted with the enzyme to form a thioether, as does the 6-chloro analog of AMP (Brox and Hampton, 1968a,b), reactivation of the enzyme with mercaptoethanol would have yielded a thioether derivative of sRTP.

The choice of a thiol group as the reactive species on the enzyme seems a reasonable one since the enzyme contains a particularly reactive thiol at the catalytic site (Kemp and Forest, 1968; Kemp, 1969a,b; Mathias and Kemp, 1972). Reaction of sRTP with enzyme thiols would be expected to inactivate the enzyme since it is known that a number of treatments which modify thiols result in inhibition of the enzyme (Younathan *et al.*, 1968; Froede *et al.*, 1968; Hofer, 1970).

It is not established with certainty that sRTP is an affinity label for the catalytic site; however, there is some evidence that this is the case. First, the monophosphate analog, sRMP, is a poor inactivator of the enzyme in comparison to the triphosphate. Second, only 6–8 mol of sRTP are bound per 380,000 g of phosphofructokinase, whereas there are at least 32 mol of available thiol (Bloxham, 1973). Third, the properties of sRTP inactivation of phosphofructokinase are not consistent with the possibility that sRTP binds to the site that is responsible for inhibition by ATP. Thus, the inhibition by ATP is minimal at pH 8 (Uyeda and Racker, 1965), whereas sRTP readily inactivates phosphofructokinase at this pH.

Two pieces of evidence are not consistent with the suggestion that sRTP is an affinity label for the active site. First, the association constant (K_I) for sRTP and phosphofructo-

kinase in the inactivation reaction (6–8 mM) is much higher than the kinetic constant for sRTP in the catalytic reaction. Second, the presence of 10 mM MgATP (50 times true K_M) decreases the rate of inactivation by 3 mM sRTP by only 60–70%.

These results could be rationalized by several alternative mechanisms as follows.

(1) Inactivation involves the binding of two molecules of sRTP near the active site. Inactivation of the enzyme could then result from the formation of sRTP disulfide and an exchange reaction between the disulfide and a thiol group on the protein. A similar mechanism has been proposed by Tokiwa and Morales (1971) to explain the inactivation of myosin by sRTP. In the present work we have noted that phosphofructokinase enhances the oxidation of sRTP and that the disulfide can inactivate the enzyme. A possible candidate for the second site of sRTP binding would be the site normally occupied by fructose 6-phosphate. If this is the case, then it would be expected that sRTP would have a low affinity for this site and this would explain why K_I for sRTP is much higher than the kinetic constant. This would also explain why ATP does not afford as good protection as would be expected from its known dissociation constant with the catalytic site of the enzyme. If this mechanism is correct then it follows that fructose 6-phosphate should be more efficient in protecting against inactivation than ATP which was the observed result (Figure 4). Furthermore, in the presence of fructose 6-phosphate only the catalytic site should be available to sRTP and the enzyme should be in the active conformation and this was also observed (Figure 1).

(2) Inactivation involves the binding of one molecule of sRTP at the active site; however, in order to inactivate the enzyme sRTP must be bound in a different conformation from the one in which it participates in the catalytic reaction.

(3) Inactivation involves the binding of sRTP to a thiol which is essential for catalytic activity but is not strictly located at the active site. In this case, protection by ATP and fructose 6-phosphate could result from gross changes in the conformation of the protein. A mechanism of this type has been proposed to explain the inactivation of phosphofructokinase by selective reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (Kemp and Forest, 1968; Kemp, 1969a,b; Mathias and Kemp, 1972).

The complete inactivation of phosphofructokinase is associated with the binding of 6–8 mol of sRTP per 380,000 g of enzyme. Studies by Kemp and Krebs (1967) revealed that the total number of ATP binding sites was of the order of 12 per 380,000 daltons of enzyme. Therefore, it is apparent that sRTP does not react with all of the available sites and this is consistent with the concept that sRTP reacts only at the catalytic site. It can be concluded that there should be six–eight catalytic sites and four–six regulatory sites per 380,000 daltons of enzyme. Studies of fructose 6-phosphate binding to rabbit muscle phosphofructokinase (Kemp and Krebs, 1967) revealed that there were only four binding sites per 380,000 daltons of enzyme. This is a lower value for the number of catalytic sites compared to the value calculated for sRTP binding. The pattern of fructose 6-phosphate binding can be fairly complex (Lorenson and Mansour, 1969; Setlow and Mansour, 1972), and it is possible that there is some degree of error in this measurement. Alternatively, if the product of the reaction between sRTP and phosphofructokinase is a disulfide, then a disulfide interchange reaction could lead to binding of the nucleotide at sites other than the catalytic site.

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