Involvement of Tryptophan(s) at the Active Site of Polyphosphate/ATP Glucokinase from *Mycobacterium tuberculosis*†

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ABSTRACT: The glucokinase (EC 2.7.1.63) from *Mycobacterium tuberculosis* catalyzes the phosphorylation of glucose using inorganic polyphosphate (poly(P)) or ATP as the phosphoryl donor. The nature of the poly(P) and ATP sites was investigated by using *N*-bromosuccinimide (NBS) as a probe for the involvement of tryptophan in substrate binding and/or catalysis. NBS oxidation of the tryptophan(s) resulted in fluorescence quenching with concomitant loss of both the poly(P)- and ATP-dependent glucokinase activities. The inactivation by NBS was not due to extensive structural changes, as evidenced by similar circular dichroism spectra and fluorescence emission maxima for the native and NBS-inactivated enzyme. Both phosphoryl donor substrates in the presence of xylose afforded ~65% protection against inactivation by NBS. The K_m values of poly(P) and ATP were not altered due to the modification by NBS, while the catalytic efficiency of the enzyme was decreased, suggesting that the essential tryptophan(s) are involved in the catalysis of the substrates. Acrylamide quenching studies indicated that the tryptophan residue(s) were partially shielded by the substrates against quenching. The Stern–Volmer quenching constant (K_{SV}) of the tryptophans in unliganded glucokinase was 3.55 M^{-1}, while K_{SV} values of 2.48 and 2.57 M^{-1} were obtained in the presence of xylose+poly(P)$_5$ and xylose+ATP, respectively. When the tryptophan-containing peptides were analyzed by peptide mapping, the same peptide was found to be protected by xylose+poly(P)$_5$ and xylose+ATP against oxidation by NBS. The two protected peptides were determined to be identical by N-terminal sequence analysis and amino acid composition. It is proposed from these results that one or both of the tryptophans present in the protected peptide may be located at a common catalytic center and that this peptide may constitute part of the poly(P) and ATP binding regions.

Polyphosphate glucokinase (EC 2.7.1.63), first reported to be present in *Mycobacterium phlei* by Szymona (1957), catalyzes the phosphorylation of glucose using polyphosphates, poly(P)s[1] (linear polymers of orthophosphates linked by phosphoanhydride bonds) (reaction I), or ATP (reaction II) as the phosphoryl donor. Glucokinases that utilize inorganic

$$\text{poly(P)}_n + \text{glucose} \rightarrow \text{poly(P)}_{n-1} + \text{glucose 6-phosphate} \quad \text{(I)}$$

$$\text{ATP} + \text{glucose} \rightarrow \text{ADP} + \text{glucose 6-phosphate} \quad \text{(II)}$$

poly(P)s to phosphorylate glucose are relatively rare and have been reported to be present in the phylogenetically ancient bacteria belonging to the order Actinomycetales, according to the classification of Krasil'nikov (1949). Besides its presence in the Actinomycetales, poly(P) glucokinase has been found in *Myxococcus coralloides* (Gonzales et al., 1990), in the bacterial parasite *Bdellovibrio bacteriovorus* (Bobyk et al., 1980), and in the oligotrophic bacterium *Renobacter vacuolatum* (Nikitin et al., 1983).

The enzyme was discovered in the propionibacteria by Uryson and Kulaev (1968), and its purification and characterization have been reported from our laboratory (Phillips et al., 1993). These studies revealed that both the poly(P)- and ATP-dependent activities of the propionibacterial glucokinase were the catalytic properties of a single enzyme. We have therefore designated this polyphosphate-glucose phosphotransferase (EC 2.7.1.63) as poly(P)/ATP glucokinase. Similar studies conducted in our laboratory (Heish et al., 1993) with the enzyme from *Mycobacterium tuberculosis* have also implicated a single enzyme as responsible for both the poly(P)- and ATP-dependent glucokinase activities. In addition, inhibition studies with the nucleotide analogue, Reactive Blue 4, have indicated that there are individual binding sites for ATP and for poly(P). In order to determine the nature of the two phosphoryl binding sites, we have used the tryptophan modifier, *N*-bromosuccinimide (NBS), to probe for the involvement of tryptophan at the poly(P) and ATP binding site(s) of the enzyme. The effects of both phosphoryl donors on the quenching of tryptophanyl fluorescence by acrylamide and on their ability to protect the ATP- and poly(P)-dependent glucokinase activities against inactivation by NBS were also investigated. A peptide-containing tryptophan(s), which was protected against oxidation by NBS by both xylose+poly(P)$_5$ and xylose+ATP, has been isolated and its N-terminal sequence determined. The results from previous studies suggest that poly(P) and ATP may have separate binding sites, while the results from this investigation point to a common glucose phosphorylating (or catalytic) center for both substrates.

MATERIALS AND METHODS

Materials

N-Bromosuccinimide (NBS), ATP, AMP, xylose, glucose, glucose-6-phosphate dehydrogenase, sodium phosphate glass

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[1] Abbreviations: poly(P), polyphosphate; poly(P)$_n$, polyphosphate with *n* phosphate residues; poly(P)$_{35}$, phosphate glass type 35 (Sigma) with an average chain length of 35; NBS, *N*-bromosuccinimide; TFA, trifluoroacetic acid; CH_3CN, acetonitrile; PTH, phenylthiohydantoin; AUFS, absorbance units full scale.

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(Types 5 and 35), and hexaammonium tetrapolyphosphate (poly(P)$_4$) were from Sigma Chemical Co. *Staphylococcus aureus* V_8 protease was from Miles Scientific. All other chemicals were of reagent grade.

Methods

Purification of Poly(P)/ATP Glucokinase and Enzyme Assays. Poly(P)/ATP glucokinase from *M. tuberculosis* $H_{37}Ra$ was purified as described earlier (Hsieh et al., 1993). The poly(P)- and ATP-dependent glucokinase activities were assayed by a continuous coupled spectrophotometric method as described (Szymona, 1957).

N-Bromosuccinimide Oxidation of Poly(P)/ATP Glucokinase. For NBS oxidation, the enzyme (43 μg) was prepared in 0.1 M potassium phosphate buffer, pH 6.8. Different concentrations of NBS were added to the enzyme solutions, and the progress of the oxidation reaction was monitored at 280, 282, and 295 nm with a Shimadzu UV-160 spectrophotometer. The total number of tryptophans oxidized in the absence and in the presence of various substrates was determined by the method of Spande and Witkop (1967a). For the estimation of the number of tryptophans, a molecular weight of 33 000 (Hsieh et al., 1993) was used for the monomer of poly(P)/ATP glucokinase.

Estimation of the Total Number of Tryptophans in Poly(P)/ATP Glucokinase. The total number of tryptophan residues was estimated by the method of Edelhoch (1967) in the presence of 6 M guanidine hydrochloride.

Fluorescence Measurements. Fluorescence spectroscopy was carried out on a Perkin-Elmer LS-5B spectrofluorometer equipped with a temperature control device and microprocessor-controlled photomultiplier gain. All spectral determinations were made at 26 °C. The emission spectra were obtained by scanning from 300 to 400 nm with the spectrofluorometer in the ratio mode at an excitation wavelength of 295 nm and emission and excitation slit widths of 3 and 5 nm, respectively. The emission intensity values were corrected for dilution, self-absorption of the protein and the reagents (McClure & Edelman, 1967), and the inner filter effect (Hélène et al., 1969).

Acrylamide Quenching Studies. Fluorescence intensities were determined by continuous monitoring at 333 nm after excitation at 295 nm. The final intensity at any given acrylamide concentration was taken as a time-averaged value (n = 10). A concentrated stock solution of 4 M acrylamide was used for the additions. Corrections for absorption due to acrylamide itself at 295 nm were made according to the method of McClure and Edelman (1967) using a molar extinction coefficient of 0.29 M^{-1} cm^{-1} at 295 nm (Kumar et al., 1988). Fluorescence quenching data were analyzed according to the Stern–Volmer relation (eq 1) (Stern & Volmer, 1919):

$$F_0/F = 1 + K_{SV}[Q] \quad (1)$$

where F_0 is the fluorescence intensity in the absence of quencher, F is the fluorescence intensity at the molar quencher concentration [Q], and K_{SV} is the Stern–Volmer quenching constant obtained from the slope of the plot of F_0/F vs [Q].

Circular Dichroism Studies. The circular dichroism (CD) spectra of the unmodified and NBS-modified poly(P)/ATP glucokinases were recorded with a JASCO J600 model spectropolarimeter (JASCO, Inc., Easton, MD) equipped with a microprocessor-controlled data collection and processing system. All measurements were made with cylindrical quartz cells with a path length of 0.2 cm. The relative percentages of α-helix, β-sheet, turn, and random structures were estimated using the SSE computer program (supplied by JASCO, Inc.).

Proteolysis of NBS-Oxidized Poly(P)/ATP Glucokinase with V_8 Protease. For HPLC analysis and isolation of the NBS-oxidized peptides, about 8 nmol of poly(P)/ATP glucokinase (based on M_r 33 000 for monomeric enzyme) in 0.1 M potassium phosphate buffer (pH 6.8) was treated with 25 μM (final concentration) NBS for 5 min at 25 °C. The reaction was terminated by the addition of excess free tryptophan and dialyzed overnight at 4 °C against 0.1 M potassium phosphate buffer (pH 7.5). The modified enzyme was then treated with V_8 protease at a protease to glucokinase ratio of 1:25 (w/w) and digested for 24 h at 37 °C. Unmodified poly(P)/ATP glucokinase as well as the enzyme modified in the presence of various substrates was also processed in a similar manner for V_8 digestion.

HPLC Analysis of the V_8-Digested Enzyme Samples. V_8 peptide maps of the unmodified and NBS-modified poly(P)/ATP glucokinases were obtained with a Synchropak reverse-phase C_8 column (RP-8, 25 × 0.46 cm) using a Shimadzu LC-6A liquid chromatograph equipped with a CR-5A Chromatopak integrator. Peptides were eluted with a binary gradient using 0.1% aqueous TFA (solvent A) and 0.1% TFA in CH_3CN (solvent B), as described in the figure legends. Peptide elution was monitored at 220 and 280 nm using two Shimadzu UV–vis SPD 6AV spectrophotometers.

Amino Acid Analysis and Sequence Determination. For amino acid analysis, the peptides were hydrolyzed at 110 °C in 6 M HCl for 18 h *in vacuo* and analyzed with a Picotag column as described by Phillips and Wood (1986). Sequence analyses were carried out in an Applied Biosystems Model 470A sequencer (Hunkapiller et al., 1983). Phenylthiohydantoin (PTH) amino acids were identified by complementary HPLC systems (Glajch et al., 1985; Ericsson et al., 1977). Quantitative evaluation of the PTH amino acids was as described by Smithies et al. (1971) and by Machleidt and Hofner (1981).

RESULTS

Determination of the Total and Exposed Reactive Tryptophans of Poly(P)/ATP Glucokinase. The total number of accessible or reactive tryptophans of the poly(P)/ATP glucokinase was determined by spectrophotometric titration of the native enzyme with NBS. The number of modified tryptophans was proportional to increasing concentrations of NBS (Figure 1). From the analysis of a number of enzyme preparations, 3.5–4 tryptophans were found to be oxidized at an NBS concentration of 60–80 μM, according to the method of Spande and Witkop (1967a). At this NBS concentration, complete inactivation of the poly(P)- and ATP-dependent glucokinase activities was observed. No additional tryptophans were oxidized at higher NBS concentration. The total number of tryptophans in poly(P)/ATP glucokinase was also determined by the Edelhoch (1967) procedure in the absence or in the presence of 6 M guanidine hydrochloride and was also found to be 4 per monomer of glucokinase. These results indicate that all four tryptophans of poly(P)/ATP glucokinase are readily accessible to NBS oxidation. In the presence of xylose, under similar nondenaturing conditions, the number of tryptophans oxidized was 3.2–3.5, while in the presence of xylose+poly(P)$_5$, 2.5–3 tryptophan residues per monomer were found to be oxidized (Figure 1). These results indicate that 1–2 tryptophans may be located at substrate binding sites. Similar determinations in the presence of xylose+ATP were not possible due to the high self-absorbance of ATP at 280 nm.

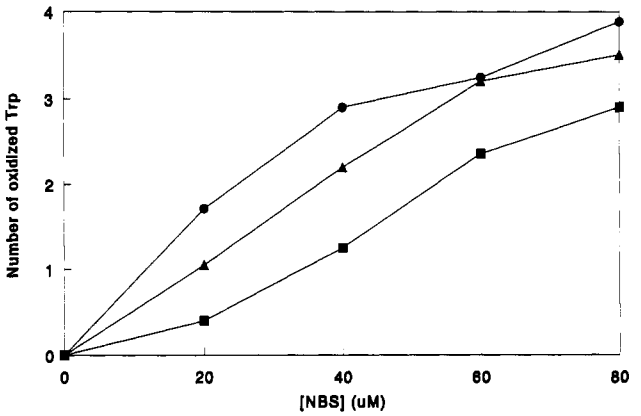

FIGURE 1: Correlation of the number of tryptophans oxidized with increasing concentrations of NBS. Varying concentrations of NBS as indicated were added to the enzyme, and the decrease in the 280-nm absorption was monitored. The number of tryptophans oxidized in the absence of substrate (●), in the presence of xylose (▲), and in the presence of xylose+poly(P)$_5$ (■) was determined as described in Materials and Methods.

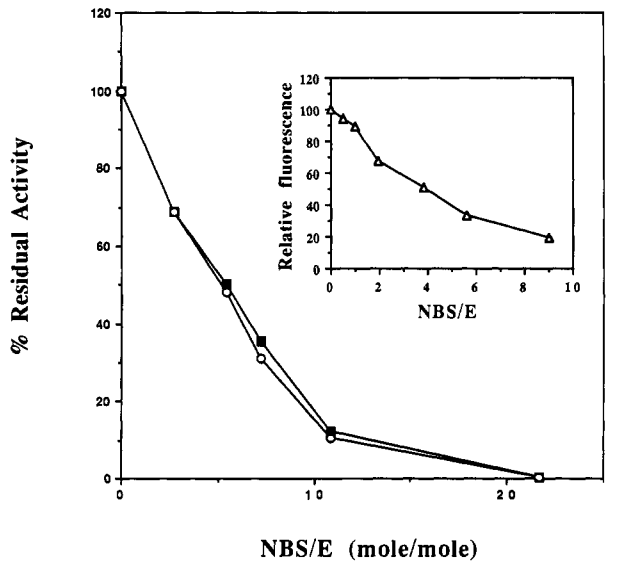

FIGURE 2: Correlation of the loss of both poly(P)- and ATP-dependent glucokinase activities and the quenching of the tryptophan fluorescence by varying concentrations of NBS. The enzyme (3.8 μg) was incubated with different concentrations of NBS (0, 5.6, 11.2, 15, 22.5, and 45 μM) in a final volume of 50 μL of 0.1 M potassium phosphate buffer (pH 6.8). After 5 min at 25 °C, the residual poly(P)- (■) and ATP- (○) dependent glucokinase activities were determined as described in Materials and Methods. The inset is a similar experiment displaying the loss of relative fluorescence (△) at a maximum emission wavelength of 333 nm with the same concentrations of NBS and enzyme.

Effect of Modification of Tryptophans on the Poly(P)- and ATP-Dependent Glucokinase Activities. Modification of tryptophan residues was carried out in order to determine whether tryptophan residues are essential for the glucokinase activity. Incubation of poly(P)/ATP glucokinase with increasing concentrations of NBS resulted in progressive inactivation of both the poly(P)- and ATP-dependent glucokinase activities (Figure 2). The NBS-dependent inactivation curve was idential for the poly(P)- and ATP-dependent activities, suggesting that the same tryptophans may be involved in both activities. The loss of both activities corresponded to the decrease in the tryptophanyl fluorescence induced by NBS oxidation (Figure 2, inset), suggesting that poly(P)/ATP glucokinase may have critical tryptophan residues essential for the activity of the enzyme. The results displayed in Figure 1 suggest that the critical tryptophan(s) may be located at the substrate binding sites, since one or two out of four tryptophans were protected against oxidation by NBS. This residue(s) could be involved in catalysis and/or binding of the substrates. Alternatively, modification of the tryptophans could have induced conformational changes that indirectly disrupted the integrity of the active site. This latter possibility is considered unlikely, as demonstrated below.

Demonstration That Inactivation of Poly(P)/ATP Glucokinase Activity by NBS Is Not Due to Extensive Conformational Changes. To determine whether NBS oxidation of the tryptophan residue(s) resulted in gross conformational changes, the CD spectra of native and NBS-modified poly(P)/ATP glucokinase were analyzed. The CD spectra of the native and NBS-oxidized enzymes were similar (Figure 3), indicating that NBS had a negligible effect on the distribution of β-sheet and α-helix in poly(P)/ATP glucokinase. Hence, the overall structure of the NBS-modified enzyme remained unchanged, and the loss of activity during NBS treatment did not appear to be correlated with structural alterations.

Moreover, when the enzyme was treated with increasing concentrations of NBS, the emission spectra of the glucokinase did not show any changes in the emission maximum (333 nm) when compared with that of the native enzyme (data not shown). If extensive conformational changes had occurred due to NBS modification, then a red shift or a blue shift in the emission maximum would be expected.

On the basis of these two criteria, it is probable that the inactivation of the poly(P)- and ATP-dependent glucokinase activities by NBS was due to oxidation of essential tryptophan(s) at the active site, which might be involved in either substrate binding and/or catalysis, rather than to modification-induced structural changes.

Substrate Protection against Inactivation by NBS. In order to confirm that the tryptophan(s) that is protected by substrate binding against NBS oxidation is critical for the glucokinase activities, different substrates (saturating levels) were tested individually or in combination for their ability to protect the glucokinase activity against inactivation by NBS. As shown in Table I, the phosphoryl acceptor substrate, glucose, or its analogue, xylose, protected 30–40% of both the poly(P)- and ATP-dependent glucokinase activities. The phosphoryl donor substrate poly(P)$_5$ afforded ~33% protection of the poly(P)-dependent glucokinase activity, while ATP (or MgATP, data not shown) did not protect the ATP-dependent glucokinase activity against inactivation by NBS. To test the effect of the sugar substrate in combination with the phosphoryl donor substrates, it was necessary to use xylose, a glucose analogue, with either poly(P)$_5$ or ATP to avoid turnover of the substrates while monitoring their protective effects. In the presence of xylose, the protection afforded by poly(P)$_5$ or ATP against inactivation of their respective activities by NBS was about 65%. It was not feasible to accurately test the effect of the phosphoryl substrate poly(P)$_5$ on the ATP-dependent reaction or the effect of ATP on the poly(P)-dependent reaction, since these substrates interfere with the spectrophotometric assay of the individual reactions. For instance, when poly(P)$_5$ is added as the protectant, as shown in row 5 of Table I, it acts as an additional substrate, resulting in erroneous measurements of the ATP-dependent reaction. Hence, the ability of the two phosphoryl donors to cross-protect each others' activity was examined with the phosphoryl donor analogues, poly(P)$_4$ and AMP. AMP was found to be a competitive inhibitor of ATP, while poly(P)$_4$ is an extremely poor substrate for the poly(P)-dependent reaction (data not shown). Thus, these two analogues were expected to mimic

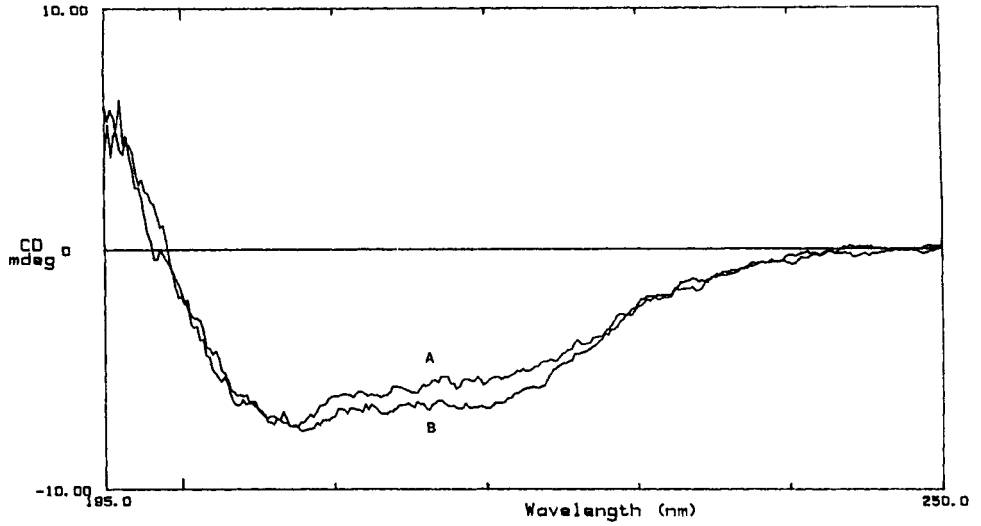

FIGURE 3: Circular dichroism spectra of the enzyme modified with NBS (A) and native enzyme (B). CD measurements were conducted with protein concentrations of 0.1 mg/mL in 0.05 M potassium phosphate buffer (pH 6.8) containing 0.2 M NaCl. The spectrum for the NBS-modified enzyme was obtained after incubating the enzyme with 25 μM NBS for 5 min at 25 °C. The % changes in the structure of the enzyme due to NBS were 1.2% for α-helix, 6% for β-sheet, 6% for turn, and 1% for random coils.

Table I: Effect of Substrates on the Inactivation of the Poly(P)- and ATP-Dependent Glucokinase Activities by NBS[a]

additions	poly(P) glucokinase activity (%)	ATP glucokinase activity (%)
control	100	100
NBS	4	3
xylose + NBS	38	34
glucose + NBS	39	32
poly(P)$_5$ + NBS	33	NA[b]
poly(P)$_4$ + NBS	25	20
ATP + NBS	NA	1
xylose + poly(P)$_5$ + NBS	68	NA
xylose + ATP + NBS	NA	62
glucose + poly(P)$_4$ + NBS	80	54
glucose + AMP + NBS	58	56

[a] Poly(P)/ATP glucokinase (3 μg) in 0.1 M potassium phosphate buffer (pH 6.8) was modified with NBS (13.6 μM, final concentration) in the absence or presence of substrates or analogues, at 25 °C for 5 min. The residual glucokinase activity was determined at 30 °C by the spectrophotometric assay as described in Materials Methods. The final concentrations of the added ligands were as follows: glucose, 90 mM; xylose, 90 mM; poly(P)$_5$, 8 mM; poly(P)$_4$, 9 mM; ATP, 50 mM; AMP, 50 mM. All substrates used were at saturating levels. [b] NA, not applicable. These activities could not be determined as the phosphoryl donor ligand added as a protectant acted as a substrate.

the phosphoryl donor substrates and also not to cause any turnovers when used in combination with glucose. When poly-(P)$_4$ was used in combination with glucose, 80% protection of the poly(P)-dependent activity was observed. Interestingly, poly(P)$_4$ in the presence of glucose also afforded 54% protection of the ATP-dependent activity. Similarly, AMP in the presence of glucose conferred 58% protection of the poly-(P)-dependent activity in addition to protecting 56% of the ATP-dependent activity. These results suggest that the critical tryptophan(s) is located at a site common to both substrates, perhaps at a single phosphorylating center.

Assessment of the Microenvironment of Tryptophans Using Acrylamide Quenching in the Absence and Presence of Various Substrates. The emission maxima of the native and denatured (in 8 M urea) poly(P)/ATP glucokinases were found to be 333 and 352 nm, respectively (data not shown). This result suggests that the tryptophan residues in the enzyme are partially buried. Hence, the changes in the microenvironments of the tryptophans of poly(P)/ATP glucokinase as a result of substrate binding were further assessed by acrylamide

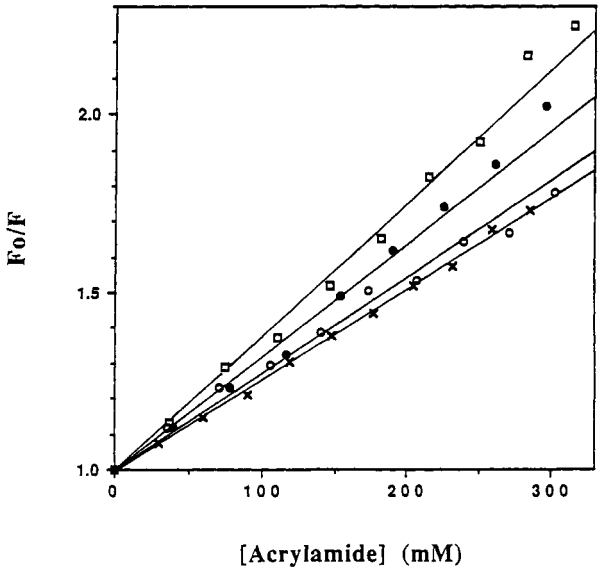

FIGURE 4: Stern–Volmer plots of quenching of tryptophan fluorescence of poly(P)/ATP glucokinase by acrylamide. The glucokinase (0.15 mg/mL) in 0.01 M potassium phosphate buffer (pH 6.8) containing 0.2 M NaCl was used in these experiments. The quenching of the tryptophan fluorescence (●) was followed by addition of varying concentrations of acrylamide. The quenching was also monitored in the presence of 90 mM xylose (□), 90 mM xylose + 10 mM poly(P)$_5$ (×), and xylose + 1.6 mM ATP (○). The collisional quenching constants (K_{SV}) calculated from the slopes of the above plots are as follows: native enzyme, 3.55 M^{-1}; in the presence of xylose, 3.98 M^{-1}; xylose+poly(P)$_5$, 2.48 M^{-1}; and xylose+ATP, 2.57 M^{-1}.

quenching experiments (Lehrer, 1971). The enzyme was titrated with acrylamide in the absence or presence of the different substrates. As shown in Figure 4, acrylamide was found to be an effective quencher of the tryptophan fluorescence of poly(P)/ATP glucokinase and gave linear Stern–Volmer plots for the four different conditions. From the Stern–Volmer plots, the quenching constants, K_{SV}, for the various substrates were determined.

The linearity of the Stern–Volmer plot with increasing concentrations of acrylamide suggests that the microenvironments of the multiple tryptophans contributing to the fluorescence emission were essentially homogeneous. The Stern–Volmer quenching constant for the native enzyme was 3.55 M^{-1}, while in the presence of the substrates the (K_{SV})

Table II: Kinetic Properties of Chemically Modified Poly(P)/ATP Glucokinase[a]

concentration of NBS (μM)	poly(P) as the variable substrate[b]		ATP as the variable substrate[c]	
	V_{max} (units)	K_m (μM)	V_{max} (units)	K_m (mM)
0	11.86 ± 0.35	16.15 ± 1.08	6.13 ± 0.19	1.77 ± 0.08
2.8	8.14 ± 0.9	13.65 ± 2.96	4.75 ± 0.3	1.67 ± 0.17
5.6	6.56 ± 0.2	14.73 ± 0.9	3.72 ± 0.1	1.72 ± 0.07
8.4	3.31 ± 0.06	16.8 ± 0.52	2.14 ± 0.035	1.69 ± 0.04

[a] Poly(P)/ATP glucokinase (0.065 mg/mL) was treated with different concentrations of NBS at 25 °C for 3 min in potassium phosphate buffer (pH 6.8). The reaction was then terminated by adding 5 μL of free tryptophan (5 mg/mL), and it was assayed for poly(P)- and ATP-dependent glucokinase activities. One unit of activity is defined as the amount of enzyme required to produce 1 μmol of glucose 6-phosphate per minute. [b] The V_{max} and K_m values for poly(P)-dependent glucokinase activity were determined by using a variable concentration of poly(P) (40, 20, 10, and 5.71 μM) and a fixed glucose concentration (13 mM). [c] Determination of the V_{max} and K_m for ATP-dependent glucokinase activity was carried out in the presence of a variable concentration of ATP (4, 1, 0.8, and 0.5 mM) and a fixed concentration of glucose (13 mM). The data were then fit with a nonlinear regression program (Enzfitter, by R. J. Leatherbarrow from Elsevier-Biosoft) to calculate the values for V_{max} and K_m (mean ± SE).

values were decreased. The quenching constants for xylose+poly(P)$_5$ and xylose+ATP were 2.48 and 2.57 M^{-1}, respectively, suggesting that one or more tryptophans may be sterically shielded from acrylamide quenching by poly(P)$_5$ and ATP, since in the presence of xylose alone the quenching constant was slightly increased to a value of 3.98 M^{-1}. The linearity of the plots as well as the similar intercepts also indicates that the reduced K_{SV} values may be due to steric shielding (Kumar et al., 1988).

Effect of Modification of Tryptophan Residue(s) on the Kinetic Properties of Poly(P)/ATP Glucokinase. In order to determine whether the tryptophan residue(s) was involved in binding of the phosphoryl donor substrates or in catalysis, the kinetic constants of the modified enzyme were determined and compared with that of the unmodified enzyme. The results shown in Table II indicate that the K_m values for both ATP and poly(P) substrates were not altered, whereas V_{max} values significantly decreased on modification of the tryptophan residues at various concentrations of NBS. These results suggest that the tryptophan residues may not be involved in the binding of the substrate, but are necessary for the catalysis of the enzyme.

Isolation and Sequence Analysis of the Tryptophan-Containing Peptide at the Poly(P) and ATP Binding and/or Catalytic Site(s). The finding (Table I) that both poly(P)$_5$ and ATP in the presence of xylose conferred protection of the glucokinase activities against inactivation by NBS suggested the involvement of tryptophan(s) at the active sites. In order to identify the peptide(s) containing the critical tryptophan, the NBS-oxidized as well as the untreated enzymes were digested with V_8 protease and fractionated by reverse-phase HPLC. The tryptophanyl-containing peptides were detected by monitoring their absorptions at 280 nm. The strategy utilized in the isolation of the active site tryptophan-containing peptide exploits the fact that the indole chromophore of tryptophan, which absorbs strongly at 280 nm, is converted to oxindole, a much weaker chromophore at 280 nm, upon oxidation with NBS. This underlying principle was first applied by Patchornik et al. (1958) for the estimation of the tryptophan content of proteins. As seen in Figure 5B, the 280-nm absorption of the NBS-oxidized peptides was markedly reduced compared to that of the unoxidized peptides (Figure 5A). In order to identify the tryptophan peptide(s) that was

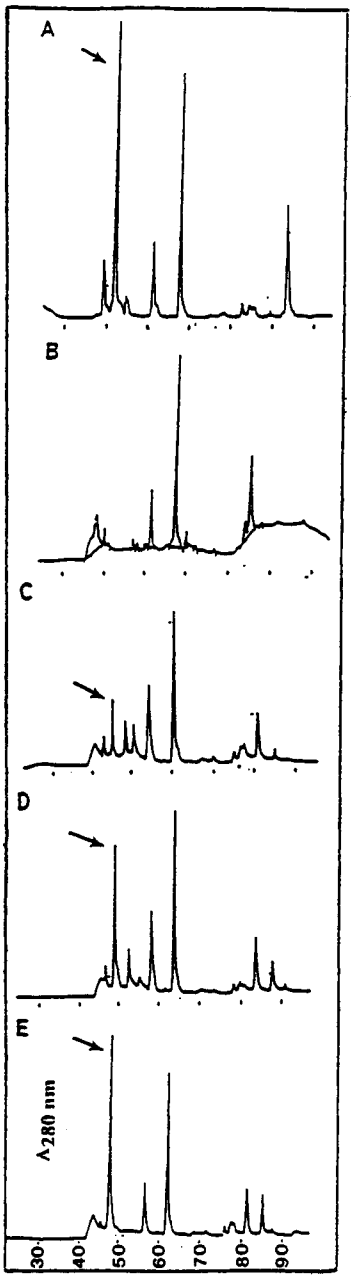

FIGURE 5: HPLC peptide maps of V_8 protease digests of poly(P)/ATP glucokinase. The enzyme was treated with NBS in the absence (B) or presence of xylose (C), in the presence of xylose+poly(P)$_5$ (D), and in the presence of xylose+ATP (E). In the control (A), the 280-nm absorption profile of the unmodified enzyme digested with V_8 protease is displayed. The modification with NBS and proteolytic digestion were carried out as described in Materials and Methods. The V_8 digests were loaded on a Synchropak C_8 reverse-phase column and peptides eluted isocratically for 15 min with 0.1% aqueous TFA at a flow rate of 0.9 mL/min. A linear gradient of 0–60% CH_3CN over 120 min was then applied by using aqueous 0.1% TFA and CH_3CN containing 0.1% TFA as the binary solvent system. Tryptophan-containing peptides were monitored at 280 nm to detect aromatic amino acid containing peptides at a sensitivity setting of 0.04 AUFS for all chromatograms. The peptide that was sensitive to NBS but protected by xylose+poly(P)$_5$ (D) and xylose+ATP (E) is indicated (→) in all of the appropriate chromatograms.

protected by the substrates, poly(P)/ATP glucokinase was modified by NBS in the presence of the various substrates, digested with V_8 protease, and subjected to peptide mapping as above. The protected peptide(s) was expected to retain its 280-nm absorption. The V_8–peptide profiles of the enzyme treated with NBS in the presence of poly(P)$_5$+xylose are shown

Table III: Amino Acid Composition of Substrate-Protected Peptides of Poly(P)/ATP Glucokinase

amino acid	control peptide[a]		xylose+poly(P)$_5$-protected peptide		xylose+ATP-protected peptide	
	analysis	sequence	analysis	sequence	analysis	sequence
Asx	0.8 (1)	1	1.1 (1)	1	0.73 (1)	1
Glx	1.8 (2)	2	1.73 (2)	2	1.79 (2)	2
Arg	1.76 (2)	2	1.71 (2)	2	1.8 (2)	2
Ser	1.7 (2)	2	1.64 (2)	2	1.7 (2)	2
Ala	1.19 (1)	1	0.6 (1)	1	1.15 (1)	1
Lys	0.88 (1)	1	0.83 (1)	1	0.98 (1)	1
Tyr	0.96 (1)	1	1.07 (1)	1	0.85 (1)	1
Trp[b]	ND	2	ND	2	ND	2
Gly[c]			1.4 (1)			
Thr[c]			0.78 (1)			
Val[c]			1.09 (1)			
totals	10	12	13	12	10	12

[a] The control peptide was purified from chromatogram A of Figure 5. [b] Tryptophan was destroyed during acid hydrolysis and was not determined (ND) in the composition. [c] These amino acids were detected as contaminants from the composition analysis.

in Figure 5D and those for enzyme in the presence of ATP+xylose are shown in Figure 5E. In both cases, the 280-nm absorption of the peptide eluting with a retention time of ~48 min was not affected by NBS treatment, while in the absence of these substrates, the absorption of the same peptide was markedly reduced in peak height (Figure 5B). These results suggest that xylose+poly(P)$_5$ and xylose+ATP protected the same tryptophan-containing peptide from oxidation by NBS. The protection was not due to xylose as such, since xylose conferred only a partial protection to the same peptide (Figure 5C).

The protected peptides from the experiments shown in Figure 5D,E were recovered manually by monitoring absorption at 220 and 280 nm. These peptides were then subjected to amino acid composition and sequence analyses. The results of these analyses are shown in Table III. The sequences of both peptides as well as that of the control (Figure 5A) were found to be the same: NH_2-Arg-Lys-Asp-Trp-Ser-Tyr-Ala-Arg-Trp-Ser-Glu-Glu-COOH. The presence of two tryptophans in the above sequence is consistent with the results obtained in Figure 1, where it is concluded that 1–2 tryptophan residues may be protected by the substrates. The results from the amino acid composition (Table III) also confirmed that xylose+poly(P)$_5$ and xylose+ATP protected the same tryptophanyl peptide, which was identical to the peptide that was isolated from the V_8 digest of the native enzyme (control) (Figure 5A), although some contaminating residues were observed in the poly(P)$_5$+xylose sample.

DISCUSSION

In this investigation, we have utilized the tryptophan-modifying reagent NBS to probe the active site of poly(P)/ATP glucokinase. The effects of NBS oxidation and the protective effect of various substrates against NBS oxidation of poly(P)/ATP glucokinase have been evaluated. From these studies, it is concluded that tryptophan(s) is essential for both the poly(P)- and ATP-dependent glucokinase activities and may be located at a common catalytic site for poly(P) and ATP.

One or more tryptophans are essential for the poly(P)- and ATP-dependent activities of the mycobacterial glucokinase, and these may be located near the poly(P) and ATP binding sites. Several lines of evidence support this conclusion and are as follows: (i) The oxidation of the tryptophan of poly(P)/ATP glucokinase by NBS is associated with concomitant loss of both the poly(P)- and ATP-dependent glucokinase activities (Figure 2). (ii) Poly(P)$_5$ and ATP in the presence of the glucose analogue, xylose, afforded similar levels of protection against the loss of the poly(P)- and ATP-dependent activities, respectively, due to modification with NBS (Table I). (iii) Inactivation of the poly(P)- and ATP-dependent glucokinase activities by modification with NBS was not due to extensive structural changes, as evidenced by the similar CD spectra of the NBS-modified and unmodified enzymes (Figure 3). However, minor conformational changes indirectly leading to catalytic inhibition would not be detected by this method. Nevertheless, the emission maximum of poly(P)/ATP glucokinase during the oxidation of the four tryptophans of the enzyme by NBS was not altered, which is further evidence against conformational changes in the enzyme due to the oxidation of tryptophans.

Additional evidence for the involvement of tryptophan(s) in the poly(P) and ATP binding site of the mycobacterial glucokinase comes from the acrylamide quenching studies. The intact enzyme exhibited a homogeneous microenvironment for tryptophans, since the Stern–Volmer plot displayed linear quenching with increasing concentrations of acrylamide. The quenching constant (K_{SV}) in the absence of substrates was 3.55 M^{-1}, while in the presence of xylose+poly(P)$_5$ or xylose+ATP the K_{SV} values were 2.48 and 2.57 M^{-1}, respectively. This decrease (~30%) in the K_{SV} value in the presence of xylose+poly(P)$_5$ or xylose+ATP suggests that the hydrodynamic properties of the tryptophans in the excited state were altered by substrate-induced conformational changes (Hélène, 1977) or by creating an unfavorable electrostatic environment for acrylamide (Omar & Schleich, 1981). In either case, the reduced K_{SV} values in the presence of substrates suggest that certain tryptophans near the substrate binding site are shielded from acrylamide quenching.

The critical tryptophan(s) may be located at a common catalytic center for the phosphorylation of glucose by either poly(P) or ATP. The observation that both the poly(P)- and ATP-dependent activities were lost in parallel to >95% due to oxidation by NBS suggested that the essential tryptophan(s) may be located at a common binding and/or catalytic center for poly(P) and ATP. Previous studies with the adenine analogue, Reactive Blue 4, showed that it was a noncompetitive inhibitor of the poly(P)-dependent glucokinase reaction with respect to poly(P), while it was competitive with ATP in the ATP-dependent glucokinase reaction (Hsieh et al., 1993). We concluded from those studies that the adenine portion of ATP and the bulk of the poly(P) moiety may have separate binding sites, although the glucose phosphorylating center may be common for both poly(P) and ATP. This conclusion is supported by parallel studies conducted with the poly(P)/ATP glucokinase from *Propionibacterium shermanii*, where Phillips et al. (1993) demonstrated that two analogues of ATP with different reactive groups displayed different inhibition patterns with respect to ATP and poly(P). The 2′,3′-dialdehyde of ATP (oATP), whose reactive group is the dialdehyde of the ribose ring, showed competitive and noncompetitive inhibition patterns with regard to ATP and poly(P), respectively, in their individual activities. In contrast, 5′-(*p*-fluorosulfonylbenzoyl)adenosine (FSBA), whose reactive sulfonyl fluoride group is related to the γ-phosphoryl group of ATP, displayed competitive inhibition patterns with both ATP and poly(P).

Additional evidence from the present study supporting the conclusion that poly(P) and ATP may share a common

phosphorylating center is the following: (i) The inactive phosphoryl donor analogue, poly(P)$_4$, protected both the poly(P)- and ATP-dependent glucokinase activities. (ii) Although poly(P)$_4$ in the presence of glucose afforded a higher degree of protection (~80%) of the poly(P)-dependent activity, it also afforded 54% protection of the ATP-dependent activity. (iii) AMP in the presence of glucose conferred 58% protection of the poly(P)-dependent activity, besides affording similar levels of protection to the ATP-dependent reaction. (iv) When the V_8-digested peptide maps of tryptophan-containing peptides of glucokinase oxidized in the absence or presence of xylose+ATP and xylose+poly(P)$_5$ were compared, it was found that xylose+poly(P)$_5$ and xylose+ATP protected the same peptide (with reference to their retention times) against loss of the 280-nm absorption (Figure 5D,E). Furthermore, N-terminal sequence analyses of the tryptophan peptide protected by xylose+ATP and xylose+poly(P)$_5$ were found to be identical. The minor differences in amino acid composition observed between ATP- and poly(P)-protected peptides were probably due to the presence of a contaminating peptide found along with the protected peptide.

The kinetic studies performed with the native and NBS-modified enzymes indicated that the values of K_m of the substrates poly(P) and ATP were not altered by the modification of tryptophan, but the catalytic efficiency of the enzyme decreased on modification. This suggests that the tryptophan residues are not directly involved in substrate binding, but are present at the catalytic site.

The results from these studies suggest that one or both of the tryptophans present in the protected peptide may be located at a common catalytic center, where the terminal phosphates of ATP or poly(P) come in contact with glucose during the transphosphorylation reaction. Owing to the variabilities in the repetitive yields of each PTH-amino acid from the sequencing cycles, it was not possible to accurately determine which of the two tryptophan residues was recovered in higher yield. The tryptophan with the higher yield would be implicated as that which had been protected by the two substrates. Nevertheless, the close proximity of the two tryptophans in the peptide suggests that the isolated tryptophan peptide may be part of a common catalytic center.

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