Further studies to locate the calcium-binding site and the initial polymerization site will be greatly facilitated by the recently published completion of the fibrinogen primary sequence (Doolittle et al., 1979).

Acknowledgments

We were ably assisted by N. Linton.

References

Blomback, B., Hessel, B., Hogg, D., & Therkildsen, L. (1978) *Nature (London) 275*, 501.
Bock, P. E., & Frieden, C. (1978) *Trends Biochem. Sci. 3*, 100.
Brand, L., & Witholt, B. (1967) *Methods Enzymol. 11*, 776.
Castellino, F. J., & Sodetz, J. M. (1976) *Methods Enzymol. 45b*, 273.
Doolittle, R. F. (1973) *Adv. Protein Chem. 27*, 1.
Doolittlle, R. F., Goldbaum, D. M., & Doolittle, L. R. (1978) *J. Mol. Biol. 128*, 311.
Doolittle, R. F., Watt, K. W. K., Cottrell, B. A., Strong, D. D., & Riley, M. (1979) *Nature (London) 280*, 464.
Dray-Attali, L., & Larrieu, M. J. (1977) *Thromb. Res. 10*, 595.
Ferguson, E. W., Fretto, L. J., & Mckee, P. A. (1975) *J. Biol. Chem. 250*, 7210.
Finney, D. J. (1964) *Statistical Methods in Biological Assay*, Griffin, London.
Greenwood, F. C., Hunter, W. M., & Glover, J. S. (1963) *Biochem. J. 89*, 114.
Heene, D. L., & Matthias, F. R. (1973) *Thromb. Res. 2*, 137.
Kudryk, B. J., Collen, D., Woods, K. R., & Blomback, B. (1974) *J. Biol. Chem. 249*, 3322.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lindsey, G. G., Brown, G., & Purves, L. R. (1978a) *Thromb. Res. 13*, 345.
Lindsey, G. G., Brown, G., & Purves, L. R. (1978b) *Thromb. Res. 12*, 467.
Lorand, L. (1972) *Ann. N.Y. Acad. Sci. 202*, 6.
Marguerie, G. (1977) *Biochim. Biophys. Acta 494*, 172.
Nussenzweig, V., Seligman, M., Pelmont, J., & Grabar, P. (1961) *Ann. Inst. Pasteur Paris 100*, 377.
Olexa, S. A., & Budzynski, A. Z. (1979) *J. Biol. Chem. 254*, 4925.
Plow, E. F., & Edgington, T. S. (1975) *J. Clin. Invest. 56*, 1509.
Purves, L. R., Lindsey, G. G., & Franks, J. J. (1978a) *S. Afr. J. Sci. 74*, 202.
Purves, L. R., Lindsey, G. G., Brown, G., & Franks, J. J. (1978b) *Thromb. Res. 12*, 473.
Rodbard, D. (1974) *Clin. Chem. (Winston-Salem, N.C.) 20*, 1255.
Vogel, R., Trautschold, I., & Werle, E. (1968) *Natural Proteinase Inhibitors*, p 76, Academic Press, New York.
Walsh, K. A. (1970) *Methods Enzymol. 19*, 42.

Reaction of Brain Hexokinase with a Substrate-like Reagent. Alkylation of a Single Thiol at the Active Site†

Ghanshyam Swarup and Umakant W. Kenkare*

ABSTRACT: An analogue of the substrate glucose, *N*-(bromoacetyl)-D-glucosamine (GlcNBrAc) inactivates bovine brain mitochondrial hexokinase completely and irreversibly in a pseudo-first-order fashion at pH 8.5 and 22 °C. The rate of inactivation of hexokinase by this reagent does not increase linearly with increasing reagent concentration but exhibits an apparent saturation effect, suggesting the formation of a reversible complex between the enzyme and the reagent prior to the inactivation step. The pH dependence of the rate of inactivation suggests that a group on the enzyme with pK_a = 9.1 is being modified by this reagent. At pH 8.0 the rate of inactivation by this reagent is very slow, and it can be shown to be a competitive inhibitor of the hexokinase reaction with respect to the substrate glucose. The substrates glucose and ATP strongly protected the enzyme against the inactivation reaction. The inactivation of the enzyme was found to be accompanied by the alkylation of two sulfhydryl residues as shown by the formation of ~2 mol of *S*-(carboxymethyl)-cysteine/mol of inactivated enzyme. Treatment of the enzyme with ^{14}C-labeled reagent results in the incorporation of ~2 mol of reagent/mol of inactivated enzyme. However, the enzyme protected by glucose still shows the incorporation of ~1 mol of the labeled reagent/mol of the enzyme. From a tryptic digest of the enzyme inactivated by this reagent, two labeled peptides were obtained, one of which was absent if the labeling reaction was carried out in presence of glucose. These results indicate that the affinity reagent reacts with two thiols, only one of which is crucial for the activity of the enzyme and is located in the region of its active site.

Previous reports from this laboratory provided evidence for the presence of thiol groups at or near the active site of bovine brain mitochondrial hexokinase, type I (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1). This evidence was mostly based on an investigation of the inactivation kinetics of the enzyme using 5,5′-dithiobis(2-nitrobenzoic acid) and tetranitromethane as protein modification reagents (Redkar & Kenkare, 1972, 1975; Subbarao et al., 1973; Subbarao & Kenkare, 1977a). Further documentation for the presence of an essential thiol at the active site of this enzyme came from experiments in which the enzyme was inactivated by an affinity reagent, 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate, which is an analogue of ATP (Subbarao & Kenkare, 1977b).

Our search to identify glucose analogues as affinity reagents for brain hexokinase led us to explore the use of haloacetyl

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0006-2960/80/0419-4058$01.00/0

derivatives of glucosamine as affinity reagents for brain hexokinase (Subbarao et al., 1973). In this paper we present kinetic and chemical evidence that a glucose analogue, *N*-(bromoacetyl)-D-glucosamine, functions as a substrate-like affinity reagent for brain hexokinase, modifying an essential thiol at the active site of the enzyme. An isomer of this glucosamine derivative, *N*-(bromoacetyl)-D-galactosamine, has been recently reported to function as an affinity reagent for yeast hexokinase (Otieno et al., 1975, 1977). A preliminary report of our work was presented earlier (Kenkare & Swarup, 1978).

Materials and Methods

Chemicals. D-Glucosamine hydrochloride, D-galactosamine hydrochloride, and *N*-acetyl-D-glucosamine were obtained from Sigma Chemical Co. Worthington Biochemical Corp. was the source of L-(1-tosylamino-2-phenyl)ethyl chloromethyl ketone treated trypsin. Glucose-6-phosphate dehydrogenase, $NADP^+$, ATP, and glucose 6-phosphate were purchased from Boehringer, Mannheim, West Germany. 2-Mercaptoethanol was a product of E. Merck. Bromoacetic acid was supplied by Riedel-DeHaen AG and was crystallized from petroleum ether before use. Iodoacetic acid (Merck) was also crystallized from petroleum ether. Bromoacetic anhydride was obtained from ICN Pharmaceutical, Inc. Ultrapure grade urea was a product of Schwarz/Mann. D-[$^{14}C_1$]Glucosamine hydrochloride was obtained from Radiochemical Centre, Amersham, England.

Preparation of GlcNBrAc.[1] GlcNBrAc was prepared by bromoacetylation of D-glucosamine by bromoacetic anhydride essentially as described by Otieno et al. (1975). The product (mp 148–150 °C with decomposition) was found to be homogeneous by TLC on a silica gel G plate in methanol–acetone (1:10 v/v) and in butanol–acetic acid–water (4:1:1 v/v). It gave a strongly positive test in the modified Morgan–Elson reaction for *N*-acylhexosamine (Reissig et al., 1955). Anal. Calcd for $C_8H_{14}BrO_6N$: C, 32.1; H, 4.7; Br, 26.6; N, 4.7. Found: C, 31.24; H, 4.98; Br, 29.93; N, 4.82.

Preparation of the Enzyme. Type I hexokinase from bovine brain mitochondria was purified essentially by the procedure of Redkar & Kenkare (1972) but with the following modification. The second DEAE-cellulose column chromatography was omitted, and, instead, the enzyme preparation obtained after the first DEAE-cellulose chromatography was further purified by affinity chromatography on a phosphocellulose column. Hexokinase was specifically eluted from this column by 50 μM glucose 6-phosphate (Swarup, 1979). The enzyme purified in this manner showed only one protein band on polyacrylamide gel electrophoresis. The enzyme was at least 95% pure and had a specific activity of 80–90 units/mg.

Enzyme Assay. Hexokinase was usually assayed spectrophotometrically as described by Subbarao & Kenkare (1977a). The enzyme was also assayed fluorometrically in some experiments for better sensitivity (Maitra & Lobo, 1971). One unit of enzyme activity is defined as the amount of hexokinase that catalyzes the conversion of 1 μmol of the substrate into the product under the reported assay conditions (30 °C; pH 8.0). The procedure of Lowry et al. (1951) was used for protein estimation using bovine serum albumin as the standard.

Experiments with GlcNBrAc. The enzyme was made free of all the ligands by exhaustive dialysis at 4 °C against 10 mM *N*-ethylmorpholine–acetate buffer, pH 8.0. The removal of glucose and 2-mercaptoethanol is accompanied by ~15–20% loss in enzyme activity. All the inactivation experiments were carried out at room temperature (22–24 °C) unless otherwise stated. A 10–20-μL aliquot of aqueous solution of GlcNBrAc was added to the hexokinase solution (0.5 or 1.0 mL) in 0.1 M *N*-ethylmorpholine–acetate buffer, pH 8.5. The enzyme activity was measured spectrophotometrically or fluorometrically by withdrawing 5-μL aliquots at intervals and diluting into the assay mixture.

Amino Acid Analysis. The hexokinase samples were hydrolyzed with 6 N hydrochloric acid at 110 °C in evacuated sealed glass tubes. Amino acid analysis of the protein hydrolysate was performed on a Technicon single-column amino acid analyzer which employs a combined pH and salt gradient for the elution of amino acids. In this system carboxymethylcysteine elutes just before aspartic acid but well separated from it.

Measurement of the Uptake of ^{14}C-Labeled Reagent by the Enzyme. Hexokinase was incubated with 1.0 or 2.0 mM *N*-(bromoacetyl)-D-[$^{14}C_1$]glucosamine in 0.1 M *N*-ethylmorpholine–acetate buffer, pH 8.5. Samples were withdrawn at various stages of inactivation and the protein was precipitated by the addition of cold 20% trichloroacetic acid. After 20 min at 4 °C the precipitated protein was filtered and washed repeatedly with 10% trichloroacetic acid on a cellulose nitrate filter. After the filter was dried, the radioactivity was measured in toluene scintillant by using a Packard Tri-Carb liquid scintillation spectrometer. The scintillation fluid contained 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene in 1000 mL of toluene.

Carboxymethylation of Hexokinase. Cysteine residues of hexokinase were carboxymethylated with iodoacetic acid in 8 M urea as described by Crestfield et al. (1963).

Trypsin Digestion. Before tryptic digestion, hexokinase samples were carboxymethylated so as to block all the remaining unmodified cysteine residues. The *S*-(carboxymethyl)hexokinase was taken in 0.1 M ammonium bicarbonate, pH 8.5, at a protein concentration of 2–5 mg/mL and incubated with trypsin (1% w/w) at 37 °C for 20 h. Another aliquot of trypsin was then added and the digestion was carried out for another 20 h.

Separation of Peptides on the Sephadex G-50 Column. The mixture of peptides obtained by tryptic digestion of the modified hexokinase was separated by gel filtration on a Sephadex G-50 superfine column (1.2 × 88 cm) equilibrated with 10 mM *N*-ethylmorpholine–acetate buffer, pH 8.0. The elution of peptides was carried out with the same buffer at a flow rate of ~4.5 mL/h and ~1.8-mL fractions were collected. Fractions were assayed for radioactivity in scintillation fluid made up of 60 g of naphthalene, 4 g of 2,5-diphenyloxazole, 0.2 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, 100 mL of methanol, and *p*-dioxane to make the volume 1000 mL. Peptides were monitored by measuring absorbance at 230 nm.

Results

Inactivation of Brain Hexokinase by GlcNBrAc. *N*-Bromoacetyl-D-glucosamine causes time-dependent inactivation of brain hexokinase at pH 8.5 and room temperature (22 °C). When the inactivated enzyme is dialyzed, the enzyme activity is not restored, suggesting that the inactivation of the enzyme is irreversible, probably due to the covalent modification of the enzyme by this reagent. The inactivation reaction follows pseudo-first-order kinetics and leads to a complete loss of enzyme activity after sufficient time (Figure 1).

The pseudo-first-order rate constant of the inactivation of the enzyme by GlcNBrAc does not increase linearly with increasing concentration of the reagent and shows an apparent saturation effect. Thus, the reaction of GlcNBrAc with brain hexokinase appears to proceed through the formation of a

[1] Abbreviation used: GlcNBrAc, *N*-(bromoacetyl)-D-glucosamine.

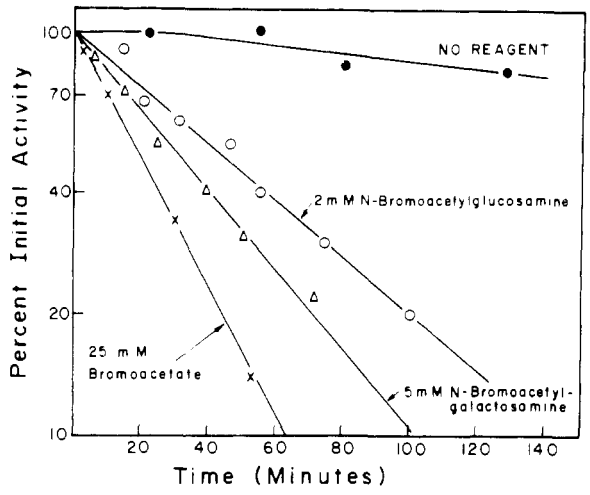

FIGURE 1: Semilogarithmic plot of inactivation of brain hexokinase by alkylating reagents. The reaction mixture, containing 2 μM hexokinase in 0.1 M *N*-ethylmorpholine–acetate buffer, pH 8.5, was incubated at room temperature (22 °C) with different reagents as indicated in the figure. No reagent was added in the control experiment.

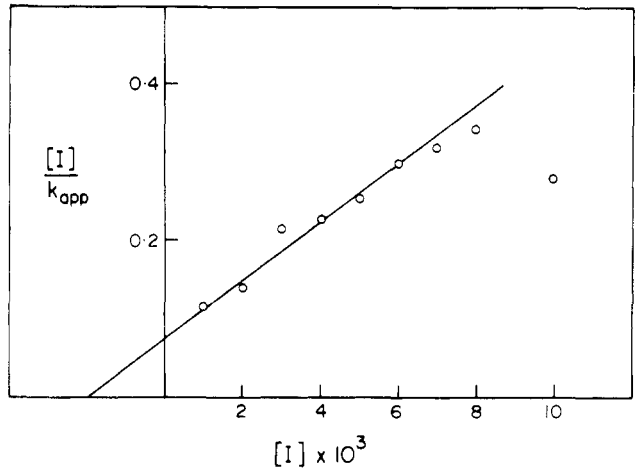

FIGURE 2: Determination of the dissociation constant of GlcNBrAc with respect to the enzyme. The pseudo-first-order rate constants were determined at various reagent concentrations from semilogarithmic plots of the type shown in Figure 1. The conditions of the experiment were the same as those described in Figure 1.

reversible complex between the inhibitor (I) and enzyme (E) prior to the inactivation step as shown:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [E{\cdot}I] \rightarrow E\text{-}I \qquad (1)$$

Here k_1 and k_{-1} are rate constants of formation and breakdown of enzyme–inhibitor complex and k_2 is the pseudo-first-order rate constant of inactivation of the enzyme–inhibitor complex to give the inactive enzyme. By use of an appropriate equation (Kitz & Wilson, 1962; Otieno et al., 1975), kinetic constants of the hexokinase–GlcNBrAc complex can be obtained as shown in Figure 2. This plot (Figure 2) gave a limiting rate constant of inactivation k_2 of 0.0266 min^{-1}. The dissociation constant K_I for the enzyme–GlcNBrAc complex as calculated from this plot was found to be 2.0×10^{-3} M. In this plot there is a deviation from linearity at reagent concentrations of 8 mM and above. This may be due to the nonspecific labeling of the enzyme at high reagent concentrations. The inactivation of hexokinase by bromoacetate or *N*-(bromoacetyl)galactosamine does not follow such a rate equation, and the inactivation rate increases linearly with the reagent concentration (results not shown). These results strongly suggest that the reagent

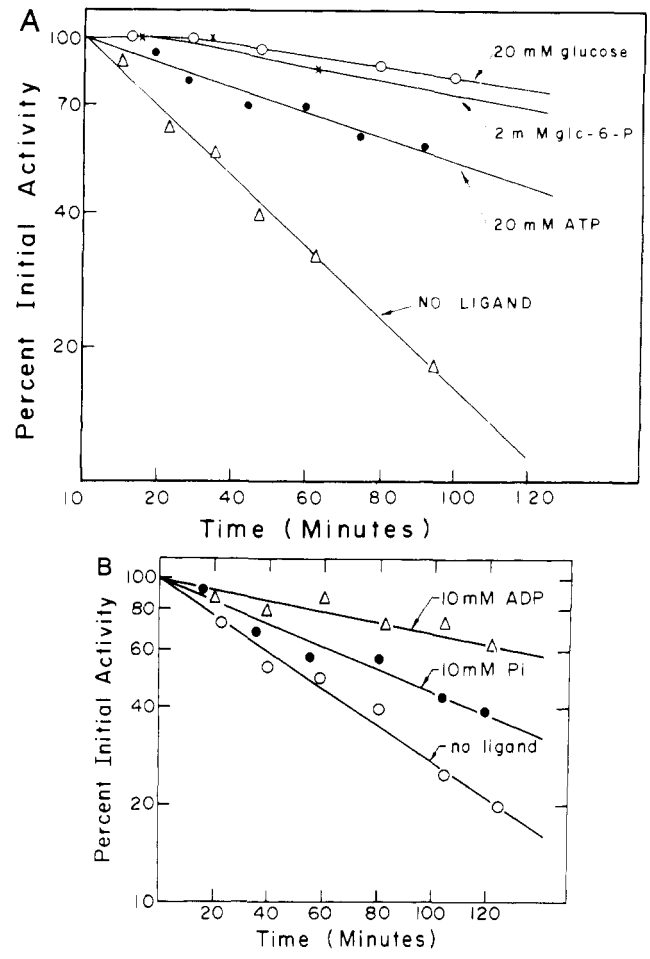

FIGURE 3: Effect of ligands on the inactivation of brain hexokinase by GlcNBrAc. The reaction mixture contained 2 μM hexokinase in 0.1 M *N*-ethylmorpholine–acetate buffer, pH 8.5, 2.0 mM GlcNBrAc, and the indicated concentration of the ligand. The reaction was carried out at 22 °C. (A) Protective effect of glucose, ATP, and glucose 6-phosphate. (B) Protection by ADP and inorganic phosphate.

GlcNBrAc interacts specifically with a site on the enzyme (presumably the active site), forming a reversible complex with it, prior to the inactivation of the enzyme.

Effect of Ligands on the Inactivation of the Enzyme by GlcNBrAc. The substrate glucose protects the enzyme against inactivation by GlcNBrAc (Figure 3A), indicating that the amino acid residue(s) modified by this reagent is likely to be present at the active site of this enzyme. ATP and glucose 6-phosphate also prevent the inactivation of the enzyme by this reagent as shown in Figure 3A. ADP provides partial protection against the inactivation reaction, and inorganic phosphate is the least effective in providing protection against inactivation of the enzyme (Figure 3B).

The protective effect of glucose against inactivation of the enzyme by GlcNBrAc could be analyzed by treating glucose as a competitive inhibitor of inactivation reaction (Figure 4). The following rate equation (Dixon & Webb, 1964; Redkar & Kenkare, 1975) can be written for such a process:

$$\frac{1}{k_{obsd}} = \frac{1}{k_2}\frac{K_{GlcNBrAc}}{[GlcNBrAc]} + \frac{1}{k_2} + \frac{1}{k_2}\frac{K_{GlcNBrAc}[glucose]}{[GlcNBrAc]K_{Glc}} \qquad (2)$$

Here k_{obsd} is the observed pseudo-first-order rate constant of the inactivation reaction in presence of glucose, K_{Glc} is the dissociation constant of enzyme–glucose complex, $K_{GlcNBrAc}$ is the dissociation constant of enzyme–GlcNBrAc complex, and k_2 is as defined in eq 1. Thus, on plotting $1/k_{obsd}$ for inactivation of the enzyme by GlcNBrAc against concentration

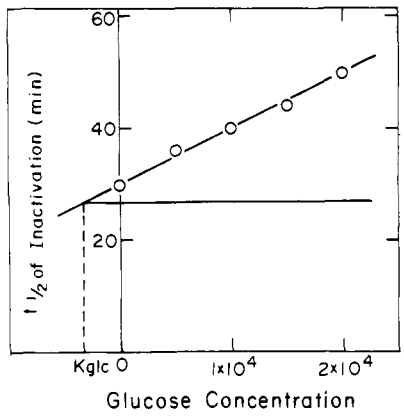

FIGURE 4: Determination of the dissociation constant of glucose with respect to the enzyme. The plot is based on eq 2. The enzyme (1.2 μM) was incubated with 4.0 mM GlcNBrAc in 0.1 M *N*-ethylmorpholine–acetate buffer, pH 8.5, with different concentrations of glucose in each incubation in a total volume of 0.5 mL.

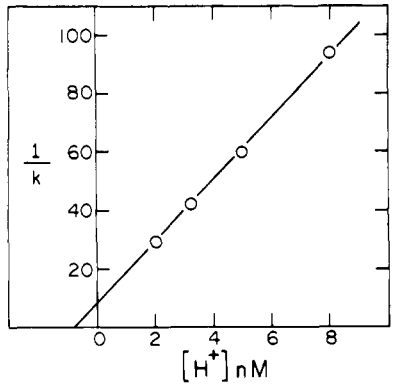

FIGURE 5: Effect of pH on the rate of inactivation of hexokinase by GlcNBrAc. The plot is based on eq 3. 2 μM hexokinase was incubated with a fixed concentration (4.0 mM) of GlcNBrAc at different pH values in 0.1 M *N*-ethylmorpholine–acetate buffer. The rate constants were determined from the semilogarithmic plots.

of glucose, we obtained a straight line as shown in Figure 4. From eq 2 it can be shown that when $1/k_{obsd} = 1/k_2$, [glucose] $= -K_{Glc}$. A horizontal line is drawn (Figure 4) through the point on the $t_{1/2}$ axis corresponding to the limiting rate constant k_2. The intersection of the lines on the negative abscissa (Figure 4) gives a K_{Glc} value of 3.2×10^{-5} M which is in good agreement with the value ($K_D = 3 \times 10^{-5}$ M) determined by direct binding measurements (Ellison et al., 1974).

Effect of pH on the Rate of Inactivation of Hexokinase by GlcNBrAc. The inactivation of hexokinase by GlcNBrAc was studied over a limited range of pH (from pH 8.1 to pH 8.7) at a reagent concentration of 4.0 mM (Figure 5). At pH values above 8.7, this enzyme is unstable in the absence of any ligand, and below pH 8.1, the rate of inactivation at this reagent concentration is very slow. The rate of the inactivation reaction increases with increasing pH of the reaction medium in this range of pH. It has been shown that for the pH range where a single ionizing group determines the reactivity, the following equation describes the pH dependence of rate constant of inactivation k (Lindley, 1962; Jones et al., 1975):

$$1/k = 1/k_{max} + [H^+]/(k_{max}K_a) \quad (3)$$

Here K_a is the ionization constant of the group involved in the inactivation reaction and k_{max} is the absolute (pH-corrected) rate constant of inactivation of hexokinase by GlcNBrAc. Thus, according to eq 3, a plot of $1/k$ against $[H^+]$ should be linear, from which k_{max} and K_a can be evaluated. Figure 5 shows that such a plot for the inactivation of hexokinase by

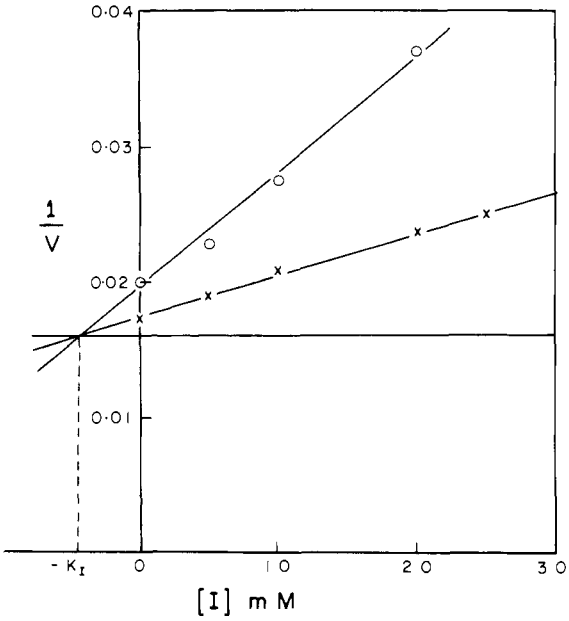

FIGURE 6: Dixon plot for GlcNBrAc as the inhibitor of the brain hexokinase reaction. The reciprocal of observed velocity, V, is plotted against the concentration of inhibitor, I, at glucose concentrations of 0.1 M (×) and 0.05 mM (○). Hexokinase activity was measured fluorometrically at 22 °C in a 1.0-mL assay mixture containing 1.30 mM ATP, 10 mM $MgCl_2$, 0.5 mM $NADP^+$, and 0.35 unit of glucose-6-phosphate dehydrogenase in 50 mM *N*-ethylmorpholine–acetate buffer, pH 8.0. The required amount of glucose was added to start the enzyme assay. The horizontal line in this figure is drawn through the point on the $1/V$ axis corresponding to the maximum velocity. The behavior shown is characteristic of competitive inhibition. The intersection of the three lines on the negative abscissa corresponds to a K_I value of 0.45 mM.

GlcNBrAc is linear over the pH range studied. From this plot, a pK of 9.1 is obtained for the enzyme group involved in the inactivation reaction, and a value of 2.08×10^{-3} s^{-1} is obtained for k_{max}.

Competitive Inhibition by GlcNBrAc. At pH 8.0 the rate of inactivation of hexokinase by GlcNBrAc is very slow, and the reagent functions as a competitive inhibitor of hexokinase reaction with respect to the substrate glucose (Figure 6). The dissociation constant (K_I) of the enzyme–inhibitor complex was determined graphically by using a Dixon plot (Dixon & Webb, 1964) in a manner similar to that described above (Figure 4). From Figure 6 a K_I value of 4.5×10^{-4} M is obtained for GlcNBrAc as a competitive inhibitor of brain hexokinase. This value of K_I is somewhat lower than the value ($K_I = 2 \times 10^{-3}$ M) obtained for irreversible inhibition of the enzyme by GlcNBrAc. This difference may be due to the difference in pH in these two experiments.

Identification of Amino Acid Residues Modified by GlcNBrAc and Stoichiometry of the Inactivation Reaction. The reagent GlcNBrAc can alkylate nucleophilic groups in a protein such as the –SH group of cysteine, the imidazole group of histidine, or a methionyl residue. The amino acid analysis of the acid hydrolysate of the enzyme modified by this reagent showed the presence of *S*-(carboxymethyl)cysteine as the only alkylated residue. No carboxymethyl derivative of histidine was detected, and the number of histidine residues in the modified enzyme was the same as that in the native enzyme. The number of methionine residues in the modified enzyme was found to be 25.55 as against 26.0 in the native enzyme (average of five experiments). Table I presents the data on the correlation between the inactivation of the enzyme and the number of *S*-(carboxymethyl)cysteines formed. Extrapolating the data from the three experiments listed in Table

Table I: Inactivation of Hexokinase by Alkylating Reagents and Formation of Alkylated Residues[a]

expt no.	reagents added	% inactn	mol of CM-cysteine/ mol of enzyme
1	GlcNBrAc, 2.0 mM for 50 min	52	1.2
2A	GlcNBrAc, 1.25 mM for 65 min	55	1.4
2B	bromoacetate, 1.25 mM for 65 min	0	<0.2
3A	GlcNBrAc, 2.5 mM for 56 min	60	1.34
3B	GlcNBrAc, 2.5 mM, plus 10 mM glucose for 56 min	10	0.72

[a] After the noted degree of inactivation, the inactivation reaction was terminated by the addition of 2–3 drops of 2-mercaptoethanol. The samples were dialyzed immediately and subjected to amino acid analysis as described under Materials and Methods.

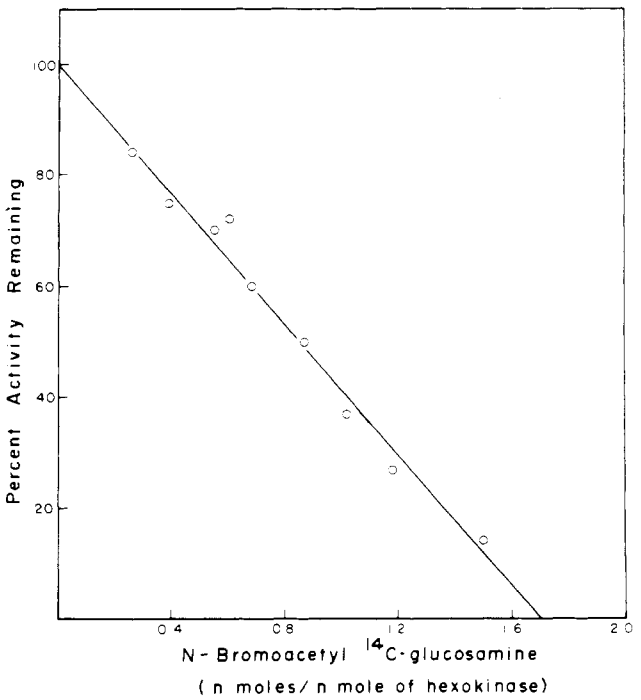

FIGURE 7: Stoichiometry of inactivation of brain hexokinase by *N*-(bromoacetyl)-D-[$^{14}C_1$]glucosamine. Brain hexokinase (11 mg in 5.0 mL) was modified with 2 mM *N*-(bromoacetyl)-D-[$^{14}C_1$]-glucosamine in 0.1 M *N*-ethylmorpholine–acetate buffer, pH 8.5, at 22 °C. At various stages of inactivation, a 0.5-mL aliquot was withdrawn from the reaction mixture and uptake of the radioactive reagent was measured after Cl_3AcOH precipitation of the protein as described under Materials and Methods.

I, we find that 2.35 ± 0.13 cysteine residues are alkylated per mol of the inactivated enzyme. Correcting for some nonspecific carboxymethylation of cysteine residues that perhaps occurs even in the absence of inactivation (Table I, experiment 2B), it is reasonable to assume that about two cysteine residues are alkylated during complete inactivation of the enzyme.

In order to confirm the stoichiometry of the inactivation reaction, we prepared radioactive (^{14}C-labeled) reagent and measured the uptake of the reagent during the course of inactivation of the enzyme (Figure 7). An analysis of four such experiments showed that 2.13 ± 0.28 mol of the reagent is incorporated per mol of the enzyme for complete inactivation. Incorporation of the reagent in the enzyme continued even after complete inactivation though at only 5% of the rate

Table II: Effects of Ligands on the Uptake of *N*-(Bromoacetyl)-D-[$^{14}C_1$]glucosamine by Hexokinase[a]

% inactn	ligand	radioact incorpd (cpm)	mol of bound reagent/ mol of enzyme
45	no ligand	2459	1.02
5	10 mM glucose	1189	0.50
10	1 mM Glc-6-P	1175	0.49

[a] Inactivation was carried out by treating the enzyme with 1.0 mM *N*-(bromoacetyl)-D-[$^{14}C_1$]glucosamine in 0.1 M *N*-ethylmorpholine–acetate buffer, pH 8.5, at 22 °C for 1 h. Uptake of radioactivity was measured after Cl_3AcOH precipitation of the protein as described under Materials and Methods.

observed during inactivation (data not shown). This indicated that some nonspecific incorporation of the reagent might be occurring even during the inactivation of the enzyme. Since this nonspecific incorporation is negligible, it has not been corrected for in the values for incorporation given above.

Reasonable agreement between the values for the overall uptake of the alkylating reagent and the *S*-(carboxymethyl)cysteine content of the inactivated enzyme shows that the inactivation of the enzyme is principally the result of alkylation of two cysteine residues. Though the alkylation of some methionine during the inactivation process cannot be entirely ruled out, it is obviously negligible to merit further consideration.

It is also necessary to point out that this value of two cysteines alkylated per mol of the enzyme inactivated by the reagent has not been corrected for the presence of ~15–20% inactive molecules present in the enzyme preparation after the removal of glucose and mercaptoethanol (see Materials and Methods). Experiments with enzyme preparations inactivated to various extents after removal of glucose and mercaptoethanol showed that the inactive molecules incorporated the reagent at ~50% of the rate of the active molecules. Since only 15–20% of the molecules are inactive, any correction to account for the difference in the relative incorporation of the reagent in the active and inactive molecules would have been insignificant and has therefore been neglected.

The modification of about two cysteine residues by this reagent raised the question whether one or both of the modified cysteines are essential for the activity of this enzyme. We found that in the presence of glucose (or glucose 6-phosphate), the enzyme is protected against inactivation by this reagent but the protected enzyme still showed uptake of the reagent to the extent of 50% of that of the unprotected enzyme (Table II). The glucose-protected enzyme also showed the alkylation of about one thiol to *S*-(carboxymethyl)cysteine/mol of enzyme (Table I, experiment 3B). The data presented in Tables I and II lead to the conclusion that only one cysteine residue, which is protected by glucose, is actually essential for the activity of this enzyme. The second nonessential cysteine residue reacts with the affinity reagent both in the presence and in the absence of glucose. This conclusion is further substantiated by the isolation of labeled peptides from the tryptic digest of the modified enzyme.

Fractionation of the Tryptic Digest of Brain Hexokinase Labeled with GlcNBrAc. In order to get information regarding the nature of amino acid residues present in the vicinity of the essential sulfhydryl residue, it is necessary to isolate a small fragment of the polypeptide chain which contains the active site sulfhydryl group. For this purpose, the enzyme labeled by *N*-(bromoacetyl)-D-[$^{14}C_1$]glucosamine was first carboxy-

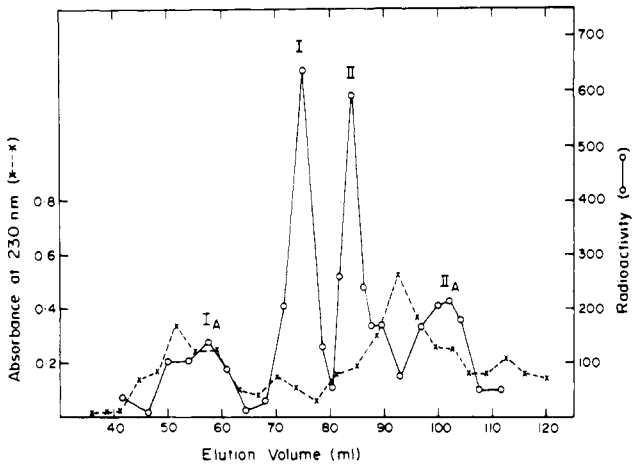

FIGURE 8: Fractionation of the tryptic digest of brain hexokinase labeled with *N*-(bromoacetyl)-D-[$^{14}C_1$]glucosamine. Hexokinase was inactivated with 0.5 mM *N*-(bromoacetyl)-D-[$^{14}C_1$]glucosamine in 0.1 M *N*-ethylmorpholine–acetate buffer, pH 8.5, at 22 °C. The reaction was stopped by the addition of 2 drops of mercaptoethanol, and the sample was dialyzed against 10 mM *N*-ethylmorpholine–acetate buffer, pH 8.0. After dialysis modified hexokinase was carboxymethylated in 8 M urea, dialyzed against 0.1 M NH_4HCO_3, pH 8.5, and subjected to tryptic digestion as described under Materials and Methods. The tryptic digest was fractionated on a Sephadex G-50 column as described under Materials and Methods.

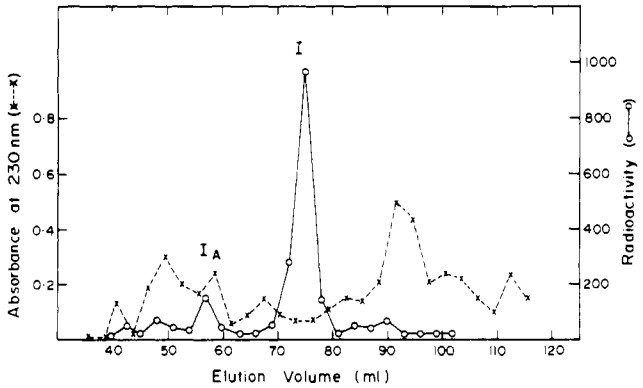

FIGURE 9: Fractionation of tryptic digest of hexokinase labeled with *N*-(bromoacetyl)-D-[$^{14}C_1$]glucosamine in the presence of 10 mM glucose. Hexokinase was incubated with 0.5 mM *N*-(bromoacetyl)-D-[$^{14}C_1$]glucosamine in presence of 10 mM glucose in 0.1 M *N*-ethylmorpholine–acetate buffer, pH 8.5, at 22 °C. This sample was then processed as described in the legend to Figure 8.

methylated with iodoacetate so as to block the remaining sulfhydryl groups and then subjected to tryptic digestion as described under Materials and Methods. This tryptic digest was fractionated by gel filtration on a Sephadex G-50 column. Two major peaks (I and II) of radioactive peptides were obtained (Figure 8) which is in line with the stoichiometry of the inactivation reaction. In order to find out which one of the two radioactive peaks contains the peptide with the essential sulfhydryl group, the affinity labeling reaction was carried out in the presence of a saturating concentration of glucose so as to prevent the essential sulfhydryl group from reacting with this reagent. The enzyme modified in the presence of glucose was then subjected to carboxymethylation, tryptic digestion, and gel filtration in the same way as the enzyme modified in the absence of glucose. As expected, the glucose-protected enzyme showed only one radioactive peak as shown in Figure 9. From Figures 8 and 9 it is clear that radioactive peak II does not show up in presence of glucose and therefore this peak contains the peptide containing the essential sulfhydryl group. The small peak I_A which is seen in Figures 8 and 9 appears to be an incompletely degraded enzyme since the molecular weight of this peptide is ~10 000. Another small peak, II_A (Figure 8), may be a degradation product of peak II since both peaks II and II_A do not appear in presence of glucose.

We have purified the labeled peptide from peak II by ion-exchange chromatography, followed by gel filtration, with at least 80% recovery at each step.[2] This shows that most of the radioactivity in peak II is associated with only one peptide, containing the essential sulfhydryl group.

Discussion

The protection by substrates against the inactivation of hexokinase by GlcNBrAc and the formation of a reversible enzyme–reagent complex prior to the actual inactivation step strongly suggest that this reagent reacts with an essential group within or close to the active site of the enzyme. This conclusion is supported by the observation that the same reagent at a somewhat lower pH functions as a competitive inhibitor of hexokinase reaction with respect to the substrate glucose. It seems quite reasonable to assume that this reagent binds at the same site while functioning as a competitive inhibitor or an irreversible inhibitor of hexokinase.

There are 20 cysteine residues in the enzyme (Subbarao & Kenkare, 1977a), and two of these are alkylated by this reagent although there is only one glucose binding site in this enzyme per monomer of 100 000 molecular weight (Ellison et al., 1974). However, only one of the modified sulfhydryl residues appears to be essential for the activity of this enzyme since the modification of only one sulfhydryl group is prevented by glucose. The second modified sulfhydryl group is apparently not essential for the activity of this enzyme since it reacts with this reagent even in the presence of glucose without causing inactivation of the enzyme. No other amino acid residue of the enzyme appears to be modified by this reagent to any significant extent. An ideal affinity label is expected to react with only one amino acid residue per active site of the enzyme. It is not clear why the second, nonessential sulfhydryl group which is modified by GlcNBrAc has higher reactivity toward this reagent than the remaining 18 cysteine residues.

The pH dependence of the rate of inactivation of the enzyme by GlcNBrAc shows that an enzyme group of pK = 9.1 is alkylated by this reagent. This supports the earlier conclusion that the modified amino acid residue is a sulfhydryl group. However, the information about the pK of a group is not sufficient to reveal its identity since the α-amino group of the N-terminal residue of the enzyme may have a similar pK.

Recently it has been reported that type II hexokinase from rat muscle is inactivated by GlcNBrAc, due to modification of an enzyme group of pK_a = 8.9, presumably a cysteine residue (Connolly & Trayer, 1979). However, yeast hexokinase is not inactivated by GlcNBrAc, but the isomeric reagent *N*-(bromoacetyl)galactosamine caused inactivation with the modification of two sulfhydryl groups per subunit, one of which appears to be nonessential (Otieno et al., 1975, 1977). Thus, an active-site sulfhydryl group appears to be present in all the hexokinases investigated so far, as pointed out by Connolly & Trayer (1979). It will be interesting to determine and compare the amino acid sequence around the essential sulfhydryl group in various hexokinases, which might throw some light on the evolutionary relationship among these enzymes.

Possible Role of the Essential Sulfhydryl Group. The presence of an essential sulfhydryl group at the active site of

[2] G. Swarup and U. W. Kenkare, unpublished results.

hexokinase indicates that this residue may be involved in catalysis or substrate binding either directly or indirectly. Protection by both substrates, ATP and glucose, against inactivation of brain hexokinase by GlcNBrAc suggests that the essential sulfhydryl residue occupies a central position at the active site. Subbarao & Kenkare (1977a) had suggested that one essential sulfhydryl residue was necessary for the catalytic function of hexokinase. However, the data available at present for brain hexokinase are not sufficient to assign unambiguously a catalytic role for this essential sulfhydryl group. A nucleophilic group at the active site of an enzyme can play an important catalytic role such as the removal of a proton from a substrate molecule. Such a possible role can be postulated for the essential sulfhydryl group of hexokinase in the transphosphorylation reaction. In the ionized form, the sulfhydryl group can act as a strong base by removing a proton from the 6-hydroxyl group of glucose. Removal of a proton from the 6-hydroxyl group of glucose will enhance its nucleophilicity and facilitate its attack on the γ-phosphorus of ATP. The ionization of the sulfhydryl group may be facilitated if a negatively charged group (e.g., carboxylate anion) is present in the vicinity of the sulfhydryl group. The presence of a negatively charged group in the vicinity of sulfhydryl group is expected to lower the pK of the latter group. However, it is quite possible that the negatively charged group comes close to the sulfhydryl group only after binding of the substrates to the enzyme. In such a case the pK of the sulfhydryl group in the free enzyme would be unaffected.

The presence of an essential sulfhydryl group at the active site of yeast hexokinase has also been suggested by Otieno et al. (1975, 1977). The essential cysteine residue has been located in the polypeptide chain of yeast hexokinase B at 80% of the length from the N terminus (Otieno et al., 1977). X-ray crystallographic studies of yeast hexokinase B complexes with sugars at 2.1-Å resolution have revealed that the cysteine residues (Cys-372 and Cys-378) are located in the sequence ~20% from the C terminus of the chain (Anderson et al., 1978). Cys-372 and Cys-378, both are ~20 Å from the active site. Thus, the essential cysteine residue located by Otieno et al. (1977) at 80% of the length from the N terminus in the polypeptide chain of yeast hexokinase is unlikely to have any catalytic role. However, two sulfhydryl groups (Cys-243 and Cys-244) are present in the vicinity of glucose binding site (Anderson et al., 1978). None of these cysteine residues is directly interacting with the bound glucose molecule, and the closest approach of a sulfhydryl residue (Cys-243) is 5.5 Å from the 3-hydroxyl group of glucose. These observations led these workers to suggest that no cysteine residue is directly involved in catalysis or substrate binding. However, one cysteine residue (Cys-243) appears to be essential for the maintenance of an active structure of the enzyme since it stabilizes the proper orientation of the amide groups of three asparagine residues that do interact directly with bound glucose (Anderson et al., 1978). This interpretation of Steitz and co-workers does not appear to be unambiguous, and a direct role of sulfhydryl group in the catalytic mechanism of yeast hexokinase is not ruled out at this stage due to the following reasons. In the crystals of yeast hexokinase B, the ATP binding site (inferred from adenosine binding site and model building) is such that direct interaction between the γ-phosphate of ATP and the 6-hydroxyl of glucose is not possible without a large conformational change which might result in reorientation of the functional groups at the catalytic center. Such a conformational change might bring a sulfhydryl residue close enough to the 6-hydroxyl group of glucose to be able to participate in the catalytic process. A conformational change upon ATP binding to the glucose-bound yeast hexokinase is well documented (Roustan et al., 1974; Danenberg & Cleland, 1975; Peters & Neet, 1978). Also, Campbell et al. (1978) have pointed out that though the models built on the basis of X-ray diffraction data may give the gross features of protein structure, they are not necessarily to be trusted when structure is to be used in detail to discuss function. Even if it is assumed that yeast hexokinase has no functional sulfhydryl residue at the active site, one need not extrapolate from this assumption to brain hexokinase. The active site in the case of the latter enzyme may have undergone some changes with respect to the amino acids participating in the reaction, the general character of which must have been retained in the process. In this connection it is relevant to note that the brain enzyme has several more sulfhydryl residues per 100000 molecular weight than the yeast enzyme though the amino acid compositions of the two enzymes with respect to the other residues is quite similar.

References

Anderson, C. M., Stenkamp, R. E., McDonald, R. C., & Steitz, T. A. (1978) *J. Mol. Biol. 123*, 207.

Campbell, I. D., Dobson, C. M., & Williams, R. J. P. (1978) *Adv. Chem. Phys. 39*, 55.

Connolly, B. A., & Trayer, I. P. (1979) *Eur. J. Biochem. 93*, 375.

Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622.

Danenberg, K. D., & Cleland, W. W. (1975) *Biochemistry 14*, 28.

Dixon, M., & Webb, E. C. (1964) *Enzymes*, p 328, Longmans, Green and Co., London.

Ellison, W. R., Lueck, J. D., & Fromm, H. J. (1974) *Biochem. Biophys. Res. Commun. 57*, 1214.

Jones, J. G., Otieno, S., Barnard, E. A., & Bhargava, A. K. (1975) *Biochemistry 14*, 2396.

Kenkare, U. W., & Swarup, G. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1311.

Kitz, K., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245.

Lindley, H. (1962) *Biochem. J. 82*, 418.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Maitra, P. K., & Lobo, Z. (1971) *J. Biol. Chem. 246*, 475.

Otieno, S., Bhargava, A. K., Barnard, E. A., & Ramel, A. H. (1975) *Biochemistry 14*, 2403.

Otieno, S., Bhargava, A. K., Serelis, D., & Barnard, E. A. (1977) *Biochemistry 16*, 4249.

Peters, B. A., & Neet, K. E. (1978) *J. Biol. Chem. 253*, 6826.

Redkar, V. D., & Kenkare, U. W. (1972) *J. Biol. Chem. 247*, 7576.

Redkar, V. D., & Kenkare, U. W. (1975) *Biochemistry 14*, 4704.

Reissig, J. L., Strominger, J. L., & Leloir, L. F. (1955) *J. Biol. Chem. 217*, 959.

Roustan, C., Brevet, A., Pradel, L. A., & Van Thoai, N. (1974) *Eur. J. Biochem. 44*, 353.

Subbarao, B., & Kenkare, U. W. (1977a) *Arch. Biochem. Biophys. 181*, 8.

Subbarao, B., & Kenkare, U. W. (1977b) *Arch. Biochem. Biophys. 181*, 19.

Subbarao, B., Chakrabarti, U., Redkar, V. D., & Kenkare, U. W. (1973) *Proc. Symp. Control Mech. Cell. Processes*, 113, Bhabha Atomic Research Centre, Bombay, India.

Swarup, G. (1979) Ph.D. Thesis, The University of Bombay.