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Essential Thiols of Yeast Hexokinase: Alkylation by a Substrate-Like Reagent†

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ABSTRACT: It is demonstrated that *N*-bromoacetyl-D-galactosamine acts as a substrate-like reagent for yeast hexokinases A and B, producing affinity labeling. At the order of 10^{-3} *M* reagent concentrations, rapid inactivation of the enzyme is produced: the kinetics are consistent with dependence upon a reversible inhibitor–enzyme initial complex, with a dissociation constant of 3.5×10^{-3} *M* for hexokinase B at 35°, pH 8.5. The glucose analog is 30-fold less effective, presumably due to self-protection. The inactivating reaction is an order of magnitude faster than that with bromoacetate. All of the alkylation of hexokinase B was shown to occur at two thiol groups per subunit, associated stoichiometrically with inactivation. Unlike the reaction there of simple alkylators, two nonessential thiols per subunit are left unattacked when this inactivation reaction is complete. Protection against the affinity alkylation is exerted by the substrates glucose, mannose, fructose, glucose 6-phosphate, fructose 6-phosphate, ATP·Mg, and ADP·Mg, in proportion to their affinities for the active center. Free ATP also protects. Mg^{2+} alone has no influence, and Mn^{2+} gives a slight acceleration, when correction is made for a slow inactivation that occurs when the enzyme is incubated at 35° with Mn^{2+} alone. Galactose, virtually a nonsubstrate, has no influence on the affinity alkylation, but *N*-acetylgalactosamine, a nonsubstrate and a weak inhibitor of the enzymic reaction, has an accelerating effect. An interpretation is made in terms of binding to a site that influences the active center. This affinity label should provide a means of isolating a peptide containing active-center-related groups.

It has been established that yeast hexokinase, in the form of the pure isoenzyme hexokinase B, possesses two classes of SH groups, each comprising four per dimeric molecule of 104,000 molecular weight (Lazarus et al., 1968). It has been shown (Lazarus et al., 1968) that two SH groups per monomer subunit become available, both to mercaptide formation and to alkylation by iodoacetate and iodoacetamide, after the first two have been derivatized. This reaction of the second group of thiols is associated with the loss of all activity. Further evidence (Jones et al., 1975) shows that this inactivating carboxymethylation at one-half of the total thiols per subunit can also be obtained, in suitable conditions, when the other set of thiols are free. (Similar results were also noted, in Abstract form, with the A isoenzymic species (Jones, 1970).) It was also shown (Jones et al., 1975) that substrates protect from this inactivating alkylation, to the extent of their active site binding. It was explicitly pointed out (Lazarus et al., 1968; Jones et al., 1975) that this type of evidence does not establish that all, or any, of those latter SH groups are in the active center of the enzyme, although the observations are compatible with such a situation.

Interest in the possible requirement of this enzyme for

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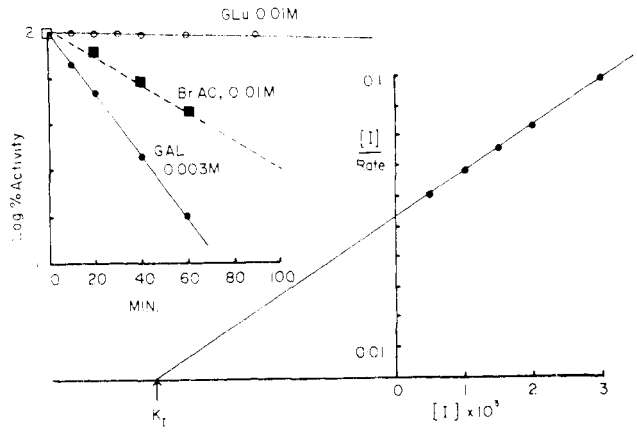

FIGURE 1: Plot based upon eq 2, for the reaction of *N*-bromoacetylgalactosamine at molar concentrations [I]. The pseudo-first-order rate constant was derived in each case from a plot of the type shown in Figure 1, and used in the denominator on the ordinate. Insert: Semilogarithmic plots of the inactivation of hexokinase B by *N*-bromoacetylgalactosamine (●), bromoacetate (■), and *N*-bromoacetylglucosamine. (○). Each was at pH 8.5, 35°, $I = 0.1$. Note that the concentrations of the latter two reagents were each 3.3 times that of the galactosamine derivative.

certain thiol groups is heightened by the progress being made in the crystallographic determination of the structure of hexokinase isoenzyme B (Steitz et al., 1973). That structure is now available at 3-Å resolution (Bates et al., 1974). The methylmercuric derivative has been one of those examined in the crystal state in the latter investigation, and the sites of occupation have been located in the overall structure. Studies in solution on the relation of the thiol groups to the active center hold the possibility, therefore, of an interpretation in direct structural terms.

We have sought to design an active-site directed alkylating agent, to test the hypothesis that an SH group is in or close to the active center region. Of several alkylating derivatives based upon the hexose ring that we have examined, *N*-bromoacetyl-D-galactosamine has been found to perform a specific alkylation of the enzyme, as noted briefly previously (Rustum and Barnard, 1971; Otieno et al., 1974). We describe here the characteristics of this reaction, which class this reagent as an affinity label for hexokinase.

Materials and Methods

All materials and methods were as described by Jones et al. (1975) or Ramel et al. (1971), unless specified here. D-Mannose was from Mann, *N*-acetyl-D-glucosamine was from Pfanstiehl Labs., and D-glucosamine hydrochloride and D-galactosamine hydrochloride were from Sigma. D-Galactose was Sigma "Low Glucose" grade and contained (*cf.* Derechin et al., 1972) less than 1 part in 10,000 of any other sugar. $MnCl_2$ was the Sigma 10% solution. Bromoacetic anhydride was from Pfaltz and Bauer, and was distilled before use. Bromoacetic acid (Eastman) was recrystallized from boiling petroleum ether, mp 50° (Goren et al., 1968). Propionyl anhydride was from Aldrich. Elemental analyses were by Galbraith Labs. Inc.

Hexokinase B was completely purified by the method described in detail by Rustum and Barnard (1971), using the version in which the yeast cells are broken by the Manton-Gaulin Sub-Micron Disperser. The concentrated enzyme was freed of excess salts by dialysis before use, against the medium to be employed or against distilled, deionized water. It was homogeneous by electrophoresis (Rustum et al., 1971) in starch gel.

Hexokinase assays (Lazarus et al., 1966) were on glucose as substrate at pH 8.5, 25°.

N-Bromoacetyl-D-galactopyranosylamine. This was synthesized using bromoacylation by a modification of the method used for acetylhexosamines by Roseman and Lundowieg (1954). (Thomas (1970) had also reported its synthesis, by a different procedure, at a time when the present synthesis was completed.) Dowex 50 (H^+ form) was used instead of Amberlite IR-120. This substitution of the cationic exchanger increased the yield of the final product by approximately 15-fold. D-Galactosamine hydrochloride (500 mg) was dissolved in 12 ml of water and 1.2 ml of methanol. Bromoacetic anhydride (800 mg) and Dowex 1 (carbonate form) (15 ml wet resin) were then added. The mixture was stirred for 90 min at 0–5° and filtered and the residue washed with water. The filtrate and washings were passed through a column (5 ml) of Dowex 50 (H^+ form), and this was washed thoroughly with water. The eluate and washings, combined, were heated just to boiling, and the solution was finally concentrated in vacuo by rotary evaporation, keeping the temperature below 50°. The *N*-bromoacetylgalactosamine formed was crystallized twice from absolute ethanol. After drying, the final yield was 200 mg, mp 104–110°. Anal. Calcd for $C_8H_{14}BrO_5N$: C, 33.4; H, 4.7; N, 4.7; Br, 26.6. Found: C, 35.6; H, 4.6; N, 4.7; Br, 26.8. The product gave a strongly positive response in the modified (Reissig et al., 1955) Morgan-Elson reaction, and contained no free bromide ion (by silver nitrate reaction). Thin-layer chromatography (acetone–methanol, 10:1) showed a single component (R_f 0.5). The infrared spectrum showed a prominent peak at 6.05 μ due to the carbonyl group. The nuclear magnetic resonance spectrum showed a singlet at δ3.87 due to the amide-bound methylene group.

N-Bromoacetyl-D-glucopyranosylamine. D-Glucosamine hydrochloride (2.16 g) was dissolved in 50 ml of distilled deionized water and 5 ml of methyl alcohol. Bromoacetic anhydride (3.64 g) was employed, in a procedure that paralleled that described above, up to a final crystallization from absolute methanol. The yield was 825 mg, mp 147–150°. Anal. Calcd for $C_8H_{14}O_6NBr$: C, 32.1; H, 4.7; N, 4.7; Br, 26.6. Found: C, 32.8; H, 5.0; N, 4.1; Br, 25.4. In thin-layer chromatography (as above), it gave a single component. Infrared evidence was as for the preceding compound.

N-Propionyl-D-glucopyranosylamine. This was synthesized according to the method of Roseman and Lundowieg (1954). D-Glucosamine hydrochloride (2.16 g) and propionic anhydride (1.8 g) were used. The yield was 1.06 g, mp 187.5–189°. Anal. Calcd for $C_9H_{17}O_6N$: C, 45.9; H, 7.3; N, 5.9. Found: C, 45.7; H, 7.4; N, 5.6.

Inactivation of Hexokinase. Using *N*-bromoacetylhexosamine, the reagent (50–100-μl solution) was added to a solution of hexokinase B (1 mg/ml), each being in 0.025 *M* glycylglycine buffer (pH 8.5), $I = 0.1$ (adjusted with NaCl) and preincubated separately at 35° for at least 10 min. The final volume was usually 1 ml. Enzymic activity was measured on samples withdrawn into assay medium at intervals during the incubation at 35°. The activity at zero time was taken as that of an aliquot withdrawn into assay medium as rapidly as possible after mixing (about 10–15 sec). Since the enzyme and alkylator suffered a dilution of at least 60-fold in the assay medium, and since the activity was determined from the initial velocity over the first minute of

Table I: Rate Constants for the Loss of Catalytic Activities of Hexokinases during Reaction with *N*-Bromoacetylgalactosamine and Related Agents.

Hexokinase	Reagent	Concn (m*M*)	Loss of Activity on Glucose (k, min^{-1}, × 100)[a]	Loss of Activity on Fructose (k, min^{-1}, × 100)[a]	k_2[b] (l. mol^{-1} min^{-1})
B	*N*-Bromoacetylgalactosamine	3.0	2.98	2.92	10.0[d]
B	*N*-Bromoacetylglucosamine	3.0	0.11	0.12	0.35[d]
B	*N*-Propionylglucosamine	10	0.00	0.00	
A	*N*-Bromoacetylgalactosamine[c]	3.0	1.25	1.12	
B	Bromoacetate				1.56
B	Iodoacetate				2.85

[a] Pseudo-first-order rate constant, for the single reagent concentration noted. For the standard deviation of the mean of the rates found in typical replicate series, see Table IV. In each case here, the activity was measured on both hexose substrates during the course of inactivation of a single sample of hexokinase. All values of B are at pH 8.5, 35°, I = 0.1. [b] Second-order rate constant for loss of activity on glucose. [c] Also at pH 8.5, I = 0.1, but at 28°, due to the lower thermal stability of hexokinase A. At 28° in these conditions, hexokinase A lost no activity when incubated alone for a period parallel to that used in measuring this reaction. [d] This rate constant is a function of the reagent concentration (see Figure 2).

assay, the alkylation reaction was in all cases quenched at the moment of assay. The residual activity as a function of reaction time was plotted semilogarithmically by computer, to give the slope fitted by least squares and thus the rate constant. The reaction rate of the amino group of glycylglycine is negligible in the periods used in these conditions, and at 25 m*M* concentration this buffer maintained the pH constant to the end of the reaction. In the case of bromoacetic acid reactions, the same final conditions were used, the reagent first being dissolved in the buffer alone and adjusted in pH.

Analyses of Derivatives. Alkylated hexokinase samples, withdrawn at various stages, were immediately gel filtered on a G-25 column (55 × 1.5 cm) in water (or in some cases, 30% acetic acid). The protein peak at the excluded volume was pooled and lyophilized, and the protein was hydrolyzed with 6 *N* HCl (Merck Ultra-Pure) at 110°, 24 hr, in an evacuated sealed glass tube. Amino acid analysis was performed on a Beckman Spinco amino acid analyzer by standard procedure. Particular care was taken to exclude air during the hydrolysis, to obtain a full yield of *S*-carboxymethylcysteine, which was determined in these amino acid analyses (Lazarus et al., 1968). Measurement of the associated number of micromoles of protein was made by means of the aspartic acid content of the sample and of a hexokinase standard (Lazarus et al., 1968). Examination for other CM-amino acids and their derivatives in acid hydrolysates followed the procedures of Goren et al. (1968), except that only ninhydrin-positive peaks were detected.

The hexose content was determined on samples of the reacted protein, gel-filtered similarly. Known amounts of the protein were hydrolyzed in sealed tubes, in 3 *N* HCl, 110°, 4 hr. Analysis on the amino acid analyzer for galactosamine followed standard procedures. Recovery was checked by hydrolyzing a known weight of *N*-bromoacetylgalactosamine with 0.25 equiv of bovine serum albumin in the same way: upon analysis, a recovery of 105% was found.

Results

Inactivation of Hexokinase by Alkylators. *N*-Bromoacetylgalactosamine (10^{-2}–10^{-3} *M*) readily inactivated hexokinase B (Figure 1). The inactivation was shown to be totally irreversible, when the reagent was removed by gel filtration on a Sephadex G-25 column in water, or by dialysis against the starting buffer. The inactivation rate, when plotted semilogarithmically, was seen to follow pseudo-first-order kinetics throughout its course (Figure 1). It proceeded to complete extinction of the activity after sufficient reaction periods. The activity of the enzyme incubated alone, under otherwise the same conditions, was completely stable. Incubation of the enzyme in the same conditions but in a control medium containing *N*-propionylglucosamine (10^{-2} *M*) also gave no inactivation (Table I).

The galactose-based alkylator shows abnormally high reactivity with hexokinase, compared with bromoacetate (Figure 1). Inactivation of hexokinase B by *N*-bromoacetylglucosamine was very slow compared to that due to the galactose derivative (Figure 1).

Reaction with the *N*-bromoacetylgalactosamine departed from first-order dependence upon reagent concentration, showing an apparent saturation effect at levels above about 10^{-3} *M*. This can be treated according to the scheme:

$$\mathrm{I} + \mathrm{E} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E{\cdot}I} \xrightarrow{k_a} \mathrm{E'} + \mathrm{HBr} \qquad (1)$$

where the inhibitor, I, complexes reversibly with the enzyme, E, with dissociation constant K_I (= k_{-1}/k_1), and subsequent alkylation of E occurs to form inactive enzyme (E′) in a first-order process with rate constant k_a. It can readily be shown (Kitz and Wilson, 1962; Main and Hastings, 1966) that for such a case:

$$\frac{[\mathrm{I}]}{k'} = \frac{[\mathrm{I}]}{k_a} + \frac{K_I}{k_a} \qquad (2)$$

where k' is the observed pseudo-first-order rate constant for inactivation at inhibitor concentration [I]. When plotted according to (2), the values fitted such a system (Figure 1), with $K_I = 3.5 \times 10^{-3}$ *M* (at 35°, pH 8.5, I = 0.1). Hence, prior reversible complexing with the reagent is indicated. In contrast, the reactions with iodoacetamide or iodoacetate as inactivator do not fit eq 2, but are first order with respect to reagent concentration (Jones et al., 1975).

For *N*-bromoacetylglucosamine, similar behavior was found, although the rates were much slower (Figure 1). At concentrations of this reagent above 1×10^{-2} *M* (values up to 1×10^{-1} *M* were tested) no further increase in the pseudo-first-order inactivation rate was detectable. However, the kinetics appeared more complex in this case, probably due to the reversible inhibitory binding of the reagent in another mode, in view of the known inhibition of the en-

Table II: Inactivation of Hexokinase B by *N*-Bromoacetylgalactosamine (3×10^{-3} *M*) and the Formation of Alkylated Residues.

Inactivation (%)	Moles per Subunit of Enzyme[a]	
	CM-Cysteine	Uptake of Reagent[b]
25	0.5	
46	1.00	1.06
67	1.55	
90	1.85	1.95

[a] Each row represents an analysis made upon a different reaction mixture. At the degree of inactivation noted, the protein was isolated and the content of CM-cysteine, or of galactosamine, was determined after appropriate hydrolysis (see Methods). [b] Measured by the amount of galactosamine present after hydrolysis of the derivative.

zyme by *N*-acetylglucosamine. In this case, therefore, K_I in eq 2 was not estimated.

Alkylation of Cysteine Residues. The inactivation of the enzyme by *N*-bromoacetylgalactosamine is associated with the carboxymethylation of cysteine. When the inactivation was carried out until 90% of the initial activity was lost, approximately 1.9 residues of cysteine per subunit of the enzyme was found (in an acid hydrolysate) to be converted to *S*-carboxymethylcysteine. The amide bond in the hexosamine derivative is completely hydrolyzed in the conditions of protein hydrolysis, leaving the free carboxymethyl group at the site of alkylation. The extent of cysteine reaction was at each stage proportional to the inactivation produced (Table II). A maximum of one-half of the total of cysteine residues in the subunit appears, therefore, to react. No other carboxymethylated residues were detected in the hydrolysates of the inactivated protein. Since small amounts of ε-CM-lysine or of CM-methionine and its products would be measured very inaccurately by ninhydrin-based amino acid analysis (Goren et al., 1968), reliance was placed, instead, on a measurement of the hexosamine groups incorporated into the protein. Analysis for hexosamine content showed results in agreement with the number of CM-cysteine residues found after hydrolysis (Table II).

Protection of Hexokinase by Substrates and Related Agents. The effects of a series of reagents are summarized in Tables III and IV.

HEXOSES. When the alkylation by *N*-bromoacetylgalactosamine (10^{-2} *M*) was conducted in the presence of glucose concentrations of the same order, the inactivation became slight (Figure 2). When the concentration of glucose was lowered to 10^{-4} *M*, on the other hand, there was no protection. D-Mannose was an even more powerful protective agent than glucose, whereas D-fructose had very little effect. The relative inactivation rate constants are shown in Table III as a function of the concentration of the protecting hexose. Galactose, virtually a nonsubstrate (Sols et

Table III: Protection of Hexokinase B from Inactivation.[a]

Agent Added	Concn (*M*)	Relative inactivation Rate[b]		K_m (m*M*)[c]	K_d (m*M*)[c]
		BAGA	Bromoacetate		
None		1	1		
Glucose	10^{-2}	0.04	0.03	0.6[d]	1[g]
	10^{-3}	0.34			
	10^{-4}	0.99			
Mannose	10^{-2}	0.001	0.02	0.3[d]	
	10^{-3}	0.14			
	10^{-4}	0.42			
Fructose	10^{-2}	0.65		2.5[d]	
	10^{-3}	0.91			
Galactose	10^{-2}	1.05	1.0	50[e]	
Mg^{2+}	10^{-3}	0.99			
ATP	10^{-3}	0.85		0.5[f]	4[g]
	3×10^{-3}	0.25	0.46		
ADP	3×10^{-3}	0.62		2.6[f]	
ADP + Mg^{2+}	10^{-3} (each)	0.71		0.3[f]	4[g]
	3×10^{-3} (each)	0.30	0.44[h]		
ADP + Mg^{2+}	10^{-3} (each)	0.81			
	3×10^{-3} (each)	0.35	0.53		
Mn^{2+}	10^{-3}	1.93[i]	1.76[i]		
Glucose 6-phosphate	10^{-3}	0.83	0.76		
Fructose 6-phosphate	10^{-2}	1.03			
Glucose + ADP + Mg^{2+}	10^{-3} (each)	0.10			
Glucose 6-phosphate + ADP + Mg^{2+}	10^{-3} (each)	0.47			
N-Acetylgalactosamine	10^{-2}	1.73	1.47		

[a] Inactivation was by 3×10^{-3} *M N*-bromoacetylgalactosamine (BAGA) (pH 8.5), 35°. For comparison, some parallel protections using 10^{-2} *M* bromoacetate as inactivator, in the same medium, are also shown. The relative rates apply only within each alkylator series. Each protective agent added was present in the enzyme solution at 35° for 10 min prior to addition of the alkylating agent. [b] The second-order rate constant for the inactivation is expressed relative to that for a control reaction (without the added agent) performed in parallel on an aliquot of the same enzyme sample. Each is the mean for two or three replicates. For typical relative standard deviations of the mean in replicate determination and for other concentrations of *N*-acetylgalactosamine, see Table IV. [c] Michaelis constant, or dissociation constant (K_d) for the binary complex, at 25–30° (pH 8.0–8.5). [d] From Ramel et al. (1971) or E. A. Barnard (unpublished data). [e] From Sols et al. (1958), for a partially proteolyzed enzyme preparation. Hexokinase B shows behavior consistent with these values (Lazarus et al., 1966). [f] K_I (ATP^{4-}) or K_m, from Kosow and Rose (1970). [g] As cited by Colowick (1973) or Purich et al. (1973). [h] With iodoacetate as alkylator (Jones et al., 1975). [i] With 10^{-3} *M* Mn^{2+} *alone*, an inactivation occurred: this showed pseudo-first-order behavior in activity loss up to at least 120 min, at a rate one-third that of the rate with 10^{-3} *M* BAGA alone. The accelerating effect of Mn^{2+} with each reagent here is, therefore, greater than additive.

Table IV: Effects of *N*-Acetylhexosamines on the Inactivation by *N*-Bromoacetylgalactosamine.

Reagent	Concn (*M*)	k^a (min^{-1} × 100)	SD^b
N-Acetylgalactosamine	2×10^{-2}	6.50	0.21
	1×10^{-2}	5.18[c]	0.31
	3×10^{-3}	3.04	0.14
	2×10^{-3}	2.98[d]	0.07
	0	2.99	0.04
N-Acetylglucosamine	1×10^{-2}	1.08	
N-Acetylmannosamine	1×10^{-2}	2.40	

[a] Apparent (pseudo-first-order) rate constant for inactivation by 3×10^{-3} *M N*-bromoacetylgalactosamine, at pH 8.5, 35°, $I = 0.1$. [b] Standard deviation of the mean, for 3–7 experiments. [c] When extracted on Chelex 100 resin (see text) this rate in repeat experiments was increased from 5.18×10^{-2} to 8.4×10^{-2} min^{-1}. [d] An identical value was found at other lower concentrations down to 1×10^{-5} *M*.

al., 1958; Lazarus et al., 1966), gave no protection whatsoever.

The kinetics of the inactivation in the presence of strongly protecting levels of hexose were anomalous. When the reaction was greatly slowed thus, an initial lag was present (Figure 2). The length of this lag was in proportion to the overall degree of protection exerted by the hexose present. When the protection was small, as with 10^{-4} *M* glucose, the lag became negligible (Figure 2). No lag was seen with the alkylating agent alone. When the reaction was slowed by other types of protective agent (see below), the lag was either absent or very small. The behavior was not changed by omitting the routine preincubation of the enzyme at 35° (the temperature used for the reaction) in the presence of the protecting agent; precisely the same curves were obtained when the buffered enzyme solution was instead, taken from storage at 4°, mixed at room temperature with the protective agent and alkylator, and the incubation at 35° then commenced. Hence, an increase in dissociation of the protein into its subunits in the hexose medium (Derechin et al., 1972) does not seem to be the time-dependent phenomenon that causes this lag. Its origin has not been established. When the rate constants for the inactivation in hexose media were calculated, the lag segment of the time course in each case was disregarded, only the period of inactivation being employed. The reactions were pseudo-first-order in that period.

HEXOSE PHOSPHATES. Two products of the enzymic reactions, glucose 6-phosphate and fructose 6-phosphate, behaved analogously to their parent hexoses, but with lower affinity. Glucose 6-phosphate protected weakly, whereas fructose 6-phosphate had no effect up to 10^{-2} *M*.

N-ACETYLHEXOSAMINES. *N*-Acetylglucosamine is known to be not a substrate, but was reported to be a competitive enzymic inhibitor ($K_I \sim 1$ m*M*) of impure yeast hexokinase (Sols et al., 1958). *N*-Acetylgalactosamine (not previously reported upon) was tested here, and shown to be a weak competitive inhibitor of the activity of hexokinase B (Figure 3). However, contrasting behaviors were exhibited by various *N*-acetylhexosamines in the alkylation reaction (Table IV). Both *N*-acetylglucosamine and *N*-acetylmannosamine protected hexokinase B from the inactivation, the former of these much more strongly. On the other hand, *N*-acetylgalactosamine accelerated the specific alkylation to a statistically significant degree, when it was present at concentration $>5 \times 10^{-3}$ *M*.

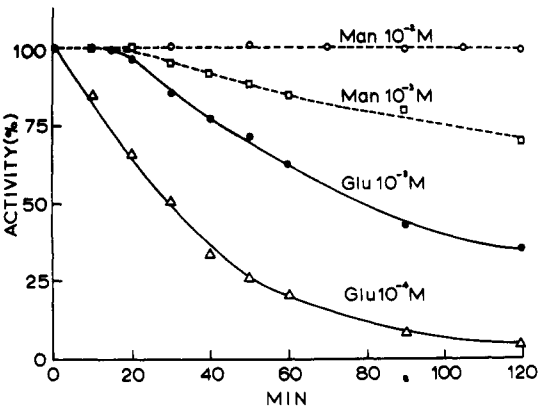

FIGURE 2: Time course of inactivation of hexokinase B by *N*-bromoacetylgalactosamine (3×10^{-3} *M*) in the presence of substrate aldohexoses. Mannose was used at 10^{-2} and 10^{-3} *M* (broken curves, fitted by eye to the points shown) and glucose at 10^{-3} *M* (●) and 10^{-4} *M* (△) concentrations. The solid curve touching the points for the 10^{-4} *M* glucose case represents the same reaction in the absence of a sugar, constructed, using the rate constant for that reaction, on the basis of pseudo-first-order behavior.

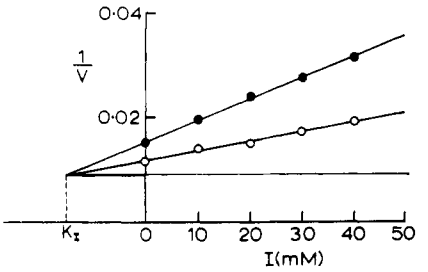

FIGURE 3: Dixon plot (Dixon, 1953) for *N*-acetylgalactosamine as inhibitor of the hexokinase B reaction (pH 8.5, 25°, [ATP] = 3.0 m*M*, total [Mg^{2+}] = 13 m*M*). The reciprocal of the observed velocity, *V*, is plotted against the concentration of inhibitor, [I], at glucose concentrations of 20 m*M* (●) and 27 m*M* (○). The horizontal line is drawn through the point on the $1/v$ axis corresponding to the maximum velocity (saturating glucose and ATP·Mg) for the fixed amount of enzyme used. The behavior shown is characteristic of competitive inhibition. The intersection of the three lines gives, on the negative abscissa, a K_I value of 14 m*M*.

DIVALENT CATIONS. Mg^{2+} alone had no effect on the alkylation. Mn^{2+} at 10^{-3} *M* and above gave an apparent acceleration of the reaction (Table III). Much of the latter effect is attributable to a distinct enzymic inactivation produced by Mn^{2+} alone, under these conditions at 35° (Table III). This effect of Mn^{2+} alone was reversible by subsequent application of 5 equiv of EDTA, or by 2 equiv of cysteine: after 43% inactivation by 10^{-3} *M* Mn^{2+} either of those treatments (at 4°, pH 8.5) gave about 90% activity after 20 min, and full activity after 12 hr. The observations suggest that Mn^{2+} slowly reacts at an initially shielded thiol to cause inactivation. Time-dependent reaction by mercurials at a thiol in hexokinase has previously been found (Lazarus et al., 1968).[1] After incubation of the enzyme with

[1] A study of the effect of Mn^{2+} and Zn^{2+} on hexokinase activity has been reported earlier by Kaji and Colowick (1965). They found an inhibition by Mn^{2+} which, however, was not time dependent (in contrast to that of Zn^{2+}), and which they ascribed to the lower effectiveness of the Mn^{2+}–ATP complex. However, in their study a partially proteolyzed form of the enzyme was used, and ATP and Mg^{2+} were present in the period of incubation with Mn^{2+} (the reaction being started later by addition of glucose). The large excess of nucleotide used would have a strongly protective effect, so that the slow reaction with Mn^{2+} was not seen. The effect with Zn^{2+} seen by Kaji and Colowick (1965) is obviously so strong that it is not masked by the nucleotide binding, and they did, in contrast, attribute the inactivation by Zn^{2+} to a slow reaction with -SH groups.

Mn^{2+} and removal of all excess metal by gel filtration in water, firmly bound Mn^{2+} (one per subunit) has been detected by neutron activation analysis (S. Otieno and C. C. Thomas, unpublished data). The effect of Mn^{2+} and the alkylating agent combined was somewhat greater than predicted for additivity of the two separate effects (Table III), so that some additional increase in the reactivity of the thiols may be produced by the metal.

ADENINE NUCLEOTIDES. ADP alone protected weakly from the alkylation and ATP alone more strongly. When the ATP·Mg complex was used, almost the same degree of protection was observed, suggesting that it is the direct nucleotide interaction with the protein that is important here. The protection by the ADP·Mg complex was stronger than that of ADP alone, and was the same as that of the ATP·Mg complex.

COMBINED SUBSTRATES. When ADP·Mg was present together with glucose 6-phosphate (Table III), the protective effect was greater than predicted from the experiments with either alone. The same was true, but not to a marked extent, with the pair glucose–ADP·Mg.

METAL REMOVAL. In view of the inactivation produced by Mn^{2+} alone, the possibility was considered that either the affinity agent, or *N*-acetylgalactosamine, was contaminated with traces of heavy metals, to account for the enhanced rates seen in certain cases. Each of these reagents was, therefore, extracted in water exhaustively with Chelex 100 (Bio-Rad Laboratories) chelating resin, freeze-dried in all-glass apparatus, and retested. The inactivation by *N*-bromoacetylgalactosamine remained, with precisely the same second-order rate constant. The inactivation by this reagent in the presence of 10^{-2} *M* *N*-acetylgalactosamine was not reduced, but on the contrary was increased (Table IV). Possibly some trace of a metal in the latter compound was, in fact, protecting the thiol somewhat. The rate enhancement phenomenon is, therefore, not due to metal effects.

Comparison with Bromoacetate Reaction. Bromoacetate was employed as a substrate-unrelated alkylating analog of the *N*-bromoacetylgalactosamine reagent. Bromoacetate inactivated hexokinase B, but at a rate about an order of magnitude slower than the alkylation by the affinity reagent (see Figure 1). The second-order rate constants for the inactivation by these two reagents and by iodoacetic acid are reported in Table I. The increase in rate with iodoacetic compared to bromoacetic acid is of the order of that normally expected with SH groups (Webb, 1966).

Using the same medium for both the bromoacetate and the affinity reagent reactions, mannose, glucose, and galactose gave protective effects with these two reagents (Table III) in a series determined in each case by the different affinities of these hexoses for the active center. The magnitude of these effects were quite similar in each case for the two reagents (Table III, and see also Table I of Jones et al., 1975). The nucleotides and their Mg complexes, and glucose 6-phosphate, also gave quite similar effects with each reagent. Good substrates were in all cases rather more protective of the *N*-bromoacetylgalactosamine reaction. This is to be expected if the enzyme–substrate complex is inert to both types of inactivating reaction, since the effect of the prior reagent–substrate complex in the affinity case introduces an additional competitive term in the usual equation [eq 3 of Jones et al. (1975)] representing the inhibition reaction.

The phenomenon of enhancement of the alkylation by the presence of *N*-acetylgalactosamine (Table IV) was seen to about the same extent in the case of bromoacetate inactivation (Table III). Mn^{2+} accelerated both types of reaction (Table III). The effect in each case was somewhat more than that predicted for additivity of the alkylation and Mn^{2+} inactivation rates.

Effect of Fructose. Since fructose was, as noted above, almost ineffective in preventing the specific alkylation, in contrast to glucose and mannose, the possibility was entertained that binding sites exist in the active center that differ for aldohexose and ketohexose substrates, and that only the site for glucose and mannose is affected by the alkylation. To test this, the loss of enzymic activity was followed separately on each hexose substrate during a given reaction with *N*-bromoacetylgalactosamine. The same was done with *N*-bromoacetylglucosamine. In each case, the loss of activity on fructose as substrate in the hexokinase reaction exactly followed that on glucose as substrate (Table I).

Further, this was examined on hexokinase A, in which the V_{max} value for fructose is three times that for glucose (in contrast to their equality in hexokinase B) (Lazarus et al., 1966; Ramel et al., 1971). Again, the two activities were lost at the same rate, in the reaction with *N*-bromoacetylgalactosamine (Table I). The results are best interpreted, therefore, as due simply to a much weaker binding of fructose than glucose or mannose at the same active center.

The data available on the binding of various substrates and competitive inhibitors to this enzyme [reviewed by Purich et al. (1973) and by Colowick (1973)] are consistent with the relative protections observed here. The actual values of the K_d values (dissociation constants for hexokinase–ligand complexes) found here would not be expected to coincide with the K_m or K_I values reported for such substrates or inhibitors, respectively. In the first place, K_m and K_I are kinetic quantities, which cannot be assumed to be equal to the thermodynamic dissociation constant K_d for the ligand in question without knowledge of the relative values of all of the rate constants for individual kinetic steps in the mechanism. In the second place, the kinetic K_m values are believed to reflect a mutual enhancement of binding by the sugar and the nucleotide substrates in the ternary complex with the enzyme (Purich et al., 1973). As pointed out by the latter authors, most of the relevant data in the literature refer to proteolytically degraded enzyme preparations or unspecified isoenzymes, and widely varying experimental conditions. Those data that relate to native hexokinase B under conditions similar to those used here are listed in the last columns of Table III. If the relative K_m values are taken to reflect the relative positions in two K_d series, one for sugars and one for nucleotides, the protection values shown follow the same series. The mutual enhancement effect is shown weakly here; it was not possible, of course, to test it with the hexose–ATP·Mg pair, where it has been deduced from kinetic data (Purich et al., 1973) to be strong. It can be shown in protection series with the β–γ amide analog of ATP, as will be discussed in full elsewhere (S. Otieno, A. K. Bhargava, and E. A. Barnard, manuscript in preparation). The extents of the protections shown in the affinity reaction (Table III) and in the iodoacetate inactivation (Jones et al., 1975) can all be understood on the basis of the two factors mentioned. The differences between K_m and K_d values are, as a matter of interest, all in the direction predicted if a random binding order of sugar and nucleotide substrates (Rudolph and Fromm, 1971) to hexokinase B holds kinetically; further studies of the various binding

constants are, however, needed to explore this further.

Discussion

In the associated paper (Jones et al., 1975), the inactivation of hexokinase B by iodoacetate or iodoacetamide was shown to be due to the alkylation of two out of the four SH groups/protein monomer (52,000 molecular weight), these having the character of "apparently essential" groups. The present study shows that the *N*-bromoacetylgalactosamine reaction is of the same character, but is much faster. This enhancement, and the fact that *N*-bromoacetylgalactosamine acts kinetically via prior reversible complexing (eq 2), lead us to conclude that the latter reagent is an active-center-directed reagent for hexokinase. Again, two SH groups are alkylated and no other groups are modified, when all activity is lost. This, and the protection from each type of these alkylations by substrates and inhibitors, to respective degrees in line with their binary complex affinities (so far as these are known) for the active center, show that the same sites are involved in each case.

It is surprising that the affinity reagent alkylates two thiols (rather than one) in the course of the inactivation. The stoichiometry for sugar binding to yeast hexokinases is one per monomer (Colowick, 1973), and this has been observed in the crystal state, too (Bates et al., 1974). The dual labeling may mean that *N*-acetylgalactosamine binds differently than glucose, at two sites per monomer; or alternatively, that one SH lies at or close to a single sugar binding site, and reaction there induces an immediate structural change which brings the second SH into a highly accessible position. Since the rate of carboxymethylation of an exposed protein SH can be much larger than that of the hexokinase SH groups with these reagents (Table III of Jones et al., 1975), such a sequential reaction could be non-rate-limiting here. There is no reason at present to conclude that *both* the SH groups alkylated here are of the apparently essential type: if the last mentioned hypothesis is true, the SH revealed could be one of the nonessential ones present (two *or* three per subunit), which are normally unreactive (Jones et al., 1975).

The facts that galactose and *N*-acetylgalactosamine are scarcely inhibitory of the enzymic activity, and that the latter compound does not protect from the affinity alkylations, but instead accelerates it at higher concentrations, tend to suggest that an allosteric binding site is involved. This is also the simplest interpretation of the fact that the simple bimolecular reaction with bromoacetate also exhibits the acceleration by *N*-acetylgalactosamine, but protection by substrates. Clearly the phenomenon here is not a simple one, and the relation of the two thiols to the active center remains an unsettled question.

The finding that *N*-bromoacetylglucosamine, although based upon a true substrate, reacts poorly in this system—more slowly, even, than the bimolecular reactant, bromoacetate—can be explained by self-interference in the reaction. In support of this view, *N*-acetylglucosamine, unlike its galactose analog, protects from the alkylation.

The binding sites on the yeast hexokinase monomer for hexoses and their derivatives will clearly need further definition, in the light of these findings. An explanation for the unexpected preference for galactose in the present case can be suggested, as a basis for such further investigation, as follows. The presence of a substituent at C-2 larger than the OH group strongly stabilizes the hexose ring in the C 1 conformation (chair equatorial: cf. Durette and Horton, 1971), or the corresponding conformation of the fructofuranose ring. When this substituent carries a good H bond donor, as in the 2-acylamino-2-deoxyhexoses, than an additional interaction there may occur on the protein surface. Evidence for this comes from the stronger inhibition of hexokinase by 2-*N*-acetylglucosamine and 2-*C*-hydroxymethyl-D-glucose than by 2-*O*-methyl-D-glucose and 2-*O*-methyl-D-mannose (Sols et al., 1958), and also by the even stronger inhibition by *N*-3,5-dinitrobenzoylglucosamine (Maley and Lardy, 1955). Crane (1962) proposed, from this and other specificity evidence, that only the C1 type of conformer is bound to hexokinases (yeast or mammalian), but to yield an inactive complex, which undergoes a structural transition to form the productive enzyme–substrate complex. Purich et al. (1973) have pointed out that the known specificity data fit the model of a complex with the C1 conformer, but do not establish that this is inactive. In fact, the form of the hexose in the active complex remains uncertain in our present state of knowledge. We can note here that *N*-bromoacetylhexosamines have a good H-bonding, bulky group attached to C-2, and that the binding of *N*-acetylgalactosamine (Table IV) is much stronger than that of D-galactose (Sols et al., 1958), or of 2-deoxy-2-fluoro-D-galactose (Bessell et al. 1972), which are both, in practice, not inhibitors at all; this is in line with the proposal that the inhibitors mentioned, at least, bind thus as the Cl conformers. The C-4-hydroxyl of D-glucose is not essential for enzymic activity: 4-deoxy-4-fluoro-D-glucose is a substrate, having a V_{max} value 10% of that of D-glucose, although its K_m is about 80 m*M* (Bessell et al., 1972). It can be proposed, therefore, that *N*-bromoacetylgalactosamine binds as the stabilized C1 conformer in the sugar-binding site but in a different orientation to that of glucose, such that the C-4-OH of the former makes an H bond, absent in the glucose series, that positions the alkylating function near an SH group. This would explain why the glucosamine analog is so much less effective. Obviously, this suggestion should be tested by comparison of further potential inhibitors.

The behavior reported serves to focus attention on one or two thiols in the active center region of the hexokinase monomer, and should aid in locating these in the protein structure. Experiments in progress show that particular alkylated peptides can be obtained for this purpose from the affinity labeled enzyme.

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Superactivation of Thermolysin by Acylation with Amino Acid *N*-Hydroxysuccinimide Esters†

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ABSTRACT: Synthesis of a series of active *N*-hydroxysuccinimide esters of aliphatic and aromatic amino acids has yielded a new class of reagents for the covalent modification of proteolytic enzymes such as thermolysin. The activities of aliphatic acyl amino acid thermolysins are from 1.7 to 3.6 times greater than that of the native enzyme when hydrolyzing furylacryloyl-Gly-Leu-NH_2, the substrate employed most widely. By comparison, the aromatic acylamino acid derivatives are "superactive," their activities being as much as 70-fold greater. Apparently, the aromatic character of the amino acid introduced is a critical variable in the determination of the functional response. The increased activity is completely restored to that of the native enzyme by deacylation with nucleophiles, such as hydroxylamine, and the rate of restoration of native activity is a function of the particular acyl group incorporated. Preliminary evidence regarding the chemical properties of the modified enzyme suggests that tyrosine, rather than lysine, histidine, or arginine, may be the residue modified. The functional consequences of successive modification with different reagents, moreover, indicate that each of them reacts with the same protein residue. The competitive inhibitors β-phenylpropionyl-Phe and Zn^{2+} do not prevent modification with these active esters. Hence, the site(s) of their inhibitory action differ(s) from that at which modification occurs. The structure of the substrate is also a significant variable which determines the rate at which each acyl amino acid thermolysin hydrolyzes peptides. Depending on the particular substrate, the activity of aromatic derivatives can be as much as 400-fold greater than that of the native enzyme, and the resultant activity patterns can be ordered in a series characteristic for each enzyme derivative.

Thermolysin from *Bacillus thermoproteolyticus* is a zinc metalloenzyme; like other metalloendopeptidases it is inhibited by metal chelating agents but is insensitive to inhibitors of thiol and serine proteases (Latt et al., 1969; Matsubara and Feder, 1971). Both its amino acid sequence (Titani et al., 1972) and three-dimensional structure (Matthews et al., 1972a,b; Colman et al., 1972) have been determined. Recently, reagents which characteristically affect the catalytic properties of this enzyme through covalent modification have been employed to implicate the involvement of specific amino acid residues in activity. Thus, diethyl pyrocarbonate[1] (DEP) is a reversible inactivator of thermolysin. It

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[1] Abbreviations used are: DEP, diethyl pyrocarbonate; FAGLA, furylacryloyl-Gly-Leu-NH_2; Dnp, 2,4-dinitrophenyl; Dns, 5-dimethylaminonaphthalene-1-sulfonyl; FDNB, 1-fluoro-2,4-dinitrobenzene; 4-DnpNH, 4-(2,4-dinitroanilino); DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, *N,N'*-dicyclohexylurea; FA, furylacryloyl; DMF, dimethylformamide; Osu, oxysuccimide. All amino acids are of the L configuration unless otherwise indicated.