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Essential and Nonessential Thiols of Yeast Hexokinase. Reactions with Iodoacetate and Iodoacetamide†

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ABSTRACT: The reaction of yeast hexokinase with iodoacetate or iodoacetamide has been investigated in detail, using pure hexokinase B. Of the four thiols in each subunit of the molecule, two (the "apparently essential thiols") are alkylated rapidly at 35°, and the enzymic activity is lost in parallel with their reaction. The other two thiols react subsequently to completion, but at a very much slower rate. In the conditions used, no other uptake of the reagent occurs elsewhere during these thiol alkylations. Electrophoretically homogeneous dialkylated and tetraalkylated protein species are formed, in the two stages of the reaction. The inactivating reaction at 35° with the apparently essential thiols is second order. The rate constant increases with increasing pH, in the range pH 7.0–8.5, in a manner consistent with control of the reaction by a group with pK_a of approximately 10. The absolute (pH independent) rate constant is of the same order as that for a normal thiol in model compounds. The availability of the apparently essential thiols appears to be associated with some conformational change in the molecule in the monomer form: it declines at high ionic strengths, is maximal at intermediate values where the dimer first dissociates, but is lowered in the dimer at very low ionic strengths. The reaction also shows a sharp temperature dependence: the dimer at 30° (in contrast to 35°) shows no availability of the apparently essential thiols. A similar transition to a state permitting fast inactivation is found with pH, above pH 8.5. The reaction of the two apparently essential thiols is strongly inhibited by glucose. ATP and ADP, and their Mg complexes, protect significantly, but less effectively than does glucose. The affinities of these substrates at the active site of the enzyme are measured in this protection system. These various reactions appear to be of value for identifying the cysteine-containing regions that are involved in the active center or in its maintenance in the structure.

There have long been indications that yeast hexokinase is a thiol-dependent enzyme. Variations in the findings of the earlier studies on this question are understandable in view of the recognition (Lazarus et al., 1966; Gazith et al., 1968; Schulze and Colowick, 1969) of the heterogeneity of previous preparations of this enzyme, which relied upon extensive autolysis of the yeast cells for its release, introducing proteolytic damage. In this laboratory, a method (Lazarus et al., 1966; Ramel et al., 1971; Rustum et al., 1971) is used which avoids such proteolysis, for the preparation of two isoenzymic forms of yeast hexokinase, A and B, in homogeneous (Rustum et al., 1971) form. Hexokinase B is the major and more active isoenzyme. (Similar material has been prepared by Colowick and coworkers: their preparation, which is by a different method, and in which they term the corresponding isoenzyme P-II, has recently been reported in detail (Womack et al., 1973).) Analysis of hexokinases A and B has established that there are eight SH groups in each molecule (Lazarus et al., 1968). The molecule has 104,000 molecular weight and consists of two identical subunits in each case (Rustum et al., 1971; Derechin et al., 1972; Schmidt and Colowick, 1973). On the basis of the inactivation of both enzymes by methylmercuric iodide, it was concluded (Lazarus et al., 1968) that four of these SH groups are not essential for catalytic activity. However, inactivation of hexokinase B with iodoacetate was, in certain conditions, associated with the carboxymethylation of all eight thiols. Earlier, Barnard and Ramel (1962), using a stable and homogeneous yeast hexokinase (but made by an earlier method, and suffering minor proteolytic damage), showed that inactivation by bromoacetate was associated with the carboxymethylation of at least one and at the most four SH groups per unit of about 50,000 molecular weight. In the present report we describe more precisely the reac-

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tion between iodoacetate (and iodoacetamide) and yeast hexokinase B, and provide evidence which shows that, under certain conditions, the loss of enzymic activity is associated with the alkylation of only two SH per molecule of 52,000 weight, and that these two SH groups are candidates for the role of active site thiols.

Materials and Methods

The preparation, concentration, assay, and definition of units of yeast hexokinase B, and any other methods not specified, were as described elsewhere (Ramel et al., 1971; Rustum et al., 1971). The specific enzymic activity of the preparations used varied from 550 to 650 units/mg (at 25°). For the pH-Stat reactions, the concentrated enzyme was transferred to 0.02 *M* glycine–NaOH–NaCl (pH 8.0; I = 0.1) medium by gel filtration on a column (25 × 1.5 cm) of Sephadex G-25 equilibrated with the same buffer. For all other reactions, the enzyme was desalted by dialysis and transferred to 0.025 *M* glycylglycine–NaOH–NaCl (pH 8.6; I = 0.1) or other stated medium.

Both iodoacetic acid (Eastman) and iodoacetamide (Calbiochem) were recrystallized twice from light petroleum ether. [1-^{14}C]Iodoacetic acid (New England Nuclear Corp.) was recrystallized from light petroleum ether in the presence of 30 mg of previously recrystallized iodoacetic acid: the final preparation was white, with a specific activity of 0.26 Ci/mol. [^{14}C]Iodoacetamide was from Amersham-Searle, and was diluted with the recrystallized iodoacetamide to a specific activity of 0.92 Ci/mol. These reagents were shielded from light during all operations. Tris was metal-free enzyme grade (Mann) and the adjustment of pH of the Tris and glycylglycine buffers was made with metal-free HCl ("Ultrex" HCl, Baker); glassware used then was first washed with 1 m*M* EDTA solution. All other chemicals were Fisher Certified or Baker Analyzed grade.

Protein was determined spectrophotometrically (Lazarus et al., 1966) using a Cary 15 spectrophotometer, or (for the determinations in relation to specific radioactivity) by the method of Lowry et al. (1951).

Inactivation Rates. For all pH-Stat experiments, the medium was 0.02 *M* glycine–NaOH–NaCl buffer, I = 0.1. A freshly prepared solution of iodoacetate at the required concentration (0.7 ml) was incubated in a 2-ml glass vessel, which was maintained at 35 ± 0.1° by water circulation through a glass jacket. The pH was adjusted to the stated value. Hexokinase B (0.2 mg except where stated), in the same buffer and at the same pH and temperature, was added to a final volume of 1.0 ml, and the pH again was checked. The adjustment and constant control of the pH (to ±0.02 unit) were performed using a radiometer TTTIC autotitrator with scale expander. The reaction mixtures were assayed for hexokinase activity at suitable time intervals by transferring samples (10–50 μl) directly into cuvets at 25° for spectrophotometric assay. Since there is at least a 60-fold dilution at this stage, and since the initial velocity in the assay over a period of 1 min was used, the inactivation reaction can be regarded as arrested at the moment of activity determination.

For the other reactions, made in buffered media without pH-Statting, the enzyme was preincubated (30 min) in the medium specified in each case, in a water bath at the temperature of the reaction, to ensure equilibration to the degree of protein dissociation characteristic of those conditions. The reaction was initiated by the addition of 25 μl of iodoacetate or iodoacetamide solution in the same medium.

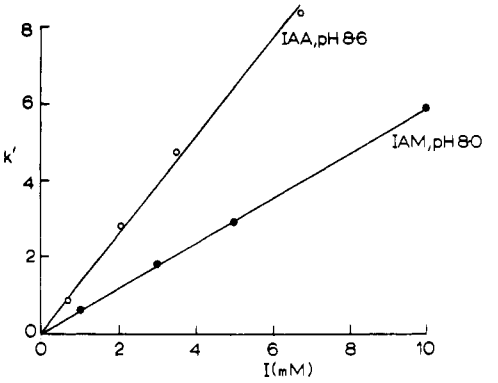

FIGURE 1: Rate constants (pseudo-first-order, k') for inactivation of hexokinase B at 35°, I = 0.1, in relation to the concentration of iodoacetate (IAA) at pH 8.6 or of iodoacetamide (IAM) at pH 8.0. A further point, off-scale, at 20 m*M* iodoacetamide lay 10% below the value predicted by the corresponding line.

Assays during inactivation were as described above. In these cases, the pH during the reaction period was constant within 0.1 unit. Rates were calculated from least-squares lines fitted to semilogarithmic plots. In every set of conditions, a control incubation of enzyme alone was run, and showed no inactivation whatsoever in the same period (except above pH 8.9).

Isotopic Measurements. Radioactive solutions were counted (Goren et al., 1968) in a Packard liquid scintillation counter, using 100-μl samples, at a quenched efficiency for ^{14}C of 83%. [^{14}C]CM-cysteine content was measured in a 6 *N* HCl (110°, 24 hr, in vacuo) hydrolysate of the ^{14}C-labeled alkylated protein, by ion-exchange chromatographic separation and measurement in a scintillation flow-cell system, by the methods described by Goren et al. (1968). The identity of the [^{14}C]CM-cysteine present was confirmed by its cochromatography with a synthetic sample (Goren et al., 1968). The corresponding weight of the protein was determined from the aspartic acid content in the amino acid analysis run at the same time (Lazarus et al., 1968). The same radioactivity chromatograms, run to completion as described by Goren et al. (1968), were used in examining the possible presence of other alkylated residues.

Electrophoresis. Polyacrylamide gel electrophoresis was performed by the method of Davis (1964), with staining for protein only, by Coomassie Blue. Cellulose acetate electrophoresis was performed (Rustum et al., 1971) at 150 V, room temperature, 2 hr, with staining by Buffalo Black.

Results

Inactivation Reactions. Hexokinase B and iodoacetate at 35 ± 0.1°, pH 8.6, I = 0.1, were mixed, and the pH was maintained at 8.60 on an autotitrator. Final concentrations of iodoacetate were in the range of 0.7–6.7 m*M*. The inactivation was measured at once on samples withdrawn at intervals. Other reactions, up to higher iodoacetate concentrations, were performed in glycylgycine buffer at pH 8.6 or 8.0, I = 0.09. In every case, semilogarithmic first-order plots for activity loss had the points all lying on straight lines passing through the origin, up to 95% inactivation where tested. The reaction proceeds to complete extinction of the enzymic activity; the absence of any residual activity is further noted below. The pseudo-first-order rate constant, derived from the plots mentioned, increases linearly with the concentration of iodoacetate in this range (Figure 1), so that a second-order rate constant, k_2, can be obtained. The

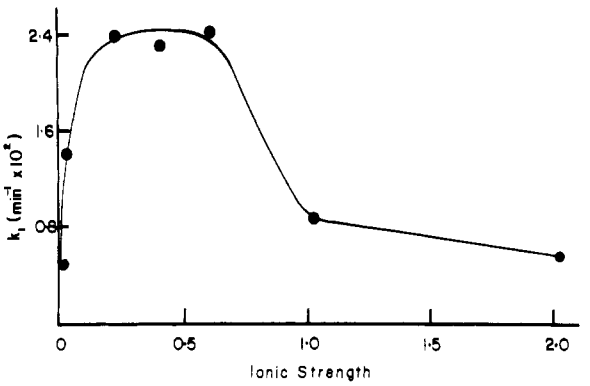

FIGURE 2: Effect of ionic strength on k_1, the pseudo-first-order rate constant, for the inactivation of hexokinase B by iodoacetamide (5 mM) at 35°. The media contained Tris (not more than 50 mM total concentration), NaCl, and HCl to bring the pH to 8.0.

same was true for the inactivation reaction with iodoacetamide. The reaction is significantly faster with the uncharged amide reagent: k_2 is 0.10 (iodoacetate) or 0.42 (iodoacetamide) l. mol^{-1} sec^{-1}, at pH 8.60, I = 0.11 in glycine–NaCl medium, 35°.

The reaction is unusually sensitive to the ionic strength of the medium (Figure 2). With iodoacetate, at I = 0.11, the rate is 3.9 times faster than at I = 0.01 in glycine–NaCl medium at 35°. At higher ionic strengths, only the amide reagent was used, to avoid the subsidiary effect of high I on the interaction of thiolate with a charged reagent. In the ionic strength range 0.01–0.4, a pronounced increase in the inactivation rate occurred with ionic strength. Paradoxically, in the range I = 1.0–2.0, strong retardation of the reaction was found (Figure 2). This effect was not attributable to heavy metal impurities in the high salt concentrations, since it was not affected by the presence of 1 mM EDTA, was found equally when metal-free "Ultrex" NaCl[1] was used, and since the enzyme incubated similarly in the same salt (2 M NaCl) medium without the iodoacetamide (and assayed in the normal medium) lost no activity, even in 48 hr at 35°.

In contrast to the present data, the effect of ionic strength increase on the iodoacetamide alkylation rate of model thiols, e.g., GSH, is relatively small and regular, being about a 1.5-fold increase for alkylation of GSH at 22° for the entire ionic strength range 0.2–2.0 (Guidotti, 1967).

Effect of pH on the Alkylation Rate. Hexokinase B (approximately 0.2 mg) was incubated at different pH values at several levels of iodoacetate concentration, and the course of the inactivation was followed. The second-order rate constant for the reaction at each pH value was determined using the usual semilogarithmic plot. At each pH value a control experiment measured the rate of spontaneous inactivation of the enzyme: this rate was negligible at pH values below 8.9. At higher pH values, however, the rate of such inactivation was significant and extremely sensitive to pH, so that estimations of the rate of alkylation are unreliable there. The second-order rate constant for inactivation by iodoacetate is shown as a function of pH in Figure 3.

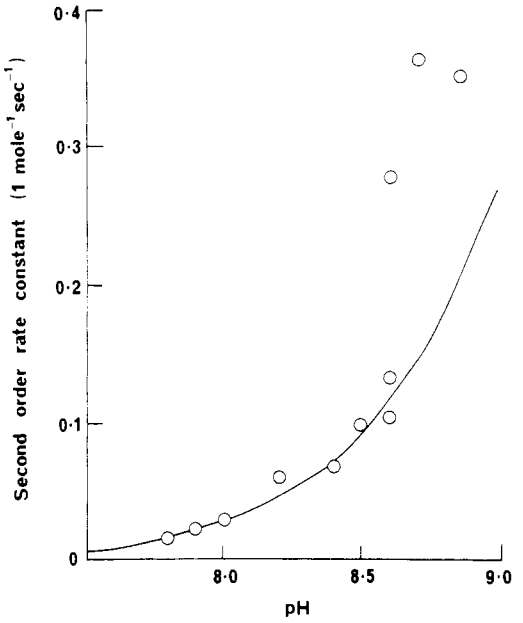

FIGURE 3: Inactivation of hexokinase B by iodoacetate at 35° at various pH values. The curve is theoretical and based on a pK value of 10.0.

If the undissociated SH groups (or any equivalent target groups) are unreactive toward iodoacetate, and if the absolute (pH independent) rate constant for the reaction between the mercaptide ion and iodoacetate is k_{max}, then the rate constant, k, for reaction at any pH value can be obtained from

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

where K is the ionization constant for the group involved. Hence, if k_{max} is known, K may be calculated. Simple behavior of this type for model thiols reacting with halogenoacetamide has been substantiated by Lindley (1962). For the pH range where a single ionization determines the reactivity, we can use eq 1 in the form

$$\frac{1}{k} = \frac{1}{k_{max}} + \frac{[H^+]}{k_{max}K} \quad (2)$$

A plot of $1/k$ against hydrogen ion concentration should then be linear, and k_{max} and K can be obtained from the graph (Lindley, 1962). As seen in Figure 4, the extrapolated line passes very close to the origin, so that precise measurements of k_{max} are not obtained thus in this case. However, for any assumed value of K the corresponding value of k_{max} may be obtained from the slope of the line in Figure 4. In this way it is possible to construct theoretical curves describing the variation of k with hydrogen ion concentration, using a series of assumed values of K. Accordingly, the best-fitting curve drawn in Figure 3, and the line in Figure 4, are theoretical and based on pK = 10 for the acidic dissociation of the target group and a corresponding k_{max} of 3.3 l. mol^{-1} sec^{-1}.

Effect of Substrates on the Alkylation. The inactivation of the enzyme by iodoacetate (7 mM, pH 8.6) was strongly retarded in the presence of glucose (Table I). The protection was essentially complete at a glucose concentration of 10 mM.

If the alkylating reagent reacts in the free enzyme with groups which are unavailable to alkylation when the en-

[1] J. T. Baker Chemical Co., Phillipsburg, N.J. When this NaCl was employed, deionized redistilled water was used, 1 mM EDTA was present, and EDTA-prewashed glassware was used for all handling of the solutions. No effect on the alkylation rate was produced by these precautions, which were not used routinely.

Table I: Effects of Substrates on the Reaction of Hexokinase B with Iodoacetate.[a]

Addition	Relative Rate of Alkylation[b]
None	1.0
Glucose (1.0 m*M*)	0.30
Glucose (10.0 m*M*)	0.03
ATP (3.0 m*M*)	0.53
ATP + Mg^{2+} (each 3.0 m*M*)	0.44
ADP (3.0 m*M*)	0.58
ADP + Mg^{2+} (each 3.0 m*M*)	0.53
Glucose 6-phosphate (10.0 m*M*)	0.54

[a] Reaction was at pH 8.5 ± 0.1, I = 0.09, 35°. [b] Ratio of observed rate in presence of the substrate to that in its absence, with the same concentration of iodoacetate in both cases, this being either 7 or 10 m*M*. Each is the mean for two or three replicates.

zyme binds its substrate, then the decrease in the measured rate constant (k) for the inactivation of the enzyme to the value (k') observed in the presence of a concentration [S] of substrate is given by

$$k' = k/[1 + ([S]/K_s)] \quad (3)$$

where K_s is the dissociation constant for the enzyme–substrate complex (Whitehead and Rabin, 1964; Mildvan and Leigh, 1964). Hence

$$\frac{1}{k'} = \frac{1}{k} + \frac{[S]}{kK_s} \quad (4)$$

The plot of $1/k'$ against [S] should, therefore, be linear, and from it K_s may be calculated. When the apparent rate constants for the alkylation of hexokinase B in the presence of different levels of glucose were plotted thus, the values lay on a straight line (least-squares slope 30.38 ± 3.12 (SD) × 10^4 sec, intercept = 13.6 *M* sec, correlation coefficient = 0.995). The dissociation constant for the enzyme–glucose complex (at 35°, pH 8.6, I = 0.1) calculated from this relationship is 4.6 × 10^{-4} *M*.

The same effect was shown using iodoacetamide (5.6 m*M*) as the alkylator, under the same conditions (but without buffer, on the pH-Stat) at pH 8.6. This gave, alone, 90% inactivation in 30 min, and a greatly slowed progress curve in the presence of 10 m*M* glucose, with only 20% inactivation being attained in 60 min.

When ATP and Mg^{2+} (each at 3 m*M*) were present together, the inactivation reaction was significantly retarded, but this substrate was several-fold less effective than glucose in its protection. The ADP·Mg complex also protected, but less effectively still (Table I). In each case, most of this protective action was also exerted by the free nucleotide.

Glucose 6-phosphate was very much less effective than glucose: some protection was obtainable at higher concentrations (Table I).

Incorporation of ^{14}C-Labeled Alkyl Groups. Hexokinase B samples (2–5 mg) were reacted with [^{14}C]iodoacetate (1–4 m*M*) at 35°. Samples (10 μl) were removed at intervals and assayed for hexokinase activity. When the activity of the enzyme had been reduced to less than 10% of its original value (expt. 1–3 of Table II), the reaction mixture was rapidly cooled in ice. Protein and unreacted [^{14}C]iodoacetate were at once separated at 4° on a column (30 × 1.5 cm) of Sephadex G-25 equilibrated with the glycine buffer at pH 8.0. Two uv-absorbing radioactive peaks, one at the

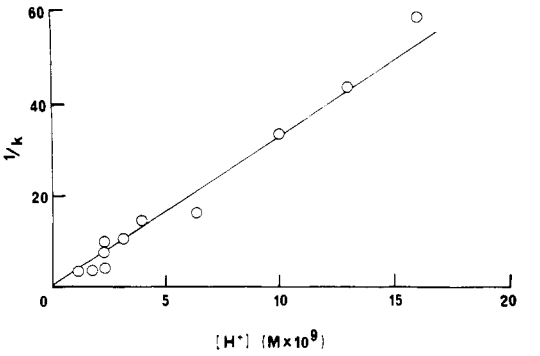

FIGURE 4: Effect of hydrogen ion concentration on the rate of alkylation of hexokinase B, plotted according to eq 2. k is the second-order rate constant for the alkylation (l. mol^{-1} sec^{-1}). The line is theoretical, with k = 3.3 and pK = 10.0.

excluded volume and the other close to the totally included volume, corresponding to the protein and iodoacetate, respectively, emerged from the column. Dialysis of the protein for 24 hr (4°) against the same buffer produced no decrease in the content of radioactivity. This material was analyzed for protein and ^{14}C content (Table II): the results show that the loss of hexokinase activity is associated with the incorporation of two ^{14}C-containing groups per monomer.

To analyze further the course of this reaction, hexokinase B was reacted similarly with [^{14}C]iodoacetate and, at intervals, 10-μl aliquots were removed for enzyme assay and 0.5-ml aliquots for estimation of the radioactivity of the protein by the gel filtration method described above. It was found thus (Figure 5) that there is a rapid incorporation of two [^{14}C]CM groups per molecule during the first hour. A slower incorporation of another two groups proceeds during the next 20 hr. During the first hour there is an approximately stoichiometric loss of enzyme activity with incorporation of ^{14}C into the protein, the reaction with two iodoacetate molecules resulting in complete loss of activity (Table II, expt 1–4). The protein in some cases was hydrolyzed and the content of [^{14}C]CM-cysteine was determined by the radioactivity in the chromatographic peak of the latter, measured by a scintillation flow-cell attached to the amino acid analyzer (Goren et al., 1968). These determinations showed that all of the ^{14}C incorporated, up to a total of 4 mol/mol of subunit, is accounted for by CM-cysteine (Table II). No free cysteine or cysteic acid was detectable in analyses of the hydrolysates of the protein carrying four [^{14}C]CM groups. Other residues were not alkylated in the conditions used.

Alkylation by [^{14}C]iodoacetamide was monitored by a similar method (Table II, expt 7–9). When a fast inactivation occurred (at I = 0.1), as in the case of iodoacetate two groups/subunit reacted irreversibly. Hydrolysis of the protein in this case yielded [^{14}C]CM-cysteine by complete hydrolysis of the [^{14}C]carboxamidomethyl derivative, again accounting for all of the radioactivity in the protein sample (Table II). After an iodoacetamide reaction (as in expt 8 of Table II) was continued further to a point corresponding to the presence of 2.8 [^{14}C]carboxamidomethyl groups per subunit, careful examination of the enzymic activity of the protein, using increasing amounts in the assay, showed that no residual activity exists at a significant level, it being below 0.1% of the native enzyme activity.

From these results, it is concluded that two "apparently essential" residues of cysteine are present per monomer, these being alkylated in the first phase of the reactions stud-

Table II: Loss of Enzyme Activity Associated with Incorporation of ^{14}C-Labeled Alkyl Groups into Hexokinase B at 35°.

Expt.	^{14}C-Labeled Alkylator Type	(mM)	pH	Inactivation[a] (%)	Equivalents of ^{14}C per Subunit	[^{14}C]CM-cysteine[b] Residues per Subunit
1	Iodoacetate[c]	2.1	8.7	95	2.2	2.0
2		1.3	8.4	90	1.9	
3		4.0	8.4	94	1.9	
4		2.1	8.7	52	1.1	
5		4.0	8.4	100	4.0	3.8
6		3.0	8.0	100	3.9	3.9
7	Iodoacetamide[d]	5.0	8.0	90	2.2	
8		5.0	8.0	95	2.4, 2.4[e]	2.4
9		5.0	8.0	100	4.0, 3.8[e]	3.8

[a] The reaction was arrested at the time when the inactivation level shown was attained, and the analyses performed then, except that the samples at 100% inactivation were further incubated with the reagent for 15–20 hr after inactivation was complete, to correspond to the last point shown for the ^{14}C uptake in Figure 5. [b] In each case the ^{14}C chromatogram (see Methods) was run to the end, but no other labeled residues were detected. [c] The first four experiments were performed on the pH-Stat, and the other two in glycylglycine buffered medium (I = 0.09). [d] In 0.05 M Tris-HCl. [e] Results of complete duplicate experiments.

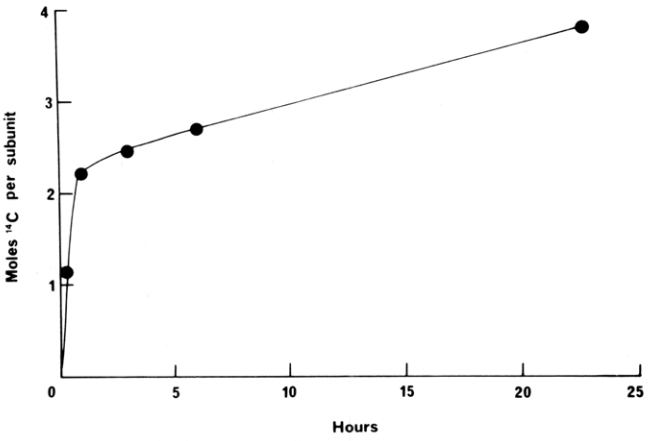

FIGURE 5: Incorporation of ^{14}C into hexokinase B (2.8 mg/ml) during reaction with [^{14}C]iodoacetate (2.1 mM) at pH 8.7, 35°.

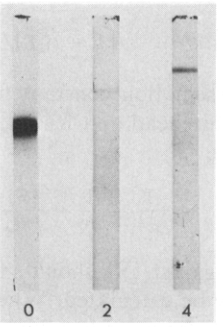

FIGURE 6: Electrophoresis in polyacrylamide gel (7.5%) of hexokinase B samples after the incorporation of 0, 2, or 4 [^{14}C]carboxymethyl groups per subunit, as indicated. The point of application (in sucrose) was at the top and migration was toward the anode at the bottom, run at pH 8.3, Tris-glycine buffer, 22°, 2.5 hr., at a constant current of 5 mA/tube.

ied, while two nonessential cysteine residues are alkylated at a much slower rate. The rate of incorporation of [^{14}C]CM groups by [^{14}C]iodoacetate reaction (as in Figure 5), *after* the uptake of 2.0 group/monomer had occurred (and inactivation was complete) followed pseudo-first-order behavior: such a semilogarithmic plot, based on five points, fitted a least-squares line passing through the origin, and based upon a second-order rate constant of 0.01 l mol^{-1} sec^{-1}. This is only 7% of the rate for the apparently essential thiols in the same conditions. A slight excess over the 2-equiv stoichiometry for inactivation was seen in the samples reacted with iodoacetamide (Table II), and is attributed to the concurrent alkylation of a small fraction of nonessential SH during the initial phase.

In the longer reaction periods used here, the carboxymethylation under the present conditions of normal, freely accessible methionine or histidine side chains would become appreciable (Goren et al., 1968). Hence, these residues are not available in hexokinase B to iodoacetate or iodoacetamide.

Electrophoresis of Alkylated Hexokinases. In cellulose acetate or polyacrylamide gel electrophoresis at pH 8.4, native hexokinase B always yielded a single, sharp band, as shown previously (Rustum et al., 1971). Samples of CM-hexokinase containing either 2 or 4 mol of [^{14}C]CM-cysteine/subunit (separated by gel filtration and analyzed as above) were also run. They gave a single, sharp band in each case, these being less anionic in character than the native enzyme. The fully alkylated protein showed the lowest mobility, both in cellulose acetate and in polyacrylamide gel (Figure 6). This apparent anomaly in mobility is presumed to be due to a considerable change of structure of the protein on alkylation, which is also reflected in a considerably lower solubility which was observed for the alkylated protein in media of low ionic strength. The behavior observed supports the interpretation made in terms of discrete dialkylated (inactivated) and tetraalkylated (reacted to completion) subunits being formed at the two stages of reaction considered.

Discussion

The results presented here confirm the earlier finding (Lazarus et al., 1968) that intact hexokinase B contains four SH groups per monomer (subunit) of molecular weight 52,000. The combined results show that the thiols of hexokinase B fall into two quite different classes, each comprising two per subunit. It is possible to alkylate two SH groups per subunit with stoichiometric loss of enzymic activity, whereas the remaining two SH are alk-

ylated subsequently at a very much lower rate. It seems reasonable to deduce that these latter SH groups are those nonessential ones (4 per dimeric molecule) which are preferentially titrated with limiting amounts of methylmercuric iodide at very low ionic strength (Lazarus et al., 1968).

The other two thiols per subunit can be classed as "apparently essential" groups. The inactivation that parallels the alkylation of these two thiols, and that also parallels their reaction with methylmercury (Lazarus et al., 1968), and the inaccessibility of these groups to alkylation in the substrate-liganded enzyme, do not, of course, establish that they participate in the active center, although compatible with that situation. It is always possible that they have an essential structural role elsewhere in the protein, and that the structure of the complex is tightened by indirect effects of the binding interaction with the substrate, so as to bury these cysteines. Or, only one may be truly essential, the second being revealed and highly reactive only upon alkylation of the first.

Increased ionic strength might be expected to accelerate somewhat the alkylation by iodoacetate, due to electrostatic stabilization of the reaction transition state. The effect seen here (Figure 2) is obviously much more than this. A large rate increase is found in the range 0.01-0.2 I, and this is true even when the uncharged iodoacetamide is used as the reagent. For the alkylation of model thiols, as noted earlier, this factor is very much smaller, being (by interpolation from the data of Guidotti (1967)) an increase of 50% for the iodoacetate reaction rate and only 6% for iodoacetamide, over the ionic strength range just cited. With these hexokinase thiols, even with iodoacetamide the corresponding increase is 900%. Another predicted effect of elevated ionic strength would involve the electrostatic free energy change of the protein molecule, modifying the pK of the reacting thiolate group; the hydrogen ion concentration in eq 1 must then be multiplied by the electrostatic correction term $e^{-2Z\omega}$, where Z is the net charge on the protein molecule and ω is the usual electrostatic potential parameter (Tanford, 1962). With usual values of ω (Tanford, 1962), this term would also be far too small to account for the acceleration produced here by increase in ionic strength. Even more anomalous in terms of simple solvent effects is the inhibition of the alkylation in media of higher ionic strength, even with the uncharged reagent (Figure 2). The dimeric molecule undergoes dissociation that is affected by ionic strength (Derechin et al. (1972)), and the precise contributions of monomer and of dimer in the range of the maximum alkylation (I = 0.1-0.4) at 35°, pH 8, will have to be defined by a separate study. The decline in alkylation at high I suggests that a conformational change in the monomer at high I removes the apparently essential thiols from availability to the alkylator.[2]

The alkylation of the apparently essential pair of thiols is inhibited by the binding of substrates. In the case of glucose, the extent of this protection at various substrate concentrations is large enough to suggest that in the liganded enzyme these two thiols have no alkylation reactivity. If that is the case, the value of K_s (eq 3) obtained by protection should be equal to the dissociation constant (K_d) obtained by direct measurement of the binding of labeled glucose to the protein. This affinity for glucose varies with the different forms (isoenzymic or proteolytically damaged) of yeast hexokinase: Colowick (1973) notes that for hexokinase B, K_d is, by dialysis measurements, about 10^{-4} M for glucose (at high concentrations of glucose). This value, however, refers to higher protein concentration and lower temperature than we have used. The subunit–subunit interaction appears to be competitive with the subunit–glucose interaction (Derchin et al., 1972), so that for the purposes of an exact comparison, K_d must be determined for the low protein concentration (and the 35° temperature) used in the alkylation protection measurement. The (more difficult) evaluation for K_d for such conditions is not at present available, but the value of 4.6×10^{-4} M found here for K_s for glucose is probably close to the true K_d value.

The protection by ATP·Mg and ADP·Mg (Table I) is weaker than that by glucose, but is significant. The affinity of these species in forming binary complexes with hexokinase in the absence of the sugar is too low to measure easily (Womack and Colowick, 1968; Noat et al., 1969), but appears to approximate the value of K_m for Mg·ATP^{2-} in the ATPase reaction (Colowick, 1973). That K_m value for a chromatographed yeast hexokinase at pH 8.5, 30°, is about 5×10^{-3} M (Noat et al., 1969). The degree of protection exerted by ATP·Mg here would be consistent with such a complex being inert to the alkylation. The nucleotide without Mg^{2+} has, also, a significant affinity for hexokinase B (Table I). This is to be expected, since it has been found that ATP^{4-} is a competitive inhibitor with respect to MgATP^{2-} in the hexokinase reaction (Kosow and Rose, 1970; Rudolph and Fromm, 1971).

The effect of pH on the rate of alkylation of hexokinase B is rather complex. At pH values below 8.5, the increase in the rate of inactivation of the enzyme with increasing pH seems to reflect the ionization of protein groups with an average pK of approximately 10. These ionizing groups might be the SH groups themselves, the reaction rate being determined by the amount of thiolate anion, as found by Lindley (1962) for the carboxamidomethylation of model thiols. These groups could have this rather high apparent pK value if they are close to carboxyls. However, several possible difficulties can complicate the interpretation of this apparent pK value, such as alternative microscopic pathways for the ionization of linked groups (Lindley, 1962), or pH-dependent structural changes elsewhere in the protein determining the alkylation rate of the thiol. In fact, at pH values above 8.5 the rate of alkylation increases faster than a titration curve for a simple ionization (Figure 4). While either of the possibilities cited could explain this, it seems likely that the anomaly arises from a partial unfolding of the molecule at the higher pH values. A progressive unfolding of the molecule with increasing pH would account for the rapid spontaneous inactivation of the enzyme observed (at 35°) at pH values above 8.9. However, it is also possible that the thiols do not ionize within the pH range studied, and that the entire pH profile reflects the progressive unfolding of the molecule as a result of the ionization of other groups in the protein. The nonionization of SH within this pH range has been reported for another kinase enzyme, creatine phosphokinase (Watts and Rabin, 1962; Rabin et al., 1964) and for alcohol dehydrogenase (see Table III). In neither of these cases was there any significant change of the iodoacetamide reaction rate with ionic strength.

It should be noted that the maximum (pH-corrected)

[2] A comparable case is the reaction of iodoacetamide with two thiols of deoxyhemoglobin, which shows a similarly steep rise in rate with initial increase in I, correlated with the protein dissociation by salt into half-molecules (Guidotti, 1967). In that case, however, there is no inhibition of the alkylation at high ionic strengths.

Table III: Absolute Rate Constants (k_{max}) for the Alkylation Reactions of the Apparently Essential Thiols of Hexokinase Compared to Some Other Thiols.

Location of Thiol	Temp (°C)	k_{max} (at I = 0.1–0.2) Iodoacetate (l. mol^{-1} sec^{-1})	k_{max} (at I = 0.1–0.2) Iodoacetamide (l. mol^{-1} sec^{-1})	Ref
Hexokinase B	35	3.3	14[a]	This work
Glutathione	22	4[b]	20[b]	Guidotti (1967)
N-Acetyl-L-cysteine[c]	25	4.3	33.0	MacQuarrie and Bernhard (1971)
Alcohol dehydrogenase (yeast)	25		0.6[d]	Whitehead and Rabin (1964)
Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle)	25		230[b]	MacQuarrie and Bernhard (1971)
Creatine phosphokinase (monkey)[e]	30		22[d]	Kumudavalli et al. (1970)
+ ADP·Mg + creatine			1300[b]	
Papain, ficin	25–30		300–400 (estimated)[f]	Holloway et al. (1964); Wallenfels and Eisele (1968); Chaiken and Smith (1969)

[a] The pH-dependence curve in a given medium was not determined separately for iodoacetamide: the value here is obtained from the rate constants, k, for iodoacetamide and for iodoacetate at pH 8.6 in the same medium, and the assumption that the same value of K determines the relationship between k and k_{max}, according to eq 1, for the two reagents. [b] Estimated approximately from data given by the author(s) cited. [c] Other model thiols also usually give k_{max} values of the same order. Thus, dimethylcysteamine gave similar reactivities with iodoacetate, and with iodoacetamide, to those of *N*-acetyl-L-cysteine (MacQuarrie and Bernhard, 1971). The k_{max} values for glutathione, L-cysteine, L-cysteinyl-L-asparagine, glycyl-L-cysteine, glycyl-L-cysteinylglycine, and β-mercaptoethylamine were compared by Lindley (1960, 1962) using chloroacetamide, at 30°, I = 0.1, and were all in the range 0.26–0.43 l. mol^{-1} sec^{-1} for that alkylating agent. [d] The rate noted was found to be essentially unchanged with pH up to pH 10, so that the absolute (k_{max}) value for the mercaptide form could not be obtained. For other species reported upon (human, rabbit, mouse), k is rather lower, in the range 7.8–13.6 (at 20°, without substrates), again being pH independent. [f] This has been investigated with chloroacetamide or D-α-iodopropionamide, where the corresponding absolute rate constants are 10–15 times greater than the k_{max} values for the same reagent with cysteine.

rate of iodoacetate reaction with the hexokinase apparently essential thiols is of the order of k_{max} for model thiols (and the same is approximately true for the apparently essential thiols of creatine phosphokinase, when substrate is absent) as shown in Table III. The difference in rates between the iodoacetamide and iodoacetate inactivations of hexokinase at I = 0.1 is similar (Table III) to that found with model thiols for these two reagents. The latter difference largely disappears at $I \sim 2$ (Guidotti, 1967), but, as we have seen, there are other complications there in the case of hexokinase. Other readily available thiols in proteins also often show the same order of alkylation rate as the model thiols, although much higher rates are known in a few proteins, where special environments in the site must be inferred (see examples in Table III).[3] Hence, we prefer at present the interpretation that ionizable thiols of normal reactivity constitute the apparently essential groups in hexokinase, these becoming accessible upon dimer dissociation and an associated conformational change. This conclusion is in agreement with the finding that the rates of inactivation of yeast hexokinase by mercurials, iodoacetate, and iodoacetamide (at low ionic strengths) are very low at temperatures below 30° and increase abnormally sharply over the temperature range 30–37° (Lazarus et al., 1968; Barnard and Ramel, 1962). At high ionic strength, a further unfolding that screens these apparently essential thiols is inferred.

From the present results on the specific carboxymethylation of two apparently essential thiols, we suspect that alkylation of the enzyme at ionic strength ~ 0.1 (at 35°) may prove useful as a method of labeling cysteine residues which are either in the active center or in a critical structural situation. Conversely, alkylation at very high ionic strength at lower temperature should identify the nonessential cysteines. Studies to be reported elsewhere show that specific [^{14}C]CM-cysteine-containing peptides can indeed be isolated, after reactions of the type described in this report.

[3] The alkylation rates of a number of other accessible thiols in proteins have been determined, but usually the pH dependence and k_{max} values were not determined (or were complex). In many cases only a single pH value was used; since K (eq 1) for the controlling group can differ by two or more orders of magnitude from K for a given model thiol, a rate comparison (as in Table III) with the latter would then be meaningless.

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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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