Evidence for a Single Essential Thiol in the Yeast Hexokinase Molecule†

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ABSTRACT: In yeast hexokinase B, two thiols per monomer appeared to be essential when enzymic inactivation was produced by the concurrent alkylation of both of them, by several reagents including the affinity reagent *N*-bromoacetyl-2-D-galactosamine. However, it is shown that only one of these thiols is actually essential. Three of the four thiols present can be blocked by alkylation in the presence of a substrate in appropriate conditions, without loss of enzymic activity. Subsequently, in the absence of substrate, the affinity reagent reacts at the one remaining thiol, with complete inactivation. The same behavior can be obtained by reaction with iodoacetamide or by the formation of the -SCN group. The affinity reagent inactivates hexokinase B faster than does the isomeric glycosidic compound (glycosides being nonsubstrates), although the latter has twice the reactivity of the former toward glutathione. The reactions with alkylating agents, with or without substrate present, are used to classify the four thiols in the monomer. The temperature dependence of the alkylation of the essential thiol provides evidence for a transition in the molecule at about 31 °C. The inactive monomer containing the -SCN group can regenerate, by thiolysis, active enzyme with the thiol free. It can also perform an intramolecular cleavage of the chain. The latter reaction was used to locate the essential cysteine residue in the chain, at 80% of the length from the N terminus.

In previous studies (Otieno et al., 1975) of the role of thiol groups in the yeast hexokinase molecule, it was shown that *N*-bromoacetylgalactosamine is an affinity reagent for this enzyme and alkylates two thiol groups per subunit in the dimeric protein. Two other thiols are present per subunit and are left unmodified when this inactivation reaction is complete. It was also shown (Jones et al., 1975) that alkylation by iodoacetamide or by iodacetate can occur at the first pair of thiol groups, again inactivating completely. With the latter reagents, the second pair of thiol groups per subunit can, however, be alkylated in addition, in a subsequent, slow reaction. Substrates protect the enzyme from inactivation by any of these reagents. It was suggested, to explain the uptake of two molecules of the affinity reagent per monomer, that this reagent binds at the sugar substrate binding site on the monomer to alkylate one thiol that is close to that site, and that the covalent attachment of that reagent molecule leads to a structural change which exposes a second thiol of high enough reactivity to be rapidly alkylated.

We report here evidence to show that alkylation of a single thiol per monomer of the hexokinase B isoenzyme is, indeed, sufficient to cause complete inactivation, and that the other three thiols per monomer are truly nonessential. The region of the polypeptide chain where the essential cysteine is located is indicated. Evidence is also presented for a change in the availability of this thiol in a temperature transition exhibited by hexokinase B.

Materials and Methods

The preparation of homogeneous hexokinase B, and all other materials and methods, were as described by Otieno et al. (1975) and Jones et al. (1975), unless specified here. All assays were by the spectrophotometric Cresol red titration method employed in those studies, except at pH 7, where the coupled assay procedure using glucose-6-phosphate dehydrogenase (Sharma et al., 1963) was used, in 0.02 M cacodylate–NaOH buffer. *N*-[^{14}C]Bromoacetyl-2-D-galactosamine was prepared, and characterized as pure, by the methods used previously for the unlabeled compound (Otieno et al., 1975) but using 1-[^{14}C]bromoacetic anhydride. For labeling experiments it was diluted with the unlabeled compound to a specific activity of 0.33 mCi/mmol. *N*-Bromoacetyl-1-D-β-galactosamine was a gift from Dr. M. Meisler (Buffalo), being made, and characterized as pure, as described by Thomas (1970). NTCB[1] was prepared by the method of Degani and Patchornik (1971). It was characterized as pure by its melting point, and by spectrophotometric titration of the stoichiometric amount of thionitrobenzoate ion produced by its reaction with glutathione at pH 7 (Jacobson et al., 1973). DTNB was from Pierce, and glutathione from Sigma.

Alkylations with [^{14}C]*BAGA.* [^{14}C]BAGA (3 mM) was reacted with hexokinase B (6 mg/mL) in 0.025 M glycylglycine buffer (pH 8.6), I = 0.1, at 35 °C, and the inactivation was followed, as described previously for BAGA (Otieno et al., 1975). After the period specified, the reaction mixture was placed on a column (90 × 2 cm) of Sephadex G-25 at 4 °C, equilibrated in the same buffer. By monitoring A_{280nm} and radioactivity in 1-mL fractions, the protein was shown to be completely separated in a single peak from the excess reagent present. The protein-containing fractions were pooled and concentrated in an Amicon B15 cell with a PM10 membrane. The protein was reacted again in the conditions specified for each experiment, and finally separated from the reagents on a Sephadex G-25 column (30 × 1.5 cm) equilibrated in 0.05 M ammonium acetate.

Analysis for CM-Cysteine Content. The peak tube of the

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[1] Abbreviations used: BAGA, 2-(*N*-bromoacetyl)-D-galactosamine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NTCB, 2-nitro-5-thiocyanobenzoic acid; Tris, tris(hydroxymethyl)aminomethane; Gdn·HCl, guanidine hydrochloride.

fractions containing ^{14}C-labeled alkylated protein, separated on a G-25 column as noted above, was used in each case. The sample was mixed with an equal volume of 12 N HCl (metal-free, "Ultrex", Baker) and hydrolyzed in vacuo at 110 °C, 22 h. The pH was then adjusted with 50% NaOH to 2.2 without an evaporation stage, and the sample was applied to an amino acid analyzer column whose effluent passed to a flow-cell scintillation-counting system, recording and identifying all the peaks of radioactive products from [^{14}C]CM-containing amino acid residues, as described by Goren et al. (1968). The CM-cysteine content of the original protein sample was calculated from the radioactivity present at the position of *S*-CM-cysteine (as shown by a synthetic marker), plus that in the peak of its radioactive breakdown product emerging at 60% of its elution volume.[2] To some samples of the protein, instead, thioglycolic acid was added to give 1% (v/v) solution prior to hydrolysis, and the hydrolyzate in these cases was finally rotary evaporated to dryness in the usual way and analyzed as just described. The [^{14}C]CM-cysteine content of a given protein sample was found to be identical after these alternative treatments. However, the avoidance of drying, thereby removing the need to add thioglycolic acid to prevent any air oxidation of CM-cysteine, was preferred, because of an observation made of an artefact with the thioglycolate system. When the latter method was tested on a sample of [^{14}C]CM-ribonuclease A rich in [^{14}C]CM-methionine residues (Goren and Barnard, 1970), it was found that the *S*-CM-homocysteine produced from the latter in hydrolyzates (Goren et al., 1968) was destroyed by reaction with the thioglycolic acid present during the hydrolysis. This means that any [^{14}C]CM-methionine present in the ^{14}C-labeled alkylated hexokinase would not be detected. The products characteristic of [^{14}C]CM-methionine after acid hydrolysis (Goren et al., 1968) were, on the other hand, fully conserved in the alternate treatment in which evaporation is avoided, and this treatment was used for all of the analyses of labeled proteins that are reported. It was found thus that no CM-methionine residues were detectable in any of the samples of alkylated hexokinase produced in the present experiments.

Reaction with Iodoacetamide. Hexokinase B (about 5 mg/mL) in 0.01 M Tris-HCl buffer, pH 8.5 (unless specified otherwise), was reacted with 1-[^{14}C]iodoacetamide (final concentration 0.005 M) at 35 °C. Monitoring of the inactivation and other details were as described by Jones et al. (1975). After the period specified, the mixture was placed on a column (60 × 2 cm) of Sephadex G-25 in 0.05 M ammonium acetate, pH 7, and eluted with the same buffer, collecting 1-mL fractions.

Reaction with NTCB. Reaction of hexokinase (about 2 mg/mL) was with 3 mM NTCB in 0.05 M Tris-HCl, pH 8.0, I = 0.1, at 35 °C (except where noted), until all activity was lost (about 1.5 h). The protein was separated by gel filtration as before. Where indicated, the product was concentrated and alkylated finally with [^{14}C]BAGA (as above). In the case where cyanylation was to be followed by cleavage, the reaction was conducted with 3 mM NTCB in 4 M guanidinium chloride/0.2 M Tris-acetate, pH 8.5, at room temperature, for 2 h. Immediately upon adding the Gdn·HCl, the sample was placed for 2 min in a waterbath at 100 °C to inactivate any trace of latent protease (Ramel et al., 1971) present.

Cleavage of Cyanylated Protein and Separation of Peptides. The cyanylated protein (about 2 mg/mL) was placed in 4 M guanidinium chloride/0.2 M Tris-acetate buffer, pH 9.0. Incubation was for about 18 h at 37 °C. The guanidinium chloride was then removed by gel filtration on G-25, as above. The material with light-absorbance at 280 nm, occurring in a sharp peak at the void volume, was pooled, freeze-dried, and dissolved in the smallest possible volume of 0.03 M Tris-glycine buffer, pH 8.6, containing 1% 2-mercaptoethanol. Sodium dodecyl sulfate was added to 1%, and the solution was at once held at 100 °C for 2 min, as before. Electrophoresis was in tubes of 7.5% polyacrylamide gels (12-cm long) in the same medium, run at 4 mA per tube, and in other details followed the procedures of Rustum et al. (1971). Slices were cut with a gel slicer and dissolved in 6% Digestol (Yorktown Research Inc., New Hyde Park, N.Y.) in a toluene-based scintillation counting medium at 60 °C, 1 h, cooled, and counted. For staining, the sodium dodecyl sulfate was removed by overnight treatment in 25% 2-propanol–10% acetic acid, followed by 0.04% Coomassie brilliant G250–3.5% perchloric acid (Reisner et al., 1975). For calibration, the cleaved products were also run with standard proteins, i.e., bovine serum albumin, hexokinase B, ovalbumin, whale myoglobin, and ribonuclease A. A plot was made of the logarithm of molecular weight against mobility and was linear with all of the points touching the least-squares line, as illustrated previously (Rustum et al., 1971) for such a calibration.[2]

Alkylation Rates on Glutathione. Using a fresh solution of glutathione in deaerated water, the final incubation medium at 0 °C was: glutathione, 0.25 mM; alkylating agent, 3 mM; glycylglycine 25 mM, plus HCl to pH 8.5; NaCl added to I = 0.1. At intervals, aliquots (100 μL) were assayed with DTNB (1 mL of 0.5 mM solution in the same buffer) at 25 °C, reading A_{412nm} (against a control DTNB solution as reference) as a measure of the free glutathione present. It was confirmed that A_{412nm} in these conditions is proportional to glutathione concentration over the range involved.

The data were plotted semilogarithmically according to the equation, $[2.303/(b-a)] \log [a(b-x)]/[b(a-x)] = k_2 t$, where a and b are the initial amounts of the reactants, and x is the amount reacted at time t, to obtain k_2, the second-order rate constant.

Other Methods. Radioactivity of solutions was determined using a xylene-based scintillation fluid containing Triton X-100 (Anderson and McClure, 1973). Counting was on a Packard liquid scintillation counter, Model 3385; efficiency for carbon-14 was 95% in these media. Rate constants for inactivation reactions were determined by linear least-squares computer fitting of semilogarithmic plots.

Results

Incorporation of ^{14}C-Labeled Alkyl Groups into Hexokinase by Reaction with [^{14}C]BAGA. The ^{14}C-labeled affinity reagent preparation used was shown to inhibit hexokinase B, at pH 8.5, 35 °C, irreversibly, with a second-order rate constant identical with that found (Otieno et al., 1975) for the original preparation of the unlabeled reagent.[2] In the course of this inactivation, a maximum of two alkyl groups were incorporated per 52 000 molecular weight subunit (Table I, expt i). Hydrolysis and analysis for labeled products of CM-amino acids (Goren et al., 1968) showed that all of the radioactivity was in [^{14}C]CM-cysteine. Techniques used to preserve and detect the products of [^{14}C]CM-methionine, if formed (see

[2] Data provided with this paper for the scrutiny of reviewers, and not included in the text but obtainable upon request from E. A. Barnard, included the kinetic plot for the [^{14}C]BAGA reaction that led to the rate constant mentioned for it (correlation coefficient 0.9997), a recording of the radioactivity in an ion-exchange column chromatogram of the hydrolyzate of hexokinase alkylated by [^{14}C]BAGA, showing the products of alkylation at cysteine residues (only), and the plot of migration positions in gel electrophoresis (with sodium dodecyl sulfate present) of the marker proteins and the products of the cleavage referred to in the text.

TABLE I: Groups Alkylated in the Reaction of Hexokinase B with [^{14}C]BAGA in Various Sequences of Alkylation.

Expt	Reagent sequence[a] Step 1	Step 2	[^{14}C]Alkyl Groups/Subunit Total[b]	CM-Cys (%)[c]	Recovery (%)
(i)	[^{14}C]BAGA				
(a)	1.5 h		1.91	100	*d*
(b)	2.0 h		1.98	100	*d*
(ii) (a)	BAGA +0.005 M Man, 1 h	[^{14}C]BAGA	0.94	100	97
(b)	BAGA +0.005 M Man, 12 h	[^{14}C]BAGA	0.98	100	94
(iii)	[^{14}C]BAGA +0.005 M Man, 12 h		1.0[e]	98	98

[a] BAGA and [^{14}C]BAGA were always at 0.003 M, pH 8.5, 35 °C, I = 0.1; Man = mannose. In step 2, the reaction was conducted until inactivation was complete. [b] Mean from at least two alkylations, determined after the final step. [c] The radioactive products in a hydrolyzate were analyzed by ion-exchange chromatography (see Materials and Methods); the percentage of the total radioactivity in the chromatogram that is contributed by [^{14}C]CM-cysteine residues is recorded. Also given (next column) is the radioactivity recovered in this chromatogram, as a fraction of that applied to the analyzer column. [d] Not determined. [e] Final enzymic activity unchanged from initial value.

TABLE II: Groups Alkylated in the Reaction of Hexokinase B with Iodoacetamide.

Expt	Reagent sequence[a] Step 1	Step 2	Act. (%)	[^{14}C]Alkyl groups per subunit Total	CM-cysteine (%)
(iv)	[^{14}C]IA +0.006 M Man, 6 h		100	3.0	100
	12 h		100	3.15	95[b]
	+0.007 M Man, 2 h		100	3.0	100
(v)	IA + 0.006 M Man, 6 h	[^{14}C]IA	0	1.02	100
(vi)	As in v	[^{14}C]BAGA	0	1.1	

[a] IA = iodoacetamide; used at 0.005 M, pH 8.5, 35 °C, I = 0.1. Other details, and ^{14}C recovery, as in Table I. [b] The remainder was [^{14}C]CM-methionine products.

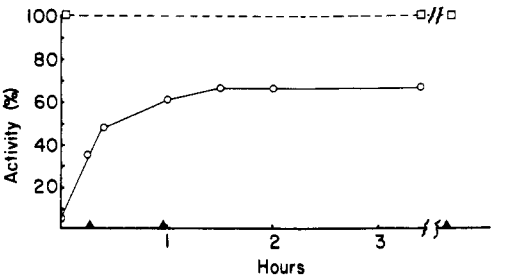

FIGURE 1: Removal of -CN from -SCN at the essential SH group of hexokinase by 0.025 M mercaptoethanol (in 0.05 M Tris-HCl, pH 8.6, 22 °C) as shown by restoration of activity (○) from cyanylated hexokinase. No effect was found on native hexokinase of 0.025 M mercaptoethanol (□). There was also no reversal by mercaptoethanol on hexokinase completely alkylated by BAGA (▲).

Materials and Methods), showed that no significant level of methionine reaction had occurred.

Further evidence that alkylation of methionine was not responsible for any of the loss in activity was obtained by treating the alkylated protein with mercaptoethanol (Figure 1, triangles). Naider and Bohak (1972) have shown for proteins and peptides alkylated at methionine residues (including the case of alkylation there by *N*-bromoacetyl-1-β-galactosamine, an isomer of BAGA), that mercaptoethanol (under the conditions used here) can bring about a quantitative removal of the alkyl group by thiolysis, with regeneration of the methionyl residue, and of the enzymic activity in cases where enzyme inactivation had initially ensued. It is seen here that mercaptoethanol, at concentrations up to 0.14 M and exposure for 1 day, brings about no reversal of the alkylation by BAGA of hexokinase.

In the conditions used in the reaction with [^{14}C]BAGA, 90–95% of the enzymic activity was always removed in 1.5 h. After 2 h, when two groups had been incorporated, no trace of activity remained. Hence, the observation made previously with the unlabeled alkylating agent that inactivation is concurrent with the alkylation of 2 cysteine residues per subunit (Otieno et al., 1975) was confirmed, and additional noninactivating reactions at other types of residue were excluded.

In another experiment, hexokinase B was reacted, instead, with unlabeled BAGA in the conditions described for [^{14}C]-BAGA but in the presence of a saturating level of mannose; no activity loss was now found up to 12 h. After the reagents were removed by gel filtration (see Materials and Methods), the activity still being fully present, the protein was realkylated with [^{14}C]BAGA in the standard conditions to inactivation. It was then found that only one alkyl group per mol of subunit was now introduced, again exclusively onto cysteine (Table I, expt ii). Consistent with this, it was shown that, in the first stage of this reaction (in the presence of mannose, for 1–12 h), 1 mol of cysteine per subunit becomes alkylated, exclusively (Table I, expt iii). Hence, the alkylation of the 2 reactive cysteines in the native enzyme can be separated into 2 stages, one alkylation leaving the enzyme fully active, and the other completely inactivating it.

Incorporation of [^{14}C]Labeled Alkyl Groups into Hexokinase by Reaction with [^{14}C]Iodoacetamide. Previously (Jones et al., 1975) it was shown that iodoacetamide, also, introduces 2 alkyl groups per subunit onto cysteine residues. Experiments using protection with mannose, as above, were performed now with [^{14}C]iodoacetamide as alkylating agent.[3] These showed (Table II, expt iv) that 3 cysteine residues (rather than 1 as with BAGA) account in their reaction for all of the alkylation in the enzyme–hexose complex. The fourth thiol can then be specifically labeled by a subsequent, inactivating reaction with iodoacetamide after the sugar is removed (expt v). The same was true when BAGA was used for the final step (expt vi). Hence, the first 3 alkyl groups introduced thus remove no activity, but the last one completely inactivates.

Enzymic Activity of Trialkylated Hexokinase. After reaction of hexokinase at 35 °C with iodoacetamide (0.005 M)

[3] The values of the rate constants for the inactivation reaction of hexokinase B by iodoacetamide in various ionic strength and buffer conditions at 25 and at 35 °C were provided with this paper and can be obtained on request,[2] as can the enzyme kinetic plots referred to in the text.

TABLE III: Hexokinase Inactivation and Glutathione Reaction Rates for Five Alkylating Agents.[a]

Reagent	Hexokinase[b] k_2 (L mol^{-1}) at 35 °C	Glutathione k_2 (L mol^{-1} min^{-1}) at 0 °C
Iodoacetamide	25.2	47.6[c,d]
Iodoacetate	6.0	9.5[c,e]
BAGA	10.0	39.7
N-Bromoacetyl-1-β-galactosamine	7.0	87.6
N-Bromoacetyl-2-glucosamine	0.35	35.7

[a] Second-order rate constants are given, at pH 8.5, $I = 0.1$. [b] All these values are obtained from the rates at 0.003 M reagent concentration. With BAGA, k_2 is a function of the reagent, due to its affinity behavior (Otieno et al., 1975). [c] This value approaches that for the hexokinase essential thiol still more closely when corrected for the somewhat different degrees of thiol ionization in the two cases: the corrected, pH-independent rate constant for hexokinase inactivation (Jones et al., 1975) is 70–80% of the corresponding constant for glutathione reaction. [d] This rate constant agrees exactly, when corrected for temperature and pH (Jones et al., 1975) with that obtained, by another method, for the same reaction with glutathione by Guidotti (1967). [e] See Jones et al. (1975). Note also (from Table III of those authors) that the comparisons in this table do not depend upon the choice of glutathione for a model thiol since similar values of alkylation rates were reported for glutathione and a number of other thiol-containing small molecules and peptides.

in the presence of saturating mannose, an active trialkylated derivative is formed, as seen in expt iv of Table II. This was isolated (at 7 mg/mL) by dialysis against 0.004 M glycylglycine–0.10 M NaCl, pH 8.5, and diluted into 0.02 M cacodylate, pH 7.0, for activity determination (at 25 °C). A Lineweaver–Burk plot was constructed using velocities measured on glucose in the range 0.0001 to 0.005 M (six concentrations); this was linear and coincided precisely with the line obtained for native hexokinase B in the same medium (see footnote 3). K_m was 0.0002 M in each case. Hence, the catalytic center of the enzyme appears unchanged when the three nonessential thiols are alkylated.

Relative Reactivities of Substrate-Related and Unrelated Alkylating Agents. A comparison was made of the inactivation reactions performed by (*N*-bromoacetyl)-2-D-galactosamine, its glucose analogue, and the 1-β isomer of the galactose derivative, as well as by iodoacetamide and iodoacetate. In their inactivations of hexokinase B (Table III), the glycosidic galactose reagent reacts more slowly than the 2-substituted galactose reagent, but still is much faster than the 2-substituted glucose reagent.

To interpret these differences, the chemical reactivity of the alkylating function should also be compared in this series. This was examined using the rate of reaction on glutathione in the same conditions, assaying the free glutathione with DTNB (Degani and Patchornik, 1974). The reactions were conducted to 70–90% completion and gave linear semilogarithmic second-order plots through this range. The rate constants obtained thus (Table III) showed that the sugar-containing bromo reagents are about as intrinsically reactive as iodoacetamide, or more so. The glucose compound does *not* react very slowly in the model reaction, as it does with the enzyme. The glycosidic reagent is intrinsically about twice as reactive as the others; corrected for this, BAGA is (at 3 mM concentration) about three times more reactive with hexokinase than is its glycosidic isomer.

A test was also made for an instantaneous, i.e., reversible, inhibition of hexokinase by BAGA. This possibility was examined at pH 7.0 (25 °C) using a low glucose concentration (5×10^{-4} M) to demonstrate more readily any inhibitory effect. At this pH, it was shown that the irreversible reaction of BAGA is slowed such that the assay can be completed without detectable irreversible inhibition by concentrations of BAGA up to 5×10^{-3} M. At pH 7, also, the K_m of glucose is close to (somewhat smaller than) that at pH 8.5 (0.17×10^{-3} M).[3] The enzymic activity was only slightly changed (up to 10% change at the maximum) by the addition at the moment of assay of BAGA up to 5×10^{-3} M concentration.

The result is not in good agreement with the value for K_I, the inhibition constant, for BAGA (i.e., for the reversible phase of its reaction) of 3.5×10^{-3} M, deduced from the kinetics of the inactivation treated graphically as an affinity reaction (Otieno et al., 1975), but is what would be predicted if the K_I of BAGA in the affinity reaction were close to, as expected, the K_I of *N*-acetylgalactosamine as a competitive enzymic inhibitor (14 mM in the same conditions: Otieno et al., 1975).[4]

The affinity of *N*-acetylglucosamine for pure hexokinase B was also measured, by the method used for *N*-acetylgalactosamine (Otieno et al., 1975). A K_I value (at pH 8.5, 25 °C) of 5.2×10^{-3} M was obtained.[3] This is threefold stronger than for *N*-acetylgalactosamine; in view of the results of Table III, we conclude that BAGA and its glucose analogue bind differently at the glucose site on the enzyme, the glucose-based reagent protecting the thiol as glucose itself does (Otieno et al., 1975).

Selective Cyanylation of Thiols in Hexokinase. The reagent NTCB has been shown (Degani and Patchornik, 1971; Jacobson et al., 1973) specifically to cyanylate free thiol groups in proteins, and to be of value in distinguishing truly essential thiols in various SH-dependent enzymes (Degani and Patchornik, 1974). We found that NTCB (at pH 8 or 8.5) readily inactivates hexokinase at 35 °C, and that the rate of this reaction parallels the production of the thionitrobenzoate anion from the reagent. At pH 8.0, the half-time of inactivation was 25 min with 3.1 mM NTCB. Mannose (5 mM) protected completely from the inactivation by NTCB, as it did for the alkylations; no activity was then lost after 2 h in the same treatment with NTCB at 35 °C.

When the enzyme was alkylated with BAGA for 12 h in the presence of saturating mannose (as in expt iii of Table I), without any inactivation occurring, and then was treated with NTCB as before at 35 °C, all activity was again removed.

A solution of the enzyme alkylated with BAGA under those conditions of protection by mannose was freed of the reagents and divided into halves. One-half (A) was reacted with NTCB to inactivation as just described. Both halves, after suitable dialysis, were treated with [^{14}C]BAGA until 95% inactivation of the still-active (B) sample occurred. Each was then separated from excess reagent by gel filtration (Figure 2). It can be seen that the usual labeling of one thiol occurs in the sample B. This reaction is totally prevented by the CN group present in the sample A. This indicates that the inactivation due to BAGA and that due to cyanylation occur at the same thiol. As

[4] While the plot obtained (Figure 1 of Otieno et al., 1975) from the hexokinase–BAGA reaction rates showed that the rate constant does vary with reagent concentration, as expected for an affinity reaction, the actual value of the reversible dissociation constant, K_I, obtained thus is now believed to require correction upwards. Childs and Bardsley (1975) have since pointed out for other cases that such an analysis of affinity labeling, based upon simple kinetic treatments of it (Kitz and Wilson, 1962; Main and Hastings, 1966), incurs systematic errors, and estimation of the true K_I is difficult.

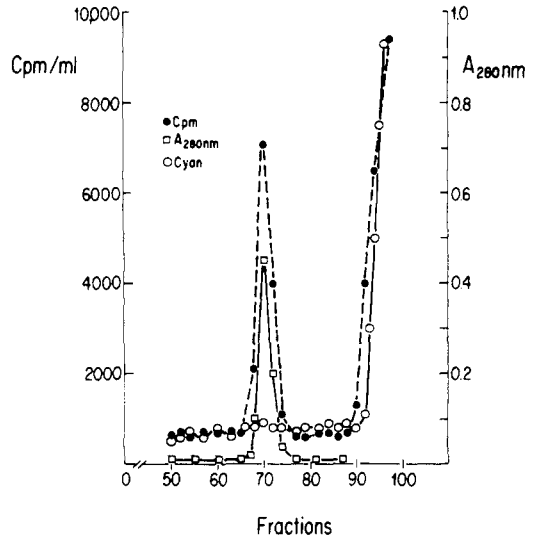

FIGURE 2: Separation on a Sephadex G-25 column of alkylated hexokinases from excess [^{14}C]BAGA. Sample A had been treated with BAGA in the presence of 0.005 M mannose, for 12 h at 35 °C, then with NTCB to inactivation, and then with [^{14}C]BAGA; its ^{14}C (○—○) and protein (□) contents were then measured. In a separate run on the same column, sample B, treated likewise with BAGA plus mannose, and then with [^{14}C]BAGA, was analyzed for ^{14}C (●—●) and for protein (coinciding with the protein peak of A). The cyanylation of the fourth thiol, in A, prevents the final labeling by [^{14}C]BAGA.

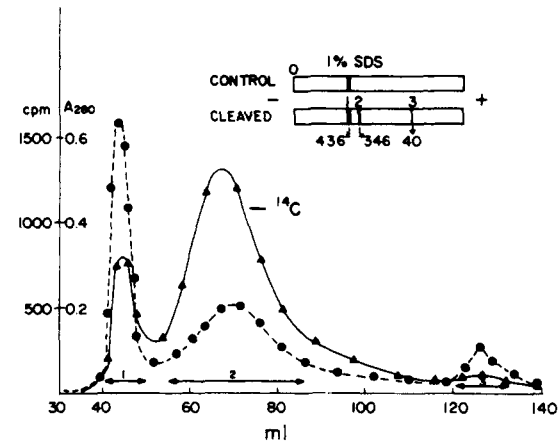

FIGURE 3: Gel filtration on a column of G-100 in 0.005 M Pipes buffer, pH 7.0 [80 × 1.5 cm, calibrated with markers (Rustum et al., 1971)], after cyanylation and cleavage of tri-[^{14}C]alkylated hexokinase. The protein or peptide concentration was measured by the absorbance at 280 nm (●), and the radioactivity due to the [^{14}C]carbamoylmethyl groups present by the cpm found in 100 μL aliquots (▲—▲). Peak 1 occupies the same position as does uncleaved trialkylated hexokinase run on this column. The peaks were pooled as indicated, for N-terminal analysis. Insert: Electrophoresis in polyacrylamide gel containing 1% sodium dodecyl sulfate (0 = origin). The upper gel shows the single stained band due to an uncleaved sample of trialkylated hexokinase. Position 1 is also the point where unmodified hexokinase B gives a single band in this system. The lower gel, run in parallel, shows the bands seen after the cyanylation and cleavage reactions. The numbers below it are the cpm measured after dissolving slices containing the stained bands. Elsewhere in this gel, the radioactivity in a slice of the same thickness was about 36 cpm. In a linear calibration plot using standard markers (see Materials and Methods), band 1 corresponded to molecular weight 52 000, band 2 to 40 000 and band 3 (a little ahead of the ribonuclease A marker) to 12 000 (all with the usual uncertainty limits of the method).

further evidence of this, 3 of 4 thiols in the hexokinase subunit were covered by carbamoylmethyl groups (see Table II, expt iv) and NTCB was again applied. The cyanylation of the last thiol again occurred with full inactivation.

Restoration of Activity by Free Thiol. The -SCN group in a protein can be cleaved by an excess of a thiol compound, to regenerate the -SH group, as noted by Vanaman and Stark (1970) in the case of the catalytic subunit of aspartate transcarbamoylase if 6 M Gdn·HCl were present. In the case of the active site of hexokinase, we found that when cyanylated this -SCN group is available to mercaptoethanol in water, where it becomes deblocked to a large extent (Figure 1, circles). This reversal occurs in the same way whether the other thiols are previously alkylated or are free. The less-than-quantitative restoration seen is attributed to a side reaction, namely, cleavage of the adjacent peptide bond (see below), which can occur during the long incubations involved in the NTCB and mercaptoethanol treatments.

Cleavage of the Polypeptide Chain. Cyanylated polypeptides can undergo an intramolecular cleavage reaction at the amino peptide bond adjacent to the CN-cysteinyl residue, upon incubation at 37 °C in slightly alkaline medium (Jacobson et al., 1973; Degani and Patchornik, 1974). If a unique thiol per monomeric molecule of hexokinase is cyanylated in all of the population, this cleavage should proceed to yield, finally, only two types of fragment. Accordingly, hexokinase B was alkylated in the presence of saturating mannose, as above, using [^{14}C]iodoacetamide, to label 3 cysteine residues per monomer; it was then, in the absence of mannose, cyanylated to inactivation at pH 8.5, and further incubated at 37 °C in 4 M Gdn·HCl, pH 9.0, 18 h.

The cleaved products were separated by gel filtration on a calibrated column (Figure 3). The first peak to emerge coincided with the elution position of the intact, trialkylated hexokinase. It carried radioactivity corresponding, in relation to the protein present, to the same degree of labeling as the starting material here. It was material not cleaved in the present reaction. The observation that only about one-half of the total protein undergoes cleavage was made in each of three similar experiments that were performed, and was attributed to inefficiency of the cleavage reaction in the whole protein. However, after the labeling experiments were concluded, it was found that this partial cleavage is due to the freeze-drying stage employed for concentration of the protein just prior to the cyanylation; some air oxidation of the remaining thiol in the trialkylated protein then occurs, preventing the cyanylation reaction in the fraction oxidized. If freeze-drying of the protein is avoided, the yield of the cyanylation and cleavage reactions, if conducted in Gdn·HCl medium, can become essentially complete.

The new products separated after the cleavage reaction (Figure 3) were in a broad peak centered at a position corresponding to about 40 000 weight for a globular protein, and a small peak of much lower molecular weight, at a position compatible with the 12 000 weight that would be indicated by subtraction from the native monomeric weight of 52 000. The 40 000 peak was well labeled, and the small-size fragment appeared to be unlabeled. Since the former peak was broad, and the latter had a low content of aromatic residues for quantitation, the relative labeling of these was further studied after they were separated electrophoretically. In gel electrophoresis with sodium dodecyl sulfate present, it was confirmed that trialkylated hexokinase gave a single band by both protein staining and radioactivity localization, in the same position as the hexokinase B, which is monomeric (52 000 weight) in this system (Figure 3, insert). After the ^{14}C-labeled alkylated hexokinase had been cyanylated and cleaved, only two new bands appeared, at positions that corresponded to 40 000 and about 12 000 weight. The larger fragment carried all of the

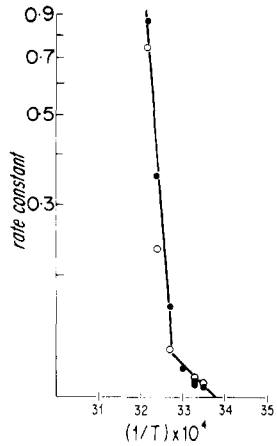

FIGURE 4: Arrhenius plot for the inactivation by BAGA of hexokinase, at pH 8.5. The open and closed circles are from experiments on two different samples of the enzyme. The ordinate is the observed first-order rate constant (min^{-1}) with 3 mM BAGA, plotted logarithmically.

radioactivity, the smaller one being now seen to be definitely unlabeled (Figure 3, insert). This experiment was repeated, but with the detergent absent prior to and during the electrophoresis of the products (not illustrated). Again the original single band became accompanied by only two other bands after the cleavage reaction; these were not characterized, but both the new products were more anionic (at pH 8.6) than the starting material.

The peak III containing the small fragment (Figure 3) was subjected to N-terminal analysis by the dansyl method (Hartley, 1970) and yielded no N-terminal dansyl derivative whatsoever. Cleavage after cyanylation produces a blocked α-NH_2 group due to ring closure (Jacobson et al., 1973). As a check, peaks I and II in equivalent amount yielded a single spot in each of the thin-layer chromatography systems of Hartley (1970), corresponding to N-terminal valine, as confirmed by running with that marker. This is the N-terminal residue of native hexokinase. Hence, the 12 000 fragment is the C-terminal end of the chain, and all of the evidence is consistent in showing that the site of cyanylation is a single cysteine residue which is the last of the 4 in the chain: it is, in fact, at about 80% of the length from the N terminus.

Effect of Temperature on the Behavior of Hexokinase. It was previously noted (Lazarus et al., 1968) that the inactivation of hexokinase B by iodoacetate or by mercurial reagents was very slow below about 30 °C, and fast at 37 °C, suggesting that a transition occurs in the structure in this range. This was further explored here, measuring the rate constant for the inactivation by BAGA at temperatures up to 39 °C. From the Arrhenius plot (Figure 4) obtained, it is seen that a sharp break occurs at about 31 °C (at pH 8.5, $I = 0.1$). The values of ΔH^*, the heat of activation for this reaction, derived from this plot are 4900 cal below 31 °C, and 60 500 cal above 31 °C. The latter very high value, and the sharp transition, indicate that a major structural change occurs to expose this thiol.

With iodoacetamide as inactivator, likewise, the rate below about 30 °C was extremely slow and was fast at 35 °C: the rate constant was 100–170 times greater at 35 °C than at 25 °C (at pH 8.0, $I = 0.03$–0.13). None of these effects is due to spontaneous thermal inactivation of the enzyme since pure hexokinase B could be incubated at 35 °C in the same media without alkylating agent for 48 h with no loss of activity, in contrast to the period, about 1 h, sufficient for complete inactivation by these reagent solutions. Increasing ionic strength in the range 0.01–0.2 accelerates greatly the iodoacetamide inactivation at 35 °C, and retards it from about $I = 0.6$ to 2 (Jones et al., 1975). These effects are not seen, however, at 25 °C, where the rate remained as low as noted above at all I, showing only the slight, regular increase with ionic strength characteristic of the reaction of this uncharged reagent with the model thiol of glutathione (Guidotti, 1967). The same result, at 25 °C, was obtained in Tris–NaCl, glycine–NaCl, or sodium phosphate buffers.[3]

Likewise, the total of the alkyl groups incorporated into hexokinase in the [^{14}C]iodoacetamide reaction was considerably greater at 35 °C than at 25 °C. Thus, 0.62 alkylated cysteine residue per subunit was found after reaction for 2 h at 25 °C, but 2.48 at 35 °C. The 25 °C reaction was accompanied by no significant inactivation, and the 35 °C reaction by complete inactivation. Hence, the essential thiol *and* a second (nonessential) thiol become unmasked at 35 °C.

A comparable phenomenon was found with NTCB as the reagent for hexokinase thiols. At 35 °C, pH 8.0 (0.05 M Tris-HCl), 3.1 mM NTCB inactivated hexokinase relatively rapidly, the half-time being 25 min, as noted above. At 25 °C in the same medium, no inactivation was detectable over 6 h. It was again found that temperatures above about 30 °C were necessary for readily detectable rates to be obtained.

Discussion

Based upon the evidence so far available on the thiols of yeast hexokinase (Lazarus et al., 1968; Jones et al., 1975; Otieno et al., 1975, and the present results), we can summarize the position as follows. There are 4 cysteine residues per identical monomer of about 52 000 weight, all present in the free thiol form. We can number these, arbitrarily, I, II, III, and IV. Thiol I is essential for activity: even the introduction of the very small, uncharged -CN group inactivates. In several other apparently thiol-dependent enzymes chemically modified at their thiols, the -SCN derivative, alone, retained activity (Degani and Patchornik, 1974), so that this modification can be of diagnostic value for nonessential thiols. The results of the cleavage, also, show that the inactivating cyanylation reaction occurs at a unique cysteine in each molecule. Bound substrates, both of the nucleotide and the hexose classes, protect thiol I, but none of the other thiols. In view of all of these phenomena and, especially, the fact that thiol I undergoes the affinity labeling with BAGA, we conclude that this thiol is close to the active center, but need not be directly *in* it. Thiol I should be near enough in the structure to the glucose-binding site for the alkylating chain attached to C-2 of the bound sugar to attack it, in the perhaps slightly different orientation in binding of C-2-substituted galactose (Otieno et al., 1975), and requiring the flexibility in the protein introduced thermally above 31 °C for this access. This cysteine is at about 80% of the length along the chain from the N terminus, and thiols II, III, and IV all precede it in the sequence.

Thiol I has, above the transition temperature (31 °C at pH 8.5), a reactivity to electrophilic substitution approaching that of the free thiol of glutathione (Table III). It is not yet known if the glycosidic galactose reagent also receives some binding assistance in its reaction at this thiol; that reagent is (Table III) only three- to four-fold less reactive at thiol I than is BAGA. It appears that the intrinsic reactivity of BAGA will account for much of its reaction rate at thiol I, but the affinity, although weak, nevertheless gives some enhancement. Below 31 °C, where this thiol is almost inaccessible, the energy of activation for its reaction with BAGA is very low, 40% of that (Guidotti, 1967) for the model thiol of glutathione reacting with iodoacetamide. Above the transition, ΔH^* is, in contrast, in the range for protein unfoldings. Hence a great loosening of this

part of the protein structure occurs in this transition at 31 °C.

Thiol II is highly reactive when thiol I has been alkylated. It reacts, therefore, with the affinity reagent (but in, it must be presumed, a normal bimolecular reaction), or with iodoacetamide or methylmercury, during the phase of inactivation at thiol I by one of these reagents. The present evidence now permits us to say that it is nonessential. It is not in the vicinity of either of the cosubstrate binding sites since it is not protected thereby and can, by a suitable strategy, be blocked without any effect on the enzyme activity. Thiol II is very reactive to BAGA in the enzyme–hexose complex, its complete reaction occurring then in 1 h (expt ii of Table I), extremely selectively (expt iii). The reaction of II is almost as fast when BAGA is at the active center thiol (expt i). The region of the molecule carrying thiol II is subject to the same transition as that which exposes thiol I. Since cysteine II can be labeled exclusively (expt iii), its position in the polypeptide chain will also be determinable, by strategies based upon selective cyanylation and cleavage at other thiols.

Thiols III and IV are also nonessential and must be well outside the binding sites. They are relatively inaccessible: even at 35 °C in the free enzyme, iodoacetamide reacts with them at only a very slow rate, and BAGA does not react with them appreciably. This shows that the transition noted above is not a general unfolding of the protein. In the enzyme–hexose complex, they both become distinctly more reactive to iodoacetamide, evidence of a far-ranging conformational change occurring in the formation of this complex. The change does not, however, render them (expt iii) reactive to BAGA: in simple bimolecular reactions, this is about as good an alkylator (Table III), so we presume this effect arises from the greater bulk of BAGA, i.e., iodacetamide penetrates much better to their shielded locations. They are, on the other hand, reactive in the free enzyme, or in the enzyme–substrate complex, toward methylmercuric iodide (Lazarus et al., 1968), a poorly dissociated molecule which may penetrate to hydrophobic regions containing these thiols.

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References

Anderson, L. E., and McClure, W. O. (1973), *Anal. Biochem. 51,* 173.

Childs, R. E., and Bardsley, W. G. (1975), *J. Theor. Biol. 53,* 381.

Degani, Y., and Patchornik, A. (1971), *J. Org. Chem. 36,* 2727.

Degani, Y., and Patchornik, A. (1974), *Biochemistry 13,* 1.

Goren, H. J., and Barnard, E. A. (1970), *Biochemistry 9,* 959.

Goren, H. J., Glick, D. M., and Barnard, E. A. (1968), *Arch. Biochem. Biophys. 126,* 607.

Guidotti, G. (1967), *J. Biol. Chem. 242,* 3673.

Hartley, B. S. (1970), *Biochem. J. 119,* 805.

Jacobson, G. A., Schaffer, M. H., Stark, G. R., and Vanaman, T. C. (1973), *J. Biol. Chem. 248,* 6583.

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

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

Lazarus, N. R., Derechin, M., and Barnard, E. A. (1968), *Biochemistry 7,* 2390.

Main, A. R., and Hastings, F. L. (1966), *Science 154,* 400.

Naider, F., and Bohak, Z. (1972), *Biochemistry 11,* 3208.

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

Ramel, A. H., Rustum, Y. M., Jones, J. G., and Barnard, E. A. (1971), *Biochemistry 10,* 3499.

Reisner, A. H., Nemes, P., and Bucholtz, C. (1975), *Anal. Biochem. 64,* 509.

Rustum, Y. M., Massaro, E. J., and Barnard, E. A. (1971), *Biochemistry 10,* 3509.

Sharma, C., Manjeshwar, R., and Weinhouse, S. (1963), *J. Biol. Chem. 238,* 3840.

Thomas, E. W. (1970), *J. Med. Chem. 14,* 755.

Vanaman, T. C., and Stark, G. R. (1970), *J. Biol. Chem. 245,* 3565.