Yeast Hexokinase. III. Sulfhydryl Groups and Protein Dissociation*

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ABSTRACT: The two enzyme species, hexokinases A and B, isolated from yeast in homogeneous form, have been analyzed by several methods for their SH group content. Each contains eight SH groups per molecule (102,000 molecular weight) and no disulfides. Four of these thiols react readily with methylmercuric iodide, without enzyme inactivation. Only after that reaction do the other four per molecule become reactive to this, and to other, thiol reagents.

The reaction of this latter four SH groups inactivates the enzyme. Sedimentation equilibrium measurements show that when reaction with mercurials occurs, the protein becomes extensively dissociated. The smaller units formed were not characterized, due to their tendency to form new, large aggregates. It is suggested, however, on the basis of the combined activity and dissociation evidence, that, firstly, mercurial mercaptide formation at the four more reactive thiols produces chiefly half-molecules which are enzymically active; and, secondly, that when the remaining, previously shielded, four thiols also become reacted, further dissociation mainly occurs to give inactive subunits, thought to be quarter-molecules. Some effects of medium composition, temperature, and reagent type on SH reactions of hexokinase have been found to be consistent with the suggestions made.

In parts I and II of this series (Lazarus *et al.*, 1966; Derechin *et al.*, 1966) there were described two forms of hexokinase, A and B (the latter of threefold higher activity), that can be obtained from baker's yeast by a new isolation procedure, apparently in undegraded form. These enzymes were each homogeneous and stable at room temperature. We present here evidence on their content of sulfhydryl groups, and the relations of these groups to the activity of these two enzymes.

While yeast hexokinase, like other kinases, has long been suspected to be a thiol-dependent enzyme, the evidence reported on previous preparations of this enzyme on the presence of and requirement for these groups has been far from consistent. Dixon and Needham (1946) noted that cysteine stabilized their preparation, while Bailey and Webb (1948) showed that a number of thiol-reactive compounds produced inactivation. Other investigators (Berger *et al.*, 1946; Fasella and Hammes, 1963; Kaji, 1966) have concluded (from mercaptide formation experiments) that the thiol groups are not essential for hexokinase activity. Barnard and Ramel (1962), using hexokinase prepared by a modification (Ramel, 1964) of the Darrow and Colowick (1962) procedure, showed that carboxymethylation of at least one, and at most four, SH per unit of 50,000 molecular weight, led to the loss of enzymic activity. This same preparation, which was homogeneous and was stable under the conditions of treatment, was shown to contain eight SH per molecule of about 96,000 weight. Different values for this total thiol content have been reported on hexokinase isolated by the unmodified Darrow and Colowick (1962) procedure: 6 SH (Fasella and Hammes, 1963; Kaji, 1966) or 7.8 SH (Kenkare and Colowick, 1965) per 96,000. The preparation used in these latter three studies has been shown (Trayser and Colowick, 1961; Kaji *et al.*, 1962) to be heterogeneous, due to proteolytic contamination (*cf.* also Lazarus *et al.*, 1966), and it seems likely that partial degradation in the earlier preparations accounts for the multiplicity of findings.

Experimental Section

Materials. All chemicals unless otherwise specified were Fisher Certified reagent grade. Hydrochloric acid for protein hydrolyses was metal-free Ultra-Pure grade (E. Merck, Darmstadt, Germany), obtained from Brinkmann Instruments, New York. Methylmercuric iodide was from K & K Laboratories Inc., and PMB[1] and reduced GSH from Sigma Chemical Co. Iodoacetic acid (Eastman) was purified as described below. Hexokinases A and B were prepared by the method of Lazarus *et al.* (1966): only rechromatographed specimens were used, with specific activities at 25° of 200–210 units/mg in the case of hexokinase A and 650 units/mg or higher in the case of hexokinase B. The enzymes were concentrated on a small column of DEAE-cellulose (Lazarus

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[1] Abbreviations used in this work that are not listed in *Biochemistry 5*, 1445 (1966), are: PMB, *p*-mercuribenzoate; DMF, dimethylformamide.

et al., 1966) and dialyzed against 5 mM sodium succinate (pH 6.0) at 4°, prior to use. Other materials were as specified by Lazarus *et al.* (1966).

Methods and Procedures. All reactions were carried out in 2-ml glass tubes, with temperature control (by circulation through glass jackets) to ±0.1°. The pH adjustments and control were by a Radiometer TTTlC titrator, with appropriate temperature compensation applied to the electrode.

All spectrophotometric studies were performed on a Cary 15 instrument with matched cells (1 cm) used in thermostatted cell holders. Hexokinase assays were as described by Lazarus *et al.* (1966), as were the determinations of protein content, and any other methods not specified. The arbitrary activity unit used in Figures 2–7 represents an initial rate of about 0.25 μmole of substrate reacted/min at 25°.

Titration with CH_3HgI. This was a modification of the procedure of Edelhoch *et al.* (1953). CH_3HgI solutions in DMF were standardized against GSH, using a stock solution (always freshly prepared and kept at 4°) containing 61.4 mg of GSH in 20 ml of degassed water. Aliquots were titrated at 2°, pH 8.5, with CH_3HgI, in the presence of 0.05 ml of 10% sodium nitroprusside as indicator. CH_3HgI was always added from an Agla micrometer syringe (Burroughs-Wellcome, England) calibrated to 0.2-μl intervals. For titrations of total SH groups, 0.5–2.0 mg of protein was used in 0.5 ml of 5 mM succinate solution, at 2°. Solid guanidine hydrochloride (600 mg) was added, with stirring, to give a final volume of 1 ml and a concentration of 6.0 M. The pH was adjusted with the titrator to 8.5 by the addition of 0.01 N NaOH, and titration made as noted above. In all CH_3HgI titrations, magnetic stirring was maintained throughout, under a nitrogen blanket. The visual end point of titration is extremely reproducible in these conditions.

Activity titrations of native hexokinase A were carried out routinely at 25°, while those of hexokinase B were at 35°, always in a thermostatted reaction vessel on the titrator. To the protein (0.5–1 mg) in 5 mM succinate (pH 6.0) buffer (about 0.6 ml) 0.01 N NaOH and water were added to pH 8.0 and total volume 1 ml. Accurately known aliquots of CH_3HgI were added, as above, at 10-min intervals. Subsequent calibration of the CH_3HgI solution used by titration with the standard GSH solution (as above) was made, showing that each addition was in most experiments 0.93, and in the rest 1.00, equivalent of mercurial per mole of protein present. The enzymic activity was assayed immediately after each addition of reagent. Control incubations were performed with the mercurial omitted: addition of the same volumes of its vehicle (DMF) at the same intervals produced no detectable effect on the activity of the enzyme.

Titration with PMB. The procedure used was based on that of Boyer (1954) and Benesch and Benesch (1962), modified as follows. For total SH group titration, 6 M guanidinium chloride was used. The PMB solution used was standardized by titration against GSH (prepared as noted above, but in 6 M guanidine hydrochloride) at 2°, when it was shown that the method was equally applicable in this medium. To an exactly known amount of hexokinase in solution in 5 mM succinate (pH 6.0) was added 600 mg of solid guanidine hydrochloride, and the succinate medium to 1 ml. This solution was titrated at 250 mμ with the PMB solution. The reference cell contained the same guanidine hydrochloride medium without protein. Aliquots of PMB were added to both cells. The reaction was considered complete when further aliquots of PMB failed to produce an absorbance increment (Figure 1).

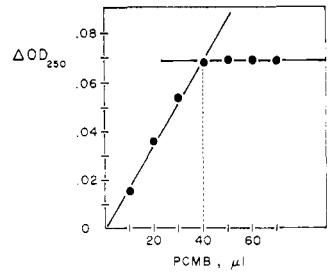

FIGURE 1: Spectrophotometric titration of hexokinase B (0.25×10^{-1} mole) (5.15×10^{-4} M) in 6 M guanidinium chloride, 2°. Absorbance difference values (ΔOD_{250}) were read on 0–0.1 slide-wire of Cary 15 spectrophotometer.

Amino Acid Analysis for Cysteic Acid. Oxidation of SH followed the method of Hirs (1956). Known amounts of hexokinase in 5 mM succinate were placed directly in hydrolysis tubes and dried over P_2O_5 *in vacuo*. The oxidations were performed in these tubes at −8° for the periods stated (trials having shown that the reaction on hexokinase at −12° was too slow for convenience). After the performic acid treatment, the reagents were removed by lyophilization and the samples were hydrolyzed in 6 N HCl at 110°, 20 hr. The HCl solutions were first deaerated by bubbling washed nitrogen through them, followed by degassing at an oil pump, and sealing under vacuum. Amino acid analysis was performed on the Beckman–Spinco (Model 120B) analyzer using the procedures of Spackman *et al.* (1958), but with the 60-cm column, 52°, procedure. After analysis, the cysteic acid peak (appearing well before aspartic acid) was integrated and converted into micromoles of cysteic acid using a calibration factor determined from adjacent analysis of a standard amount of pure L-cysteic acid (Calbiochem). The number of micromoles of protein associated with the sample analyzed was determined from the aspartic acid content, as measured on the chromatogram, using calibration values of this content from other analyses of accurately known amounts of native hexokinase.

Carboxymethylation of Hexokinase. Iodoacetic acid was recrystallized from the ligroin–diethyl ether mixture and dissolved in 0.2 M sodium acetate (pH 4.03) to give 0.62 M reagent. This (5 μl) was added to water (150 μl) in a 2-ml reaction vessel, thermostatted at 35°, and protected from light. The pH was maintained at 8.0 while adding 1 mg of protein, and the final volume was brought with water to 1 ml. The concentration of iodoacetate represents a 300–400-fold excess over that of the enzyme. Hexokinase activity was measured on aliquots at fixed intervals. For final analysis, the reaction mixture was transferred to a Diaflo ultrafilter (Amicon

TABLE I: Sulfhydryl Groups per Molecule Determined by CH_3HgI Titration in 6 M Guanidine Hydrochloride at 2°.

Enzyme	Amt of Protein		CH_3HgI Required		SH/Molecule[a]
	mg	Moles	μl	Moles	
Hexokinase A	1.43	1.40×10^{-8}	52	11.40×10^{-8}	8.1
	0.68	0.67×10^{-8}	24	5.28×10^{-8}	7.9
Hexokinase B	1.05	1.03×10^{-8}	38	8.10×10^{-8}	7.9

[a] Molecular weight 102,000.

Corp., Cambridge, Mass), type UM 1 with attached syringe unit, and washed with water until no more material absorbing at 265 mμ could be detected in the effluent. The protein was flushed from the ultrafilter with water quantitatively into a hydrolysis tube, where it was freeze dried and hydrolyzed (as above). Aliquots were taken for amino acid analysis. Amounts of protein were determined by reference to the aspartic acid content as in the cysteic acid determination. A pure sample (Calbiochem) of *S*-CM-cysteine (which emerged just before, and resolved from, aspartic acid) was used (in an adjacent analysis) for color value calibration of this peak in the protein analyses. To correct for any effects of the presence of mercury in the hydrolysis (in the CH_3HgI-treated samples), a known amount of cysteine was allowed to react with a deficiency of CH_3HgI, and the product was added to a standard solution of *S*-CM-cysteine (at concentrations of the latter and the mercaptide similar to those found in the experimental samples): this mixture was subjected to the usual hydrolysis conditions in 6 N HCl. Subsequent analyses showed that no reduction in the amount of *S*-CM-cysteine occurs in this treatment.

Sedimentation Measurements. These were made in a Spinco Model E ultracentrifuge equipped with a phase plate as a schlieren diaphragm. Double-sector cells (12-mm optical path) were used routinely for velocity experiments. Unless otherwise stated, all experiments were at known temperatures in the range 4–7°. Sapphire windows were used in experiments at higher speeds and quartz windows at lower speeds. The plates were analyzed using a Nikon Model 6 microcomparator, which we have equipped with 6-in. diameter micrometer drums (Shardlow Micrometer drums, Shardlow Micrometers, Ltd., Sheffield 4, England) giving readings at 2 μ on each division.

The sedimentation equilibrium experiments were made using 3-mm solution columns. In most experiments interference optics were used and the changes in concentration throughout the cell were followed by a procedure[2] that involves interposing a fine wire mesh in front of the photographic plate and taking photographs at suitably short intervals throughout most of the experiment. Thus, the fringe displacement could be followed from the start of the experiment and the analysis of the plot at equilibrium could be made with precision. Molecular weight determination by this method was considered necessary in view of the tendency of the protein to form aggregates.

The molecular weight was determined by making use of Svedberg's equation (with the usual symbolism)

$$M = \frac{RT}{(1 - \bar{v}\rho)\omega^2} \frac{\mathrm{d} \ln c}{\mathrm{d}r^2}$$

Other experiments used schlieren optics, and molecular weights were then obtained from graphs of $(1/r)(dc/dr)$ *vs.* c (Van Holde and Baldwin, 1958), where c is the protein concentration (in arbitrary units, obtained by integration of the area between the concentration gradient curve and the base line) and r is the distance from the center of rotation.

Densities of solutions employed were determined by pycnometry, in a bath maintained at 20 ± 0.01°. Protein concentrations were determined in the ultracentrifuge making use of a standard double-sector synthetic boundary cell (capillary type); $\bar{v}$ was assumed to be 0.740.

Unless otherwise specified, in all buffers the named reagents were at concentrations such as to contribute a total of 0.02 to the ionic strength (I), with NaCl added to give the total ionic strength quoted.

Results

Molecular Weights of Hexokinases A and B. The molecular weight of native hexokinase A was determined at pH 5.5 (sodium acetate buffer, at 2–5-mg/ml initial protein concentration) by sedimentation equilibrium measurements (using the method of Van Holde and Baldwin (1958)) and gave a range of values from 102,000 to 105,000. The mean of five determinations was 104,000. No evidence of heterogeneity was detected in the plots. The previous corresponding determination (Derechin *et al.*, 1966) of the molecular weight of hexokinase B was reexamined after finding a small error in the original computation (that gave the value of 99,000) and now gives M_z = 102,000. This is now taken as the molecular weight of hexokinase B. Since the difference in the two values (104,000 and 102,000) for A and B may not be significant, we have taken both molecular weights to be 102,000, when determining equivalents in the SH measurements.

[2] M. Derechin, manuscript in preparation.

TABLE II: Spectrophotometric Titration of Hexokinase B (0.25×10^{-8} mole) with PMB in 6 M Guanidine Hydrochloride.

Sample	PMB Required		SH Groups/ Molecule[a]
	μl	Moles	
1	37.0	1.90×10^{-8}	7.6
2	38.0	1.96×10^{-8}	7.8
3	40.0	2.06×10^{-8}	8.2

[a] Molecular weight 102,000.

Total Number of Sulfhydryl Groups per Molecule. Pure specimens of hexokinases A and B, rechromatographed on TEAE-cellulose, were used to determine the thiol contents by each of four different methods. Each hexokinase in 6 M guanidine hydrochloride solution (pH 8.5) at 2° showed, on addition of sodium nitroprusside, the presence of a considerable quantity of free SH, whose number was determined by titration with CH_3HgI. The results (Table I) showed that eight SH groups per molecule of 102,000 weight became available in this medium, in both the A and B enzymes.

Secondly, a version of the PMB spectrophotometric titration of Boyer (1954) was used. This was applied in 6 M guanidinium chloride. The titration at 250 mμ proceeded satisfactorily in this medium, and hexokinase B was shown thus to present eight SH groups for mercaptide formation, with a sharp break at this end point (Table II; Figure 1).

Thirdly, hexokinase B was reacted at 35°, pH 0.8, with iodoacetate, when inactivation occurred (Figure 2). After full inactivation, the protein was freed from reagent by an ultrafiltration method, hydrolyzed, and the content of *S*-carboxymethylcysteine was determined on the amino acid analyzer. Again, eight thiol groups were shown to be available in these conditions (Table III, first experiment).

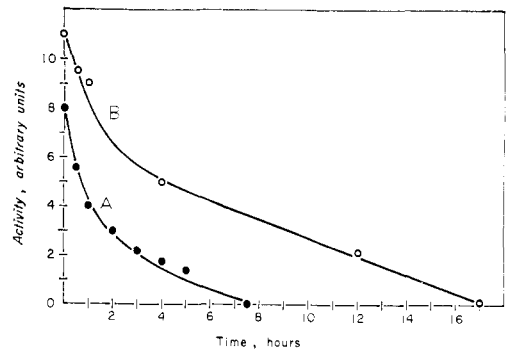

FIGURE 2: Inactivation of hexokinases A (●) and B (○) at 1 mg/ml, by 3 mM iodoacetate at pH 8.0, 35°, I = 0.015.

Finally, hexokinases A and B were oxidized with performic acid and the amounts of cysteic acid generated thus were determined in hydrolysates, on the amino acid analyzer. For hexokinase A, it was also shown that an increased period of oxidation did not produce a greater cysteic acid content. It was thus found (Table IV) that the total of cysteine and half-cystine residues was, in each protein, eight per molecule. Taken with the other results, this indicates that disulfide bridges are absent.

Activity Loss of Hexokinase B on Titration with CH_3HgI. At 35°, pH 8.0 (Figure 3), there is negligible loss of activity after the addition of 3.7 equiv of CH_3HgI. In a number of similar experiments, the greatest decrease in activity ever observed at about 4 equiv was 15%. There is, however, a marked drop in activity with the addition of 4.7 equiv of the mercurial, and complete loss of activity at about 8 equiv. The subsequent addition of excess cysteine restored the activity to control levels. Titrations at ionic strength changed from 0.015 to 0.10 by NaCl addition gave quite similar behavior, although with appreciable (23%) loss of activity at 3.7 equiv (Figure 3). On change to glycine buffer (at I = 0.10), however, inactivation began with the first

TABLE III: Reaction with Iodoacetate of Hexokinase B and of a Mercaptide Derivative.

Preparation	Protein (mmoles)	*S*-CM-cysteine (mmoles)	SH Groups Alkylated/ Molecule[b]
Hexokinase B after 100% inactivation by 3 mM iodoacetate	2.16	16.4	7.8
Hexokinase B treated[a] with 4 equiv of CH_3H_gI, and then 100% inactivated by 3 mM iodoacetate	6.50	26.4	4.1

[a] After the CH_3H_gI treatment, this sample showed 90% of the original enzymic activity remained. [b] Molecular weight 102,000.

TABLE IV: Total Cysteinyl Residues per Molecule by Cysteic Acid Determination.

Enzyme	Oxidation Period (at −8°) (hr)	Moles of Cysteic Acid/ Molecule[a]
Hexokinase A	3	7.5
	5	7.6
Hexokinase B	3	7.8

[a] Molecular weight 102,000.

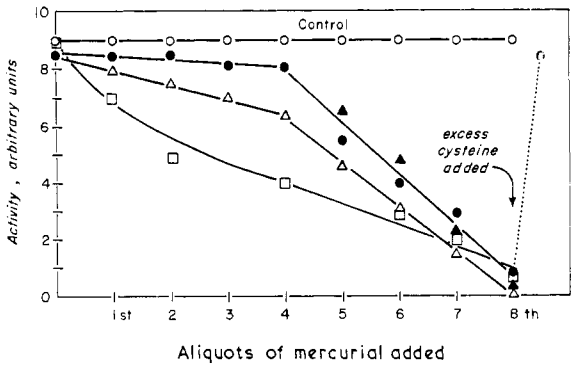

FIGURE 3: Activity titration of hexokinase B with 2.2×10^{-3} M CH_3HgI at 35°, pH 8.0. Activity was measured at 25°. Each aliquot is 0.93 mole of mercurial/mole of protein present; these were added at successive 10-min intervals. (O) Control (no mercurial added) measured at the same intervals. Activity was measured after addition of CH_3HgI aliquots, at (●—●) I = 0.015 (succinate–NaOH) or at (△—△) I = 0.10 (same medium with NaCl addition) or at (□—□) I = 0.10 (0.02 M glycine–NaOH–NaCl). (▲) Addition of successive aliquots of PMB after the fourth aliquot of CH_3HgI was added (at I = 0.015). Where shown by the dotted line, a large excess of cysteine was added to the sample (at I = 0.015) after the eight aliquot of CH_3HgI had been added.

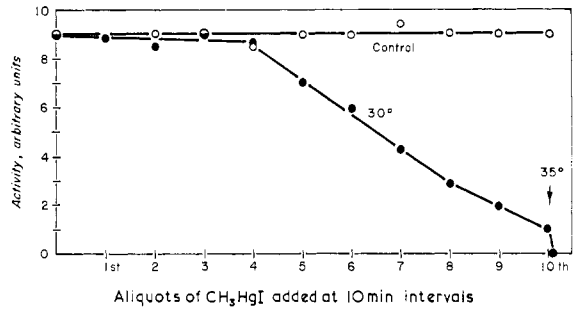

FIGURE 4: Activity titration (●) with CH_3HgI (details as in Figure 3) of hexokinase B at 30°, I = 0.015, pH 8.0. After the tenth addition of mercurial (9.3 moles/mole of protein), the temperature was raised to 35°; spontaneous loss of the residual enzymic activity then occurs.

equivalent and proceeded progressively to near completion at about 8 equiv (Figure 3).

Titration could not be performed above 40°, due to instability of the protein there.[3] At lower temperatures, *e.g.*, 30° (Figure 4), there is the same lack of effect of 3.7 equiv of CH_3HgI but the loss of activity on exceeding 4 equiv is not as great as at 35°. Even after 9.3 equiv at 30° there is still some retention of activity. When the temperature of this sample was raised to 35°, there was an immediate and complete loss of enzymic activity (Figure 4).

[3] Hexokinase B is completely stable at 35° at pH 8.0, as can be seen from the control curves in Figures 3 and 4; in fact, this preparation has been found in this laboratory to lose no significant activity up to 2 days in these conditions. At 45° (at pH 8.0), however, spontaneous inactivation occurs and is essentially complete in about 90 min. For this reason, no experiments were performed above 35°. A thermal transition appears to occur near 40°.

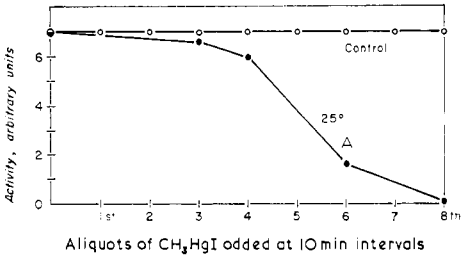

FIGURE 5: Activity (●) after treatment with CH_3HgI (details as in Figure 3) of hexokinase A at 25°, I = 0.015, pH 8.0.

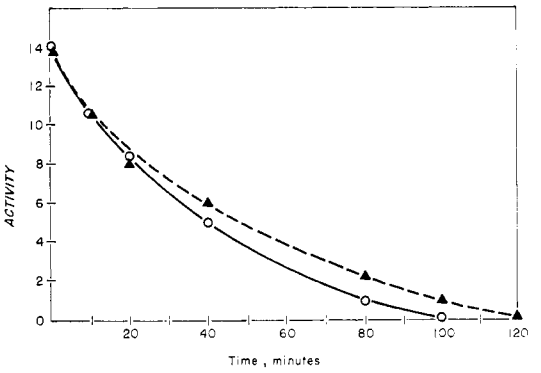

FIGURE 6: Inactivation of hexokinase B (1 mg/ml) by 5 mM iodoacetate (▲) or by 5 mM iodoacetamide (O) at pH 8.0, 35°, I = 0.10 (0.02 M glycine–NaOH–NaCl). Activity in the arbitrary units of Figure 2.

Activity Loss of Hexokinase A on Titration with CH_3HgI. The titration behavior of hexokinase A differs in several respects from that of hexokinase B (Figure 5). There is no loss of activity of the enzyme upon addition of 2.8 equiv of CH_3HgI. When 3.7 equiv is present, there is a drop in activity, on average of the order of 20% (the highest noted in repeat determinations being 25%). There is a subsequent steeper decline with titrant addition, to inactivation near 8 equiv. This titration was performed at 25°. An increase of temperature to 35° (as used for hexokinase B) causes a loss of the sharp break in activity that occurs about the fourth equivalent, but there is (in contrast to hexokinase B) precipitation of A at 35° in the presence of 2 or more equiv of mercurial, so that the reaction cannot be followed there. At 25°, obvious precipitation does not occur in A until about 8 equiv of mercurial is present. The addition of glucose (30 mM) or ATP–Mg^{2+} (5 mM) did not alter this inactivation profile.

Alkylation of Hexokinase. Both A and B enzymes showed a slow inactivation by 3 mM iodoacetate at 35°, pH 8.0 (Figure 2), which proceeded to completion. The rate of this inactivation was about ten times faster when the ionic strength was raised from 0.015 to 0.10 (Table V). About the same rate was found when iodoacetamide replaced iodoacetate as the reagent. Curves similar in form to those of Figure 2 were also found in plots of the inactivations at 0.10 ionic strength (Figure 6), so that the half-times (Table V) in all cases are a measure of the reaction rates. As noted above, the inactivation by iodoacetate is associated with the carboxymethylation of cysteines (Table III). The iodoacetate treatment

TABLE V: Inactivations of Hexokinase by Alkylation.

Hexokinase	Alkylating Reagent[a]	Ionic Strength	Mercurial Present	Half-Time of Inactivation at 35° (min)
B	Iodoacetamide[b]	0.10		27
B	Iodoacetate[b]	0.10		34
B	Iodoacetate	0.015		200
B	Iodoacetate	0.015	4 equiv of CH_3HgI	60
A	Iodoacetate[c]	0.015		72
A	Iodoacetate[c]	0.015	4 equiv of CH_3HgI[d]	36

[a] Iodoacetate concentration, 3 mM; pH 8.0, 35°; medium was 0.02 M glycine–NaOH adjusted with NaCl to 0.10 ionic strength, *or* was sodium succinate at 0.015 ionic strength. [b] Concentration, 5 mM. [c] Some protein precipitate present after reaction. [d] Mercurial was added here at 25°, then mixture was brought to 35°, and iodoacetate was added.

was repeated on hexokinase B after exposure to 4 equiv of CH_3HgI (an amount of the mercurial that is noninactivating). The iodoacetate inactivation was now, by comparison, rapid (Figure 7). After inactivation was just complete, the protein was isolated as before and analyzed; the results (Table III) show that this inactivation is associated with the carboxymethylation of four sulfhydryls.

Hexokinase A was similarly treated (Figure 7). Again a more rapid loss in activity is now produced by the alkylation; this case, unlike that of hexokinase B, is difficult to interpret since some precipitation was observed at the alkylation stage. This is clearly related to the precipitation that was observed when 8 equiv of mercurial alone was added to hexokinase A. Ultracentrifugal analysis of these mercurial-treated hexokinase A samples (see below) has confirmed that marked aggregation occurs after the stage at which 4 equiv has reacted. While, from the results shown in Figures 2, 5, and 7, the over-all behavior of the two sets of four thiols is probably similar in both A and B enzymes, it is difficult to establish this behavior for the A enzyme due to the concurrent precipitation reaction.

Availability of Sulfhydryl Groups. The total of eight SH groups found per molecule of hexokinase was established in all cases in denaturing conditions, except for the one instance of the iodoacetate reaction, and the latter (Figure 2) was very slow. It was, therefore, desired to see whether some of these SH groups were available in the native enzyme to reagents other than iodoacetate. Of the reagents used so far in this study, this trial was feasible with PMB and with sodium nitroprusside. The techniques we have employed do not permit direct detection of bound CH_3HgI (but the experiments shown in Figures 3 and 7 indicate that four SH groups in the hexokinase B molecule in water, pH 8, 35°, bind CH_3HgI stoichiometrically and preferentially).

When sodium nitroprusside was added to hexokinase B, in aqueous medium (ionic strength 0.015) but otherwise in the conditions (pH 8.5) where it gave (in 6 M guanidine hydrochloride) the color used in titration of the SH groups with CH_3HgI, no color was produced. This was true at 8, 20, 30, and 35°. In these experiments amounts of 2 mg of hexokinase B were used, which is more than adequate (*cf.* Table I) to titrate one SH group per molecule. As a check on the method, to the same solution there was then added an amount of GSH, equivalent to four SH groups per mole of the protein present. This immediately produced a strong color, and on titration with CH_3HgI a value was obtained of 1.1 SH groups/molecule of GSH present. Therefore, in conditions where free SH groups are readily detectable with nitroprusside, no significant level of these is found in hexokinase in water.

When successive equivalents of PMB were added to hexokinase B in water at pH 7.0, 25°, no significant absorbance increment at 250 mμ was observable up to about 6 equiv, and only a very slight indication of binding at 7.4 equiv (Table VI). Correspondingly, PMB treatment (7.4 equiv) left a large fraction of the

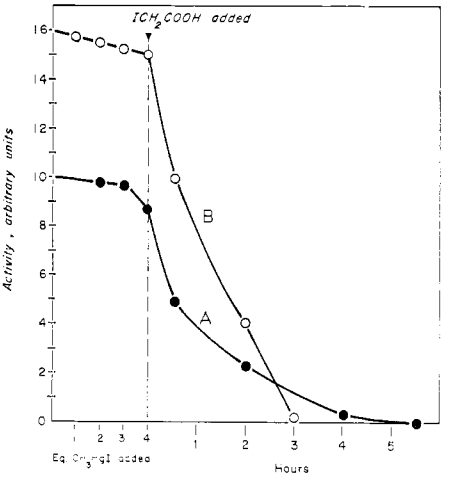

FIGURE 7: Activity of hexokinases A (●) and B (○) after treatments with up to 4 equiv of CH_3HgI (added at 10-min intervals) and then with 3 mM iodoacetate, all at pH 8.0, I = 0.015. Temperature was 35° for iodoacetate treatments and for CH_3HgI treatment of B, but 25° during CH_3HgI treatment of A.

TABLE VI: Sulfhydryl Groups in Hexokinase B Available to PMB.

Temp (°C)	Equiv of PMB Added[a]	I	Absorbance Increment at 250 mμ (%)[b]	Inactivation (%)[c]
25	3.7	0.015	0[d]	
25	7.4	0.015	9	
35	3.7	0.015		15
35	7.4	0.015		30
35	70	0.10[e]		100

[a] Added (up to 7.4 equiv) 0.92 equiv (per mole of protein present) at a time, at about 10-min intervals. [b] Increment expressed as percentage of the maximum increment expected on the binding of 7.4 equiv of PMB. This was estimated from the maximal absorbance increment found on PMB titration of hexokinase B in 6 M guanidine hydrochloride (as in Table I), corrected for the change in 6 M guanidine hydrochloride of the absorbance increment of the mercaptide (found from the ratio of the absorbance increments of the GSH–PMB adduct in the guanidine hydrochloride and water media). The maximal increment value (in water) per mercaptide group in hexokinase is near that of the GSH–PMB adduct in the same conditions (and a ratio of 1.33 was noted by Kaji (1966) for this comparison, using cysteine in place of GSH). [c] Hexokinase activity lost, expressed as percentage of the initial activity. [d] Up to 3.7 equiv, no significant change was detectable. From 3.7 to 5.5 equiv, a slight change was found, but the first accurately measurable change was present after 7.4 equiv. [e] Medium was glycine–NaOH–NaCl, pH 8.0, I = 0.1. In the other cases (I = 0.015), medium was succinate–NaOH, at pH 8.0 at 35°, or pH 7.0 at 25°.

TABLE VII: Apparent Molecular Weights at pH 8.0 (glycine medium) of Native and Mercurial-Treated Hexokinases A and B.

Protein	CH_3HgI (equiv)	Concn[a] (fringes)	M_{app}
B, native	0	20	84,600
B, native	0	13	76,000
B	3.7	13	85,500
B	7.4	13	230,000
B, native	0	6	55,700
B	3.7	6	28,300
B	7.4	6	28,300
A, native	0	20	97,000
A	3.7	20	113,000
A, native	0	10	86,000
A	3.7	10	75,000
A	7.4	10	151,000
A, native	0	6	81,000
A	3.7	6	72,000
A	7.4	6	62,000
A, native	0	4	60,000
A	7.4	4	40,000

[a] The concentration of the protein at a given point in the cell at sedimentation equilibrium is counted in fringes (42 fringes are equivalent to 10 mg/ml), and the molecular weight of the protein, M_{app}, at the same point is recorded.

activity of hexokinase B remaining even at 35° (Table VI). Stringent conditions at this pH and temperature, *i.e.*, the use of a large excess of the mercurial, in a glycine buffer which is known (see the discussion below) to promote dissociation of the protein, were required to obtain high inactivation.

Since these phenomena showed a clear difference from the effects of CH_3HgI treatment (Figure 3), an experiment using both reagents in succession was performed. Hexokinase B was treated with 3.7 equiv of CH_3HgI, producing, as usual, only small activity loss; successive equivalents of PMB were now added (Figure 3, closed triangles). PMB is now as effective in inactivating as CH_3HgI is.

Dissociation of Mercurial-Treated Hexokinase B. A series of sedimentation equilibrium studies was performed at pH 8.0 (Table VII) using hexokinase B before and after some of the treatments with CH_3HgI. The native enzyme gave a plot (Figure 8) with an upward curvature that indicated M_{app} = 84,600 near the bottom of the cell and M_{app} = 55,700 in the meniscus region of the solution. This pattern indicates the presence of an association–dissociation equilibrium in the conditions of the determination. The enzyme that had been treated with 7.4 equiv of CH_3HgI also showed the presence of an association–dissociation equilibrium (Figure 8), but here a more extensive dissociation had taken place. Thus, this material gave M_{app} = 25,000 in the meniscus region of the cell. This preparation, however, also showed the presence of heavy aggregates, which occurred to such an extent that the estimation of protein concentration could not be made in the solution at the bottom of the cell (Figure 9). Determinations relating to the region of the cell near the bottom, where concentration measurements were still possible, indicated M_{app} = 230,000 in this case. The enzyme treated with 3.7 equiv of CH_3HgI also showed the presence of a dissociation (Figure 8). Here the plot for the meniscus region was superimposable with the corresponding segment for the protein treated with 7.4 equiv of methylmercuric iodide, but no heavy aggregates were observed. In this case of 3.7 equiv M_{app} was 85,000 in the bottom region of the cell. This value is substantially higher than the molecular weight found for the native protein (also at pH 8.0) in a region of the cell having the same range of protein concentration, 10–13 fringes (Table VII).

An attempt was made to study a mercurial-treated

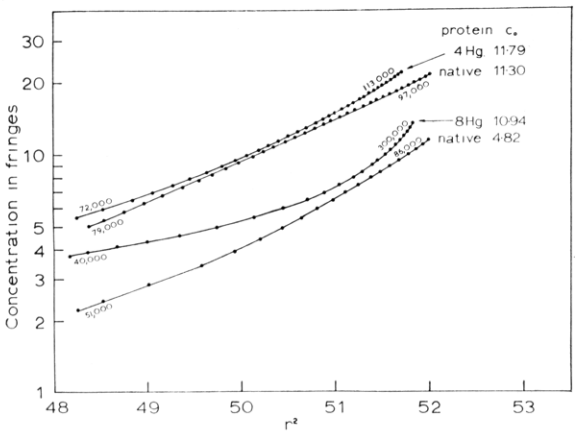

FIGURE 8: Sedimentation equilibrium plot for hexokinase B: the concentration (expressed in fringes) is plotted on a logarithmic scale *vs.* r^2 (where r is distance from the center of rotation). The initial concentration, C_0 (in fringes), is shown for the native enzyme and for the samples treated with eight aliquots (7.4 equiv) of CH_3HgI ("8 Hg") and with four aliquots (3.7 equiv, "4 Hg"). The three samples were studied simultaneously in an An-J rotor at 9307 rpm, 6°, pH 8.0. The numbers on the curves represent M_{app} in the regions indicated.

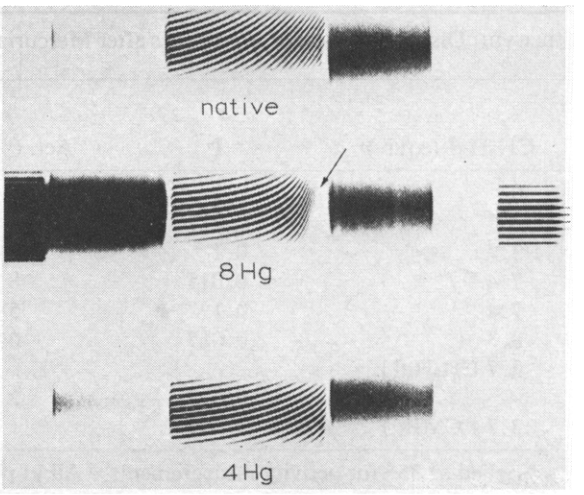

FIGURE 9: Interference patterns at equilibrium, for the solution section of the samples analyzed in Figure 8. The arrow indicates the heavy aggregates at the base of the solution treated with 7.4 equiv of CH_3HgI, in contrast to the other two samples.

hexokinase B in the same medium used in the kinetic studies described above. A protein sample treated (see above) initially with 3.7 equiv of CH_3HgI and then with 3.7 equiv of PMB was dialyzed at 4° to reach stable conditions against several daily changes of 5 mM sodium succinate, adjusted to pH 8.0 with NaOH (this being the medium used in the kinetic studies, which were conducted on a pH-Stat). The pH decreased here during the first 24 hr to pH 7.45; a few hours after each subsequent pH readjustment the pH was again 7.45 and stable. This behavior was maintained for 3 days. Thus, the conditions used in this experiment were not exactly those used in the kinetic study, but they were the best approximation that could be achieved for the present purpose. Sedimentation equilibrium experiments were made with this preparation, firstly at 18° and then at 4°. As with hexokinase B treated with 7.4 equiv of CH_3HgI, this material showed the presence of packed aggregates near the bottom of the cell and the presence of high molecular weight material. M_{app} = 450,000 could be estimated at the bottom of the solution just above this packed material. In the solution near the meniscus, M_{app} was 53,000 at 4° and 40,000 at 18°.[4]

Dissociation of Mercurial-Treated Hexokinase A. Native hexokinase A and the corresponding CH_3HgI-treated derivatives were examined similarly (Table VII), and again showed the presence of an association–dissociation equilibrium at pH 8.0. The apparent molecular weight of the enzyme varied with protein concentration (Figure 10). Thus, M_{app} was 97,000 at a concentration in the cell giving about 20 fringes (approximately 5 mg/ml) while M_{app} was 51,000 at a concentration giving about 2.5 fringes.

The protein that had been treated with 7.4 equiv of CH_3HgI differed from the native enzyme by a more extensive dissociation as well as by the presence of heavier aggregates. Thus, the mercurial-treated protein had M_{app} = 40,000 at a concentration in the cell giving about four fringes, whereas the native protein had, in regions in the cell having that same protein concentration, M_{app} of 70,000. Owing to aggregation, estimation of the protein concentration could, again, not be made in the solution zone at the bottom of the cell. In the lowest zone that could be measured toward the base of the cell, M_{app} of 300,000 was found. Hexokinase A treated with

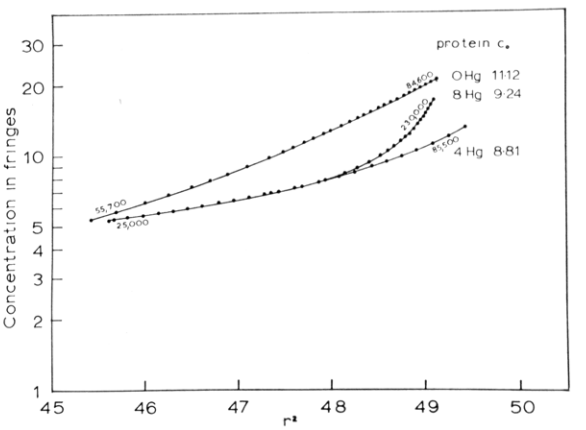

FIGURE 10: Sedimentation equilibrium plot for hexokinase A (pH 8.0, 6°). Native enzyme and the sample treated with 3.7 equiv of CH_3HgI ("4 Hg") were studied (upper two curves) at 8767 and 9335 rpm, respectively. Another concentration of the native enzyme and a sample treated with 7.4 equiv of CH_3HgI ("8 Hg") were studied at 10,589 and 8767 rpm, respectively. Other details and symbols as in Figure 8.

[4] The material could not be further analyzed at 35°. The activity experiments involved a relatively short exposure of each sample at one temperature, whereas the equilibration and sedimentation periods at 18 and 35° involved here were necessarily lengthy and, in the absence of sterility, some possibility of bacterial action rendered the results at 35° of uncertain validity.

TABLE VIII: Dissociation and Activity Loss after Mercurial Treatment of Hexokinase B.

CH_3HgI (equiv)[a]	I[b]	Act. (%)[c]	M_{app}[d] 3.3	M_{app}[d] 1.5 (mg/ml)
0	0.1	100	76,000	55,700
3.7	0.015	95		
3.7	0.1	45	85,500	28,300
7.4	0.015	8		
7.4	0.1	5	230,000	28,300
9.3	0.015	0		
3.7 CH_3HgI + 3.7 PCMB[e]	0.015	2		40,000 (18°, pH 7.5) 53,000 (4°, pH 7.5)

[a] Applied at 35° for activity measurements. [b] All at pH 8.0, in 0.02 M glycine–NaOH adjusted to I = 0.1, or 5 mM succinate–NaOH at I = 0.015. [c] Percentage of activity of native hexokinase B found on assay after treatment. [d] The same specimen was used for sedimentation equilibrium analysis at 6°. The two concentrations refer to protein at two levels in the cell (see Table VII) at sedimentation equilibrium. [e] CH_3HgI was added first, followed by PMB; the same specimen was analyzed at 18 and 4°.

3.7 equiv of CH_3HgI showed the same pattern of greater dissociation and the simultaneous presence of larger aggregates than the native protein in corresponding conditions (Table VII, Figure 10), although this trend was less pronounced than after the treatment with 7.4 equiv.

Discussion

These results clearly establish that yeast hexokinases A and B have each eight sulfhydryl groups per molecule. The stoichiometric titrations, after unfolding of the protein, both with PMB and with CH_3HgI, offer good evidence for this; confirmations of the conclusion that it is indeed SH groups that are being titrated are provided by the separation of *S*-CM-cysteine or of cysteic acid from the protein, in the predicted amounts, after appropriate treatments. Disulfides are seen to be absent.

The availability of these SH groups cannot be defined simply, but depends sharply upon the reagent used to test it. Hexokinase B is more or less inert to PMB. In contrast, all eight of the SH groups are alkylated by iodoacetate or iodoacetamide. A halfway stage of behavior is exhibited with CH_3HgI, which discriminates between two sets of four SH groups: one set reacts first, without effect on enzyme activity, while the SH groups of the second set react only subsequently and activity is removed in proportion to their mercaptidation (Figures 3 and 4). There is, also, an unusually large effect of ionic strength on the iodoacetate reaction rate (Table V).

This complex reactivity behavior should be interpreted, we propose, in terms of the dissociation of the hexokinase molecule. Both hexokinases, A and B, in solution at pH 5.5 are homogeneous, with a molecular weight of 102,000. The dissociation of hexokinase B into half-molecules has been observed (Derechin *et al.*, 1966) at alkaline pH values; it is complete at pH 10.4 (at 5°), and increases with pH in the range 7–10. Evidence in that study suggested that (at pH near 7) increase in ionic strength promotes this dissociation. At the pH (8.0) of the reactions used here, it has been further documented (Figures 8 and 10; Table VII) that hexokinases A and B (without chemical modification) are each considerably dissociated to half-molecules, in reversible equilibria. The marked effect of ionic strength on the alkylation rate (Table V), can, therefore, reasonably be explained by a decrease in the protein dissociation at very low ionic strength; this leads to the conclusion that the SH groups in the monomeric protein are much more reactive than in the dimeric protein.

Consistent with this explanation is the striking effect of mercaptide formation in inducing dissociation. In the hexokinase B samples that had reacted with CH_3HgI at ratios of reagent to protein between 4 and 8, the results show that there is a considerable formation of smaller species. The smallest species formed have a molecular weight about 26,000, and these are regarded as the subunits of this protein.[5] The results with 8 equiv of CH_3HgI is somewhat complicated by the simultaneous presence of high molecular weight aggregates, presumed to arise from an unfolding concurrent with the dissociation. In Table VIII are shown some correlations of inactivation and dissociation in samples of hexokinase B treated with mercurial reagents.

The molecular weight determinations shown in Tables VII and VIII were generally made at 6°, while the enzymic activity effects were measured after reaction at 35°; the dissociation at 35° is presumed to be equal to

[5] In recent experiments (M. Derechin and E. A. Barnard, unpublished data) on hexokinase B in 5.3 M guanidine hydrochloride at 4° (in pH 8 glycine buffer) with all SH groups covered by CH_3HgI, complete dissociation has been realized. The sedimentation equilibrium plot shows a homogeneous population with mol wt 27,500, confirming that there are four subunits in the protein.

or greater than that measured at 6°. The initial results reported for comparisons of M_{app} at 4 and 18° support this expectation.

Ionic strength and ionic type, however, have a noteworthy effect; the addition of glycine (0.02 M) with increase of I to 0.1 markedly increases the effects of CH_3HgI (Table VIII; Figure 3). We have seen that in this glycine–NaCl medium the dissociation of native hexokinase B to monomers (about 51,000 molecular weight) is extensive. In this state, the molecule after reaction with CH_3HgI at only four SH groups now undergoes considerable cleavage to subunits. These appear, from the activity titrations, to be enzymically inactive. At very low ionic strength (and absence of glycine) reaction with CH_3HgI occurs at four SH groups, but the further dissociation to subunits apparently does not occur.

We suggest that the differences in the effects of the various thiol-reactive reagents are related primarily to their polarity: CH_3HgI is undissociated (and soluble in organic solvents), whereas PMB, iodoacetate, and nitroprusside are polar.[6] It is, also, not surprising that iodoacetamide, although uncharged, acts with the latter groups of reagents, in view of its strong hydrogen-bonding and partial ionic character. The most reasonable interpretation, therefore, appears to us on present evidence to be as follows. The native hexokinase molecule is a dimer (102,000 molecular weight) composed of two loosely bound monomers, each of which carries four SH groups. In the dimer, all eight thiols are in internal situations; they have low reactivities to PMB, nitroprusside, iodoacetamide, and iodoacetate. CH_3HgI, however, can penetrate the hydrophobic environment and there reacts rapidly. The first four SH groups to react thus must be in a different situation from the latter four, which we can regard as "totally buried" when in the dimer molecule. We cannot yet state whether or not the thiols of this latter group are directly involved in active centers. The first four are clearly nonessential, however. The effectiveness of CH_3HgI in dissociating to subunits, taken together with the concurrent tendency of the subunits to form new (inactive) aggregates, suggests that the introduction of this group disrupts hydrophobic bonds in the neighborhood of the thiols in the protein structure.

The reaction rate with iodoacetate is similarly enhanced (Table V) when dissociation is promoted by higher ionic strength on the one hand and by mercurial treatment on the other, again consistent with the idea that dissociation is necessary for the reaction of these essential SH groups. This reaction rate is similar to that of some freely available SH groups of other proteins; thus Benesch and Benesch (1962) show a half-time of reaction of 25 min (compared with our 27 min at pH 8) for the two reactive SH groups of human hemoglobin reacting with 5 mM iodoacetamide (tenfold excess) at pH 7.3, room temperature.

Monomer units are active enzyme molecules (see below). A symmetric structure for the dimer molecule, *i.e.*, one in which the two monomers are equally active and each has four SH groups, two essential and two nonessential, is by far the easiest structure to reconcile with the present evidence. For example, four nonessential SH's bind CH_3HgI at low ionic strength, whereas, when extensive dissociation occurs, 4 equiv of CH_3HgI block, instead, two SH's of each type (Figure 3); several special assumptions are needed to explain this comparison if these groups are not distributed equally among the monomers.

Summarizing the proposed scheme, reaction at the four nonessential thiols leads to extensive dissociation to monomers (and further dissociation to subunits in the glycine–NaOH medium). In the monomeric form, the structure is such that the other four SH groups now become available to CH_3HgI, and also to iodoacetate (Figure 6) and to PMB (Figure 3, closed triangles). When these latter mercurial reactions occur, inactive subunits are formed.

The discrepant results obtained on previous preparations, reviewed earlier, are not surprising in view of the complexity of behavior revealed here. The number and essentiality of SH groups found in yeast hexokinase will depend upon the reagent and the conditions employed. Even when denaturing media are used, if proteolysis of the enzyme has not been excluded the number of SH groups revealed may still differ from that found for hexokinase B. For hexokinase A, aggregation and precipitation are induced by SH reaction, and for such hexokinase species this can also produce misleading quantitative results. Similar precipitation or aggregation was noted by Fasella and Hammes (1963) and by Kaji (1966) with the preparations of the enzyme used in their SH determinations.

In the only previous reported study of the physical effect of a mercurial on yeast hexokinase, Kaji (1966) notes only an aggregation. A sedimentation velocity profile is given, and shows a peak having about twice the sedimentation rate of the original enzyme. No evidence for subunit formation is reported there. However, Kaji (1966) used only PMB, and at a higher ratio to hexokinase (16:1); he also found an inactivation by PMB, not obtained in comparable studies on our material. That reaction (Kaji, 1966) was slow (15–200 min) and was not reversed by excess cysteine. We should note here that no measurable time dependence was found in our reactions using CH_3HgI or PMB. This is illustrated by the intervals used in these titrations (Figures 3–6; Table VI). Mercaptide formation at an available thiol should be very rapid. The aggregation reported by Kaji appears, therefore, to be a secondary effect, and the differences noted are considered to arise from the partial

[6] Reagent size might also be a contributing factor here. The proposal that polarity is the dominant factor leads to the prediction that fully ionic aliphatic mercuric compounds such as methylmercuric nitrate would be much less effective than methylmercuric iodide in dissociating hexokinase. The former reagent has not yet been prepared for the present studies, but has been used (Barnard and Ramel, 1962; Ramel, 1964) on an earlier, somewhat similar, dimeric hexokinase preparation. The nitrate was then less effective than the iodide now is: at pH 7, 25° (I = 0.05), 40 equiv of CH_3HgNO_3 (or $HgCl_2$) gave no activity loss, while at pH 8, they gave (contrast Figure 4 above) at 26° no inactivation when tested immediately, and at 35° a complete but time-dependent ($t_{0.5}$ = 3 min) one. These time-dependent inactivations are presumably linked to dissociation of the protein.

proteolytic attack that occurs in the course of the preparation which Kaji used (as discussed by Lazarus *et al.*, 1966), which presumably leads to an opening of the structures in which thiols are buried.

Hexokinases A and B are of the same size, but B dissociates with mercurial more readily than A (Table VII) and, consistent with this, is rather more readily inactivated by SH reagents (Figures 2, 4, and 7). Hexokinase A has only about 30% of the specific enzymic activity of B. These differences should, therefore, be related to the composition and interaction of the subunits in each of these two enzymes. These are now under active investigation.

The dissociation of hexokinase molecules to monomers cannot be assumed to occur without concomitant structural change in the monomer units, which may be a prerequisite of SH availability. Such a structural change is, indeed, suggested by the increase in the SH reactivity above about 30°. This phenomenon was prominent in a study (Barnard and Ramel, 1962; Ramel *et al.*, 1963) with a previous preparation of hexokinase, using bromoacetate or CH_3HgNO_3 at pH 8, and some similar tendency was seen here (*e.g.*, Figures 3 and 4). This effect suggests that some cooperative change occurs in the protein structure. The other data reviewed above tend to suggest that this structural change occurs readily only in the monomeric form. The entire protein structure, with the SH groups internal, is probably stabilized by the dimer formation. We do not know if the dimeric molecule has the active centers available; there is evidence on the previous preparation (Ramel, 1964; Ramel *et al.*, 1963) that the substrate mixture can dissociate the molecule. Hence, the apparent enzymic activity of the dimer may be, in fact, a property of the monomeric form only. This suggestion is reinforced in the case of hexokinase B by the evidence (Table VIII, at 1.5 mg/ml, and Derechin, unpublished results, for lower concentrations) on the spontaneous dissociation of the dimer. At pH 8.7, 4°, in glycine buffer (I = 0.1) the protein is extensively dissociated when the initial concentration is reduced to 0.2 mg/ml. In assay conditions, the medium is glycylglycine at pH 8.3, at 25–30°, and the protein concentration is of the order of 0.005 mg/ml. Spontaneous dissociation of the dimer should then be complete. The monomer molecule is, therefore, active enzymically, while the activity of the dimer is not, in fact, tested. We suggest that, in these active monomer molecules, the structural transformation that occurs above 30° to expose the critical SH groups is likely to be similar to one that occurs at any temperature in the interaction with substrates. This would permit these four SH groups to be in (or related to) the active centers, yet inaccessible to reagents even though they be applied in conditions where the enzyme would be capable of acting on a substrate if one were present.

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