Yeast Hexokinase. V. Subunit Structure and Hybridization of Isoenzymes*

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ABSTRACT: Yeast hexokinases A, B, and C were studied in homogeneous form. All three have molecular weight about 100,000. Each hexokinase was fully dissociated into inactive subunits by 8 M urea (reversibly) or by 1% sodium dodecyl sulfate. As judged by electrophoresis in starch gel containing 6 M urea, hexokinase A gave rise to a single molecular species of subunit, designated α, and the same was true for hexokinase B (β). By electrophoresis in polyacrylamide gel containing 1% sodium dodecyl sulfate, all of the subunit types present were found to have a molecular weight about 51,000, a conclusion confirmed by gel filtration in 8 M urea. Hexokinase A appears to be α_2, and B (and also C) to be β_2, in subunit structure. A protease from the yeast cell extract, which apparently binds in latent form to the pure hexokinases, can cleave the subunits into 26,000 molecular weight fragments, unless steps are taken to inactivate the protease. The known properties of a pro-proteinase which has been reported to be present in yeast cell extracts would account for the various anomalies we have observed. Ribonuclease A migrates as the dimer and trimer in the electrophoresis in dodecyl sulfate medium. Such aggregation was not found for six other proteins studied, and was excluded as interfering with the molecular weight determination of the hexokinase subunits. Hexokinases A and B can hybridize in 0.1 M sodium phosphate solution at pH values near 8. This hybrid, designated A′, has the same molecular weight as A or B, so that its structure is $\alpha\beta$. It is found in fresh lysates of yeast cells.

The isoenzymes A, B, and C of yeast hexokinase, as discussed in part IV of this series (Ramel *et al.*, 1971), have been purified by a procedure designed to remove opportunities for proteolytic attack. The absence of such damage is clearly a prerequisite for the determination of the number of polypeptide chains in these proteins.

In the forms of yeast hexokinase that have been isolated by cautious methods of that general type (Lazarus *et al.*, 1966; Schulze and Colowick, 1969; Ramel *et al.*, 1971) there is much evidence for the existence of at least two dissociable chains per molecule. In both hexokinases A and B the molecular weight of the native protein is close to 102,000 and disulfide bridges are absent (Lazarus *et al.*, 1968). Derechin *et al.* (1966) and Lazarus *et al.* (1968) found that the proteins, in sedimentation equilibrium at pH 10.4 or at pH 8.0 (at higher dilution), exist as species with M_Z[1] about 50,000; those studies, and the observation by Schulze and Colowick (1969) of M_w as a function of pH and buffer composition, gave evidence of a reversible, pH-dependent dissociation equilibrium between the native enzyme and half-molecules. However, by methylmercuric mercaptide formation at thiol groups of hexokinases A and B, evidence for the presence of smaller subunits, M_Z about 26,000, was also obtained (Lazarus *et al.*, 1968), but that conclusion was not definitive, for reasons discussed below.[2]

When earlier forms of hexokinase preparations were employed, on the other hand, the capacity to form quarter-molecular subunits (M_w ~25,000) was clearly observed, *e.g.*, by centrifugation after SDS dissociation (Ramel *et al.*, 1961) or in alkali (Kenkare and Colowick, 1965). In a more recent note, Easterby and Rosemeyer (1969), using a yeast hexokinase stated to be similar to the P-I form of Gazith *et al.* (1968), deduced from sedimentation studies at high ionic strength that dissociation occurs to quarter-molecules (mol wt 28,000). In contrast, Pringle (1970), using a commercial preparation of yeast hexokinase, has reported a molecular weight of 52,000 for the subunits separated in SDS-polyacrylamide gel electrophoresis.

The present studies (reported in part in brief preliminary form: Rustum and Ramel, 1969; Rustum *et al.*, 1970) have sought to establish, by electrophoresis and gel filtration under appropriate conditions, the number and size of the subunits present in the hexokinase molecules in the intact native state, and the identity or nonidentity of these subunits within each isoenzyme.

Materials and Methods

Unless otherwise specified, all materials and methods were as used by Ramel *et al.* (1971) and all operations were at 4°. Aldolase, LDH (type V), and ribonuclease A (type IIA) were from Sigma, commercial yeast hexokinase (crystalline, lot no. 7050598) from the Boehringer Mannheim Co. (New York), and acrylamide, Bis, TEMED, and ammonium persulfate from Eastman. SDS was "Duponol" (Dupont), and urea was Baker Analyzed grade. The molecular weight markers, bovine serum albumin, lysozyme, ovalbumin, chymotrypsinogen (six-times crystallized), and trypsin (twice crystallized), were from Mann.

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[1] Abbreviations used: TEMED, *N,N,N′,N′*-tetramethylethylenediamine; SDS, sodium dodecyl sulfate; Bis, *N,N*-methylenebisacrylamide; LDH, lactate dehydrogenase; EBT, EDTA–borate–Tris buffer (Boyer *et al.*, 1963) diluted 1:4 with water; 2-ME, 2-mercaptoethanol; M_w (or M_Z), weight average (or *Z* average) molecular weight.

[2] In a preliminary presentation of a part of this work at the Federation of American Societies for Experimental Biology, Atlantic City, April 1970 (Rustum *et al.*, 1970), evidence for subunits of mol wt 26,000 was also noted. This conclusion is now believed to be in error, for reasons cited in the Discussion of the present paper.

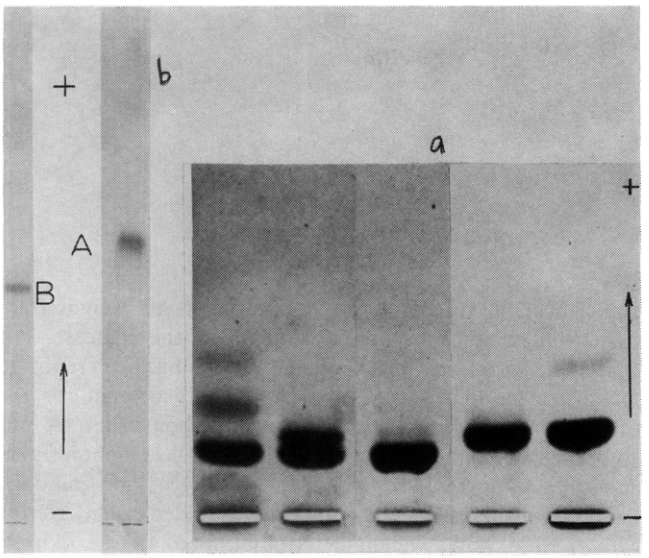

FIGURE 1: Electrophoresis in starch–urea. (a) Starch gel electrophoresis (18 hr) in 6 M urea–EBT of urea-dissociated hexokinases (staining for protein). The enzymes were, where specified, gel filtered to remove contaminating proteases at the start of the purification (Ramel *et al.*, 1971). All are rechromatographed samples. Right to left: (1) A; (2) A, gel filtered; (3) C, gel filtered; (4) A + B (both gel filtered); (5) C. Note that the samples (1 and 5) not initially freed of all proteases contain additional minor subunit species. (b) Electrophoresis as in part a, but for 72 hr, of urea-dissociated hexokinases A and B (each from a gel-filtered preparation).

Using the methods described by Ramel *et al.* (1971), hexokinases A, B, and C were isolated and finally purified by repeated rechromatography on DE-52 DEAE-cellulose. The preparations used had specific activities on glucose of at least 750 IU/mg for B, 220 IU/mg for A, and 650 IU/mg for C, at 25°, pH 8.3.

Enzyme Dissociation and Hybridization. For dissociation in 8 M urea, a sample of each of the isoenzymes was first dialyzed against EBT buffer (pH 8.6). The samples (about 5 mg of protein/ml, finally) were treated with DFP (to 1 mM) and made 8 M in urea (by addition of the solid) and 1% in 2-ME. All urea solutions used in these and other experiments were prepared immediately prior to use, to avoid artifacts due to cyanate accumulation (Stark *et al.*, 1960).

For subunit molecular weight determinations in SDS, each isoenzyme was dialyzed against 0.06 M Tris-glycine (pH 8.6). The samples (about 5 mg of protein/ml, finally) were made 1 mM in DFP (except where noted otherwise), 1% in SDS (by addition of 10% solution), and 1% in 2-ME, at room temperature.

For molecular hybridization studies, hexokinases A and B (8 mg/ml) were dialyzed, both separately and mixed, for 16 hr at 4° against 2 l. of 0.1 M sodium phosphate (pH 8.5). Alternatively (without difference in the results) the isoenzymes were obtained (separately and mixed) in 0.1 M sodium phosphate (pH 8.5) medium, simply by addition of 1.0 M sodium phosphate to them in water, to give a total protein concentration of 8 mg/ml.

Electrophoresis. For vertical starch–urea gel electrophoresis, starch (56 g) was added, with constant stirring, to EBT buffer (100 ml), followed by addition of 8.4 M urea (300 ml). The mixture was heated to incipient boiling, and degassed with a water aspirator. 2-ME was added (to 0.1%) and the mixture poured into the Buchler mold. After cooling to room temperature, the gel was left at 4° for 12–24 hr before use. The electrode buffer was EBT–0.1% 2-ME–6.1 M urea. Aliquots (40 μl) of the urea-dissociated hexokinase isoenzymes were subjected to electrophoresis at 340 V (approximately 19 mA), at 6°, 18–72 hr. The gels were then sliced horizontally and stained for protein.

Vertical polyacrylamide gel electrophoresis in the presence of SDS was accomplished in the E-C Co. apparatus, employing 7.5% polyacrylamide gel. The gels were prepared either in 0.1 M sodium phosphate (pH 7.2) according to Maizel (1966), or in 0.06 M Tris-glycine buffer (pH 8.6), as follows: 30 ml of 38% acrylamide–2% Bis, 3.0 ml of 2% ammonium persulfate, 3.0 ml of 10% (v/v) TEMED–water, 16 ml of Tris-glycine buffer (0.6 M Tris titrated to pH 8.6 with solid glycine), and 0.16 g of SDS were mixed and made up to 160 ml with water. This solution was poured slowly through cheesecloth (to trap soap bubbles) into the gel mold. Bubbles were removed and the slot former was inserted. Following polymerization, the gel was preelectrophoresed for 15–30 min at 60 mA. Each of the solutions of dissociated hexokinases and the molecular weight markers was mixed with a small volume of 1% bromophenol blue containing a few crystals of sucrose. The electrode buffers (either 0.1 M sodium phosphate (pH 7.2) or 0.03 M Tris-glycine (pH 8.6) as appropriate) contained 0.1% SDS. Electrophoresis (at 60 mA, thermostatted at 21°) was continued until the bromophenol blue marker had migrated 7–9 cm from the base of the sample slots. Electrophoresis is faster (approximately 3 hr compared to 16 hr) and more reproducible in the Tris-glycine than in the phosphate buffer system.

Cellulose acetate electrophoresis employed the Beckman Microzone apparatus and membranes, in 0.1 M sodium phosphate (pH 8.5) at 2.5 mA, for 2 hr at 23°. Similar results were obtained when these electrophoreses were conducted at 4°. The membrane was subsequently stained for protein (0.3% Amido Black–10% trichloroacetic acid), destained with 10% acetic acid in methanol, and rinsed with water.

Gel Filtration. A column (47 × 1 cm) of Agarose (Bio-Rad A-0.5m, 100–200 mesh) was equilibrated either in 0.1 M Tris-HCl (pH 8.6) or in the same buffer containing 8 M urea and 1% 2-ME. The column was calibrated with aliquots (15 mg of protein in 1.0 ml) of the molecular weight markers in the same medium. Hexokinases were treated with DFP (to 1 mM) before and after the initial dialysis against the Tris-HCl buffer. For separation of the hexokinase subunits, the dialyzed enzymes were then made 8 M in urea and 1% in 2-ME, followed by incubation at 35° for 2 hr and immediate gel filtration. In parallel experiments the DFP pretreatment was either omitted, or was followed by heating the samples (in the urea) at 85° for 5 min and immediate gel filtration.

Results

Subunits of Hexokinase Isoenzymes. Hexokinases A, B, and C were isolated from yeast in the absence of autolysis by the methods recommended by Ramel *et al.* (1971). Hexokinases A and B were homogeneous preparations by DEAE-cellulose chromatography and by starch gel electrophoresis. Hexokinase C contained a small amount (of the order of 10% by weight) of the inactive flavoprotein impurity that chromatographs with it (Ramel *et al.*, 1971) but no other form of hexokinase. Each was examined for subunit content.

Protein subunits can be dissociated and identified by electrophoresis in starch–urea gels (Chernoff and Pettit, 1964). A high-resolution vertical starch–urea gel electrophoretic procedure was developed, employing the EBT buffer system

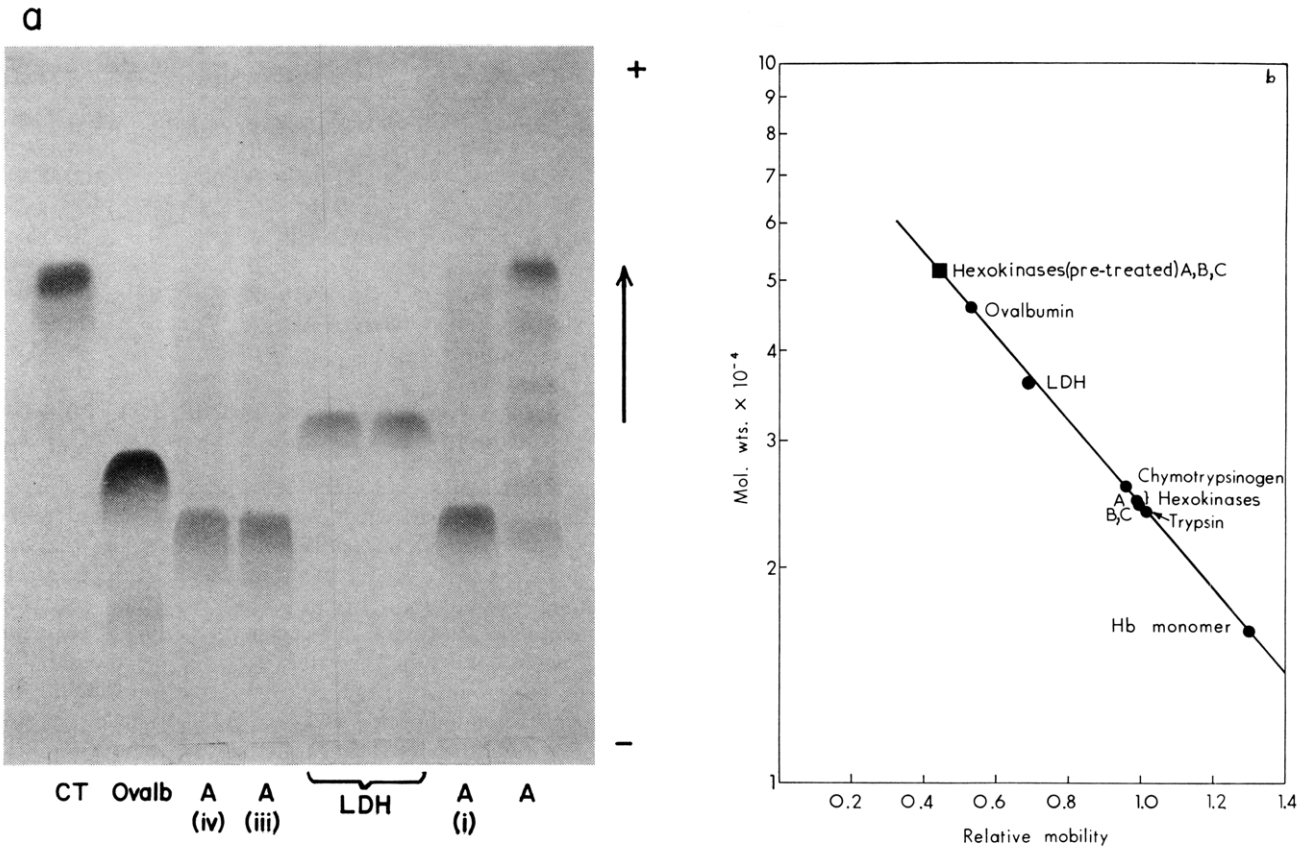

FIGURE 2: (a) Polyacrylamide gel (7.5%) electrophoresis in Tris-glycine–SDS of SDS-treated proteins. Right to left: (1) hexokinase A incubated in 1% SDS, 20 min, room temperature; (2) hexokinase A, after pretreatment i (see text); (3) LDH; (4) LDH, after pretreatment i; (5) hexokinase A, after pretreatment iii; (6) hexokinase A, after pretreatment iv; (7) ovalbumin, after pretreatment i; (8) chymotrypsinogen, after pretreatment i. (b) Calibration plot (Shapiro *et al.*, 1967), taking the mobility of trypsin as a reference point. The points (mean of three determinations each, except for hexokinase C, where only one was made) were obtained after electrophoresis as in (a). M_w was determined thus for hexokinases A, B, and C without pretreatment, or after (■) pretreatment i or iii, when A, B, and C had identical mobility.

of Boyer *et al.* (1963), to separate the hexokinase subunits. No enzymic activity remained in the hexokinases in 8 M urea medium. As shown in Figure 1, hexokinases B and C contain (by the charge criterion) the same single subunit species, different from that of the single subunit species present in hexokinase A. A single band was still obtained with A and with B, when electrophoresis was continued (Figure 1b) for 72 hr, which gives the maximum resolution attainable before severe diffusion interferes. Under these conditions, hexokinase C again behaved identically with hexokinase B. (The small amount of contaminating yellow protein in C remained at the origin in the urea system.) It was found necessary in the prolonged electrophoretic runs to maintain mercaptoethanol (at 0.1%) in the starch–urea gel and electrode buffer, to avoid formation of minor bands, ascribed to thiol oxidations.

The same results were obtained if electrophoresis of A or B was carried out (i) immediately after treatment at 22° with 8 M urea; (ii) after 2 hr at 35° in 8 M urea; (iii) after dialysis at 4° for 15 hr against 8 M urea; (iv) after heating at 85° for 2 min in 8 M urea; or (v) after 1 mM DFP treatment, prior to the addition of urea and treatment ii. (All of these enzyme samples were in EBT buffer at pH 8.6, containing 1% 2-ME). Hence, the results shown in Figure 1b are taken to represent the maximum dissociation into subunits obtainable by urea treatment.

Hexokinase A contains, therefore, a single subunit type, designated α, and B (and similarly C) another type, designated β. The latter is less negatively charged at the alkaline pH employed in the electrophoresis.

When the initial yeast extract is gel filtered on Agarose (Ramel *et al.*, 1971), DFP-resistant proteases are removed, traces of which contaminate, otherwise, the purified enzyme. The pure preparations of hexokinases that had received this early treatment each contain a single type of subunit (Figure 1). Those purified similarly, but without the gel filtration stage, contain small but definite amounts of modified subunits which migrate in the starch–urea system as sharp bands slightly faster than the corresponding α or β bands (Figure 1a). These minor bands became more significant with prolonged autolysis at 4° or if DFP was omitted in the early preparative stages. Since a difference in the specific enzymic activity or the mobility of the undissociated hexokinase was scarcely distinguishable between the two types of product, it appears that minor (but chemically significant) degrees of proteolytic damage are compatible with the active configuration of the enzyme molecules.

Molecular Weights of the Subunits. Polacrylamide gel electrophoresis, performed under conditions where charge differences are obliterated by an excess of the anionic detergent, SDS, can be a precise tool for determining the molecular weights of polypeptide chains (Maizel, 1966). Shapiro and Maizel (1969) were able thus to detect differences as small as 5% in molecular weights. This method was adopted here, but

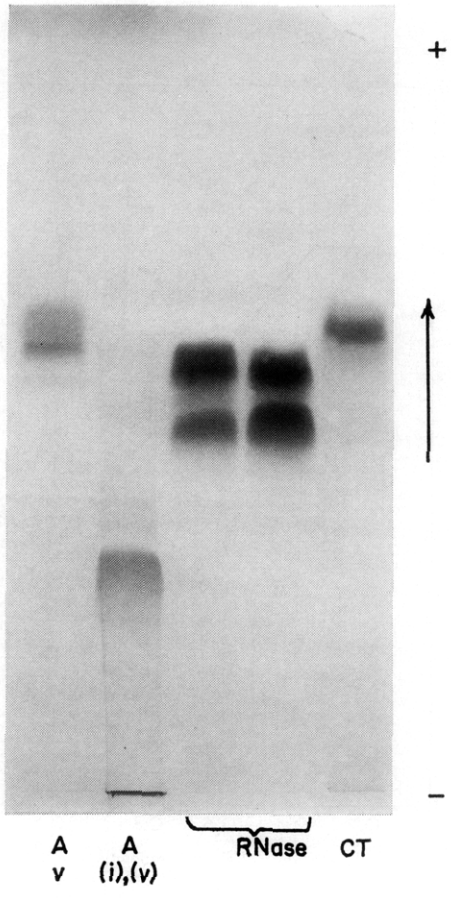

FIGURE 3: Polyacrylamide SDS electrophoresis (as in Figure 2). Right to left: (1) chymotrypsinogen; (2) ribonuclease, after pretreatment i (see text); (3) ribonuclease, after treatment v; (4) hexokinase A, after pretreatment i and treatment v; (5) hexokinase A, after treatment v only.

TABLE I: Molecular Weight Estimations by Gel Filtration.[a]

Expt	Conditions	Protein	M_w
1	0.1 M Tris-HCl, pH 8.3[b]	Hexokinase A	100,000
		B	100,000
		A + B	100,000[d]
		C	100,000
2	0.1 M sodium phosphate, pH 8.3[c]	Hexokinase A	58,000
		B	58,000
		A + B	100,000[d]
3	8 M urea–0.1 M sodium phosphate, pH 8.6 (containing 1% 2-ME)	Hexokinase A	50,000
		B	50,000
		B (DFP)[e]	50,000
		B (DFP, 85°)[e]	50,000

[a] On Agarose (see Methods) at 4°. M_w was calculated (to the nearest 2,000) using a linear calibration plot (Andrews, 1964) of V_e against log M_w for the marker proteins used, *i.e.*, serum albumin, ovalbumin, chymotrypsinogen, and lysozyme. [b] Protein concentration at the center of the eluted peak was 4 mg/ml. At 0.4 mg/ml, the values each became about 85,000 due to partial dissociation. [c] Protein concentration at the center of the eluted peak was 0.4 mg/ml. [d] A single peak was present. [e] After the pretreatments noted (see Methods).

employing a Tris-glycine, instead of the usual phosphate, buffer system containing SDS. The phosphate buffer was used in several experiments and gave the same results as reported below, but the separation was much slower than in the Tris-glycine medium.

Hexokinases A, B, and C were dissociated by 1% SDS under a variety of conditions (Figure 2). Electrophoresis in SDS showed that the subunits of A, B, and C each migrate as a single band corresponding to mol wt ≃51,000. This result was obtained only when precautions were taken to inactivate any latent protease present. The pretreatments that we have found to be effective for protease inactivation were (i) treatment at 100° (2 min) in the SDS medium (Pringle, 1970); (ii) treatment at 100° (2 min) of the hexokinase solution before addition of the SDS; (iii) addition of DFP at room temperature prior to the addition of the SDS; or (iv) exposure to 8 M urea at 35° (3 hr), followed by dialysis at 4° in the presence of 0.1% SDS–0.1% 2-ME. In each case we have also tested the effect of a subsequent treatment (v), based on the procedure of Shapiro *et al.* (1967), *i.e.*, an incubation (3 hr) at 35° in 1% SDS–1% 2-ME followed by dialysis against 0.1% SDS–0.1% 2-ME overnight at room temperature. However, it was found that the treatment v was not essential for the dissociation of these isoenzymes and could be omitted without change in the results. On the other hand, when hexokinases were treated in SDS merely by the treatment v, without one of the pretreatments i–iv above, a major band corresponding to mol wt 26,000 was always observed (Figure 3, column 5; Figure 2).

Intermediate stages could be observed in this process (Figure 2a, column 1). When hexokinase B was allowed to remain at room temperature in the 1% SDS solution before treatment i was applied, the mol wt 51,000 species was found to disappear within a few minutes. As it disappeared it was replaced by the mol wt 26,000 species and the minor intermediate forms seen in Figure 2a (column 1); the latter, however, were at all stages much less in amount than the mol wt 26,000 species. Even after 5 min at room temperature the pattern was of the type seen in column 1 of Figure 2a. After 1–2 hr at room temperature essentially only the mol wt 26,000 species was present. This pattern did not change further after 3 hr at 35° in SDS and about 15 hr at room temperature (Figure 3, column 5), so that it represents a stable end point.

Aggregation in SDS Solution. The possibility was considered that the boiling in SDS causes an aggregation of denatured hexokinase subunits. Such an aggregation was demonstrated for pancreatic ribonuclease A, which migrates as bands corresponding to the dimer and trimer (Figure 3). A cooperative micelle is apparently formed of ribonuclease molecules and SDS. The ribonuclease A sample used was not aggregated prior to solution in the SDS medium, as shown by its normal elution volume in gel filtration (Andrews, 1964) on Sephadex G-100 in aqueous buffer at pH 7. This effect in SDS was not found in similar electrophoreses in SDS medium with LDH subunits, ovalbumin, chymotrypsinogen, trypsin, or the hemoglobin α and β chains, which all traveled with their predicted monomer mobilities (Figure 2b). Aggregation upon heating in SDS seems to be excluded also for the hexokinase subunits, in view of the result of the experiment noted above in which the hexokinase was boiled before SDS treatment at room temperature, again giving the mol wt 51,000 subunits.

Analysis by Gel Filtration. Gel filtration (Andrews, 1964) was used to confirm the molecular weight of the subunit forms. Agarose (A-0.5m) was preferred as the gel, however, since the columns are more stable to hydrostatic pressure changes and because Sephadex gels were found to behave less satisfactorily for the calibration in the presence of 8 M urea.

On the Agarose column in 8 M urea–1% 2-ME (pH 8.6) (Table I), the subunits of hexokinase A or B dissociated in that medium were each in a single peak, eluted just before ovalbumin and corresponding to mol wt 50,000. The same result was obtained when the urea-dissociated enzyme was heated at 85° for 5 min in the presence of 1 mM DFP, with or without dialysis overnight.

Analysis of Crystalline Commercial Yeast Hexokinase. Several recent studies of yeast hexokinase have employed the crystalline commercial preparation, especially that from the Boehringer Co. (Pilkis *et al.*, 1968; Fromm, 1969; Kopperschläger and Hofmann, 1969; Pringle, 1970). The isoenzymes present in currently available batches thereof were compared by starch gel electrophoresis (Ramel *et al.*, 1971) with our purified preparations. Four main bands, and several minor ones, were revealed by protein staining, two of which are strongly active in phosphorylating glucose (Figure 4). A pattern of four active bands has been found by Kopperschläger and Hofmann (1969) in agar gel electrophoresis of Boehringer yeast hexokinase. In the present case, it was demonstrated that the forms present are quite different from hexokinases A, B, or C (Figure 4).

The subunits present in the commercial preparation were examined by starch gel–urea electrophoresis as in Figure 1. No bands were seen at the position of the subunits of A or B, but a series of bands running faster than either of these was present. The slowest, and major, component had a mobility about 20% faster than that of the α subunit. Several rather faster bands, not well resolved, were also seen, and a significant very fast band, with mobility almost twice that of α. This pattern was not affected by prior treatment with DFP (10^{-3} M, pH 7, 1 hr). It is known that preparations of this type are contaminated with free proteases (Lazarus *et al.*, 1966; Schulze and Colowick, 1969), and it appears, from the last-mentioned observations, that these have already modified the hexokinase molecules prior to the dissociation in urea. It was found that the Boehringer material had a specific enzyme activity of 125 IU/mg (at 25°), and a fructose:glucose phosphorylation ratio of 2.5 (suggesting that it largely consists of modified forms of hexokinase A). A second batch of Boehringer hexokinase (of different lot number) gave identical results in all the examinations noted here.

In SDS polyacrylamide gel electrophoresis of Boehringer hexokinase, after the pretreatment at 100° in SDS (see step i above), a band corresponding to about mol wt 50,000 was prominent, as has been reported recently by Pringle (1970), but in addition a band corresponding to about mol wt 25,000 was of about equal intensity, and several minor intermediate bands were also present. It is clear, therefore, that the commercial enzyme has suffered proteolysis which produces several components, one of which is close in size, but quite different in charge, to the subunits of the native enzyme.

Hybridization of the Hexokinases. Hybridization may occur between the subunits of related isoenzymes, forming new, active species (Markert, 1963). The ability of the subunits of hexokinases A and B to hybridize was tested, using cellulose acetate electrophoresis for analysis. The parent hexokinases A and B were separated slightly but reproducibly on cellulose acetate at pH 8.3. Single and multiple thawings of the

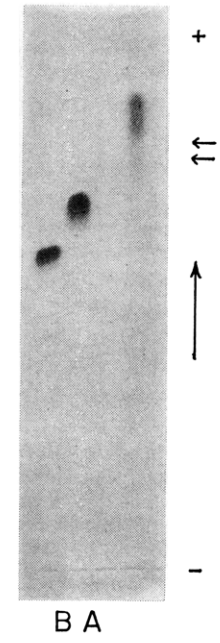

FIGURE 4: Starch gel electrophoresis (EBT buffer), with staining for hexokinase activity. Right to left: (1) commercial (Boehringer) hexokinase; (2) hexokinase A; (3) hexokinase B. The horizontal arrows in channel 1 mark the positions of additional, inactive, strong bands seen by staining for protein the other half of this gel. Three other, faint bands were also observed in channel 1 by activity staining, in addition to the two broad bands clearly discerned here.

mixtures (Markert, 1963) and also of A or B alone, were tried in various media, namely 0.1 or 1.0 M NaCl solutions (in 0.1 M Tris-HCl at pH 8.3). In each electrophoresis after these treatments the characteristic bands of hexokinases A and B (Figure 5, columns 1 and 3) were the only bands present (using protein staining), there being no detectable change from the initial constitutions of the samples (except after the freezing in urea, when only a smear from the A to B bands was obtainable). The inability of LDH isoenzymes to undergo molecular hybridization in Tris-HCl buffer is known (Markert and Massaro, 1966). However, LDH readily hybridizes in phosphate buffers containing NaCl (Markert, 1963). In a similar experiment, a form with mobility intermediate between that of A and B was observed when a 1:1 mixture of the two hexokinases in 0.1 M phosphate buffer at pH 8.5 was examined (Figure 5). Although the mobilities in the cellulose acetate system of A and B are close, the difference between them can always be discerned, and the intermediate band was invariably distinguished in many experiments of the type shown in Figure 5. This hybrid band was only seen when the mixture was maintained during electrophoresis at a high ionic strength, as in 0.1 M sodium phosphate or in 0.5 M Tris-succinate at pH 8.3, but not in 0.1 M Tris-succinate or 25 mM phosphate at the same pH, where A and B, on mixing, run separately. This hybrid could not be examined in starch gel electrophoresis. When the mixture was examined in the system used by Ramel *et al.* (1971), only the regenerated A and B bands were seen, since the ionic strength is low, while electrophoresis was impracticable in the gel at the very high current density produced when the 0.1 M phosphate medium was substituted.

It was also shown by filtration on a calibrated gel column (Andrews, 1964) that in 0.1 M sodium phosphate (pH 8.3), a hybrid of A and B exists (Table I). This has molecular weight of about 100,000, while A and B separately in the same me-

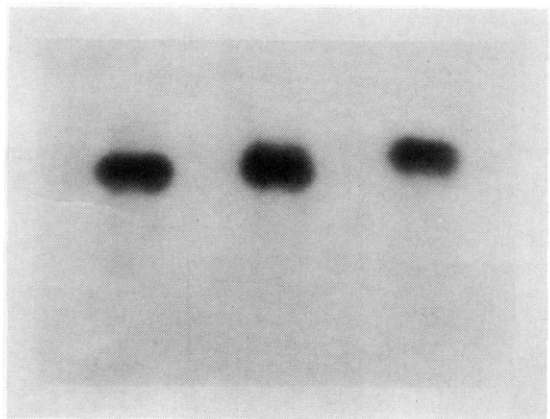

FIGURE 5: Cellulose acetate electrophoresis in 0.1 M sodium phosphate (pH 8.6). Right to left: (1) hexokinase A; (2) A + B hybridized; (3) B. Electrophoresis for 2 hr at 2.5 mA, 100 V, at room temperature, of 1.4 mg/ml enzyme in each case. Origin is at the lower edge, positive pole at top.

dium (at a protein concentration of about 0.4 mg/ml) are largely in the form of the subunits. At the same pH in Tris buffer, at about a fivefold lower ionic strength, each of hexokinases A, B, and C is in the undissociated form (Table I).

Kenkare and Colowick (1965) showed that the preparation of crystalline yeast hexokinase which they studied can readily be inactivated in solution at high pH and ionic strength, with a concomitant dissociation into quarter-molecules (mol wt ~25,000), and that the enzyme recovered its activity upon neutralization. Hybridization from such conditions was examined here. The pure hexokinases A and B, separately or in 1:1 mixture, were inactivated at pH 11.5 in 0.4 M sodium phosphate (containing 0.1% 2-ME, at 22°). Cellulose acetate electrophoresis in the same medium yielded no distinct bands but only a smear from the point of origin. When this alkali treatment was followed by adjustment to 0.025 M phosphate medium at pH 8.6, electrophoresis in the latter medium yielded in each case only the bands of the parent forms used, with no detectable hybrid. However, after adjustment to 0.1 M phosphate medium at pH 8.6 and electrophoresis in the latter medium, the same hybridization was obtained as in Figure 5. Activity (80–90% of the initial value) was recovered after this return to pH 8.6. This reactivation was slow, requiring about 15 min at 22° in the phosphate medium or 30 min in 0.1 M Tris-succinate buffer. Hence, the same single hybrid is formed after dissociation by alkali or by the phosphate medium.

Hexokinases A and B were also dissociated in 8 M urea (as above) for a period of 1 hr at 4°, mixed (1:1 or 1:3), and dialyzed at 4° against the EBT buffer (pH 8.6) to remove all the urea. This regenerated about 60% of the initial activity (in the 1:1 mixture). In starch gel electrophoresis in EBT (without urea) the bands of A and B were present but no hybrid active form of intermediate mobility was detectable. Several minor new bands of inactive protein were also generated. Hence a hybrid is not formed stably on reassociation in water of the subunits obtained from urea solution.

Discussion

Hexokinases A and B have been characterized (Ramel *et al.*, 1971) as chromatographically and electrophoretically homogeneous proteins. The electrophoretic evidence of the latter and the present study reveals that unless free protease activity is eliminated in the initial stages of the preparation, the native species are partly converted to several anionic forms. This modification can occur in several stages, producing a series of active derivatives. The mildest degree of proteolysis forms damaged subunits (Figure 1) which combine to form enzyme molecules which are difficult or impossible to distinguish from the native ones by electrophoresis, by chromatography, or by their enzymic activity. The next stage produces modified enzyme molecules separable by electrophoresis (Figure 4, column 5, of Ramel *et al.*, 1971) but not separable by the chromatographic methods employed. To avoid these two stages, it is necessary to take the various precautions described by Ramel *et al.* (1971), including the gel filtration of the initial extract to remove the free proteases. The acid protease of yeast (Hata *et al.*, 1967), for which no good inhibitor is available, readily introduces, otherwise, this limited cleavage. Further stages of proteolysis occur if significant autolysis is deliberately permitted during the preparation (*e.g.*, to increase the yield), and these stages produce enzyme species that are now distinguishable by DEAE-cellulose chromatography, *e.g.*, hexokinase D (Ramel *et al.*, 1971). Finally, if a preparation employs extensive autolysis, proteolytic attack can proceed much further to remove all molecules similar in charge to the native forms, but with retention of a fair degree of hexokinase activity, as shown by the results obtained (Figure 4) with commercial crystalline hexokinase.

The various separations in urea or SDS solutions, when taken together with the molecular weight of 102,000 for the whole proteins, show that both hexokinases A and B contain two subunits each. All the subunits are of molecular weight about 51,000. The subunits (β) of B are identical, so far as can be determined electrophoretically, as are the subunits (α) of A. The α subunits are more anionic than the β type at neutral and alkaline pH, consistent with the parallel electrophoretic difference for the parent protein A as compared to B (at pH 8).

Hexokinase C also has the structure β_2. The chromatographic difference (Ramel *et al.*, 1971) between the isoenzymes B and C must be due, therefore, to a tertiary structure difference. An explanation of this phenomenon is provided by additional studies on C, to be presented in a further communication.

Even when all active protease is removed initially, and cleavage of the native enzyme is avoided, some *latent* protease can be present, and this can produce a cleavage to half-subunits (Figure 2). This protease is latent because this attack occurs only when the hexokinase molecule is unfolded in SDS solution. This latent protease must be present in an unusual complex, since (i) no free protease activity could be measured in the hexokinase isoenzymes by release of material from hexokinase or from casein, using the methods described by Lazarus *et al.* (1966); (ii) these enzymes are completely stable at room temperature or at 4° in the absence of denaturant, no activity loss or change from the native electrophoretic mobility being detectable in tests covering several weeks storage; (iii) repeated rechromatography to chromatographic, ultracentrifugal, and electrophoretic homogeneity does not remove the bound protease; (iv) only a small percentage of the hexokinase molecules can carry this protease, as judged from the high specific enzymic activity of these hexokinases, and the absence of detectable contaminating protein when the hexokinase subunits are separated. Trace amounts of a yeast protease must be assumed to be complexed firmly in the native hexokinases, therefore, and must be (as, for example, pancreatic carboxypeptidase has been observed to be) resistant to 1% SDS at 35°.

This protease is also sensitive to boiling or to DFP treatment (Figure 2a). Since prior DFP treatment of the hexokinase had always been given routinely, it is clear that DFP can only inactivate this fraction of the protease when the latter is liberated in SDS solution from its complex. This latent protease is also inactive in 8 M urea, as seen in Figure 2a and from the observation that the mol wt 50,000 subunits were obtained in urea (Table I) whether or not the specific inactivating treatments had been applied.

An explanation can be offered that accounts for all of the observations on this complex. Hayashi *et al.* (1969) have reported that baker's yeast contains a proteinase C which exists, in carefully prepared extracts, entirely in an inactive precursor form, pro-proteinase C. The latter can be activated in various denaturing media, including SDS solution. Protease C is active in neutral and alkaline media, and (unlike the acid proteases of yeast) it is inhibited by DFP (Hata *et al.*, 1967) whereas pro-proteinase C is not. Protease C and pro-proteinase C are readily inactivated at high temperatures (Hayashi *et al.*, 1969). Pro-proteinase C is larger (mol wt $\simeq$ 81,000) than the known yeast proteinases (Hayashi *et al.*, 1969) so that this form would not be completely separated from the hexokinases by the gel filtration step we use. Since it has a similar charge (Hayashi *et al.*, 1969) to the hexokinases, small amounts of it may adhere throughout their rechromatography. No proteolytic effects would be detectable until the precursor is activated by SDS, and boiling would immediately destroy the precursor. Since the pro-proteinase form disappears with autolysis of the yeast extract (Hayashi *et al.*, 1968), preparations of hexokinase that involve this will render the proteinase C sensitive to serine–proteinase inhibitors. Ironically, the precautions that we have employed to preserve the complete integrity of the hexokinase molecules would also be more effective than in other preparations in preserving the pro-proteinase C.

This trace contamination will not affect the properties of hexokinases A, B, or C when in nondenaturing media. It can be removed by inserting in the preparative scheme (Ramel *et al.*, 1971), prior to the second chromatographic stage, a brief treatment at 35° at pH near 7 in the presence of 10^{-4} M DFP, which will release and inhibit the protease fraction concerned.

While this work was in progress, and after the appearance of preliminary reports (Rustum and Ramel, 1969; Rustum *et al.*, 1970), a relevant report was made by Pringle (1970), referring to the electrophoresis in SDS medium of commercial (Boehringer) yeast hexokinase. Many bands were observed, whereas one major species of molecular weight about 52,000 and two minor species were present after prior boiling in the SDS solution or addition of a protease inhibitor. Those results, while significant in drawing attention to the heat-sensitive yeast protease active in SDS solution, would not in themselves have led to the prediction of the present results. The commercial hexokinase is heterogeneous before dissociation (Figure 4) and, being prepared by an autolytic method, is contaminated with overt protease activity. From our analysis (with and without pretreatments) of the commercial hexokinase, and similarly from the agar gel electrophoresis of undissociated Boehringer hexokinase already reported by Kopperschläger and Hofmann (1969), it is clear that it contains a number of protein species, not all of which have hexokinase activity. All of these are quite distinct electrophoretically from the corresponding native species, when compared either in the undissociated or in the subunit states. The fact that most of the species produced therefrom in SDS medium have mol wt $\sim$50,000 is, therefore, not necessarily indicative of the situation in the native isoenzymes.

It is interesting that the cleavage produced by this complexed protease in hexokinase A, B, or C appears to be fairly specific. The mol wt 26,000 form was found in our electrophoretic analyses to be the major product in all stages when the conditions permitted this protease to act. The intermediate forms detected at short times were always minor compared to the mol wt 51,000 and 26,000 species. Hence only rather specific cleavages must occur, and the process stops at the half-subunit stage, perhaps due to the limiting amount of protease present. It should be noted, however, that yeast proteinase C has carboxypeptidase activity (Hayashi *et al.*, 1970) and this may contribute in the formation of the products observed here.

The same phenomenon of a latent protease can explain the apparent subunits of mol wt $\simeq$26,000 found in hexokinases A or B dissociated by treatment with excess methylmercuric iodide (Lazarus *et al.*, 1968); we presume that this treatment both dissociates and unfolds the protein, permitting the specific cleavage by the trace of latent protease. The observation of Ramel *et al.* (1961) that subunits of mol wt $\simeq$25,000 were seen (by ultracentrifugation) to be produced by SDS treatment of an earlier preparation of yeast hexokinase is also readily understood on the same basis. The heterogeneity that we observe after freeze-thawing of purified hexokinase A or B (Ramel *et al.*, 1971) is also likely to be due to the release of this protease: pro-proteinase C is activated thus (Hayashi *et al.*, 1969). The dissociation to subunits of mol wt 28,000 by very high ionic strength media, reported by Easterby and Rosemeyer (1969), may also be attributed to such a proteolysis.

Since the subunit structures of native hexokinases A and B are α_2 and β_2, only one hybrid form, $\alpha\beta$, is possible without molecular weight change, and this form was obtained here. In 0.1 M phosphate solution (but not in 0.1 M Tris-chloride) at pH 8.3, A exists largely as monomeric α and B as monomeric β (Table I). This is in agreement with the molecular weight data for the forms P-I and P-II in quite similar media obtained by Schulze and Colowick (1969). The hybrid $\alpha\beta$, however, is not dissociated in the phosphate medium, accounting for its specific formation therein. The reactivation experiments indicate that at high pH or in urea, also, the subunits of A and of B are reversibly separated. This hybrid form is stable only at ionic strength above about 0.3, so it is not obtained after the normal purification procedures. However, an intermediate form, hexokinase A′, was observed (Ramel *et al.*, 1971) in chromatography of the crude extract before exposure to high ionic strengths. A′ broke down to give A and B in starch gel electrophoresis, as the present hybrid form does. Since A′ contained only α and β subunits, we have identified it with the $\alpha\beta$ hybrid. While, therefore, A′ as found in the yeast crude extract might, alternatively, be formed from A and C, the facts that it can be produced experimentally from A and B, and that on rechromatography it gives rise to the A and B peaks, and not to those of A and C (Ramel *et al.*, 1971), lead us to suggest that in the intracellular state all of the hexokinase B that has not already been changed there to the C form becomes hybridized with some of the excess of hexokinase A present, forming hexokinase A′.

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Identification of the Sites of Modification of Bovine Liver Glutamate Dehydrogenase Reacted with Trinitrobenzenesulfonate*

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ABSTRACT: Bovine liver glutamate dehydrogenase has been reacted with trinitrobenzenesulfonate and the primary sites of modification identified as lysine-428 and lysine-425 in the tentative amino acid sequence proposed by Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Jr., Langley, T. J., and Mclained, M. D. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 724). Lysine-428 reacts most rapidly but only to the extent of 0.5 group/polypeptide chain (*i.e.*, three of the six subunits in the active monomer are modified). The reaction at lysine-425 proceeds more slowly but also reaches an end point of only 0.5 group/polypeptide chain. Examination of the rate and extent of incorporation of trinitrobenzenesulfonate into these residues indicates that lysine-425 may be essentially unavailable for modification until the available lysine residues as position 428 have been trinitrophenylated. Sequence analysit of the tryptic peptides containing these residues shows that the modifications at positions 428 and 425 occur on different polypeptide chains. There appears to be no polypeptide chain in which both these residues are modified. No difference in the extent of modification of lysyl residues 428 or 425 was observed when nucleotide ligands which protect against loss of catalytic activity during trinitrophenylation were added to the reaction mixture. Procedures for the purification of relatively large trinitrophenylated peptides on a preparative scale in good yields, which have been largely developed in these experiments, are described.

Bovine liver glutamate dehydrogenase (L-glutamate: NAD(P) oxidoreductase (deaminating), EC 1.4.1.3) is a complex enzyme with respect to both its regulatory and physical properties. Substrate inhibition and activation by excess NADH and NAD^+, respectively, as well as the modulation of enzyme activity by the guanosine and adenosine nucleotides have been well established (Frieden, 1959, 1965). The smallest active molecular unit (the monomer) appears to consist of six identical polypeptide chains each having a molecular weight of 56,100 (Smith *et al.*, 1970). It is well known that the active monomer undergoes a concentration dependent polymerization which has been extensively investigated (Eisenberg and Tomkins, 1968; Colman and Frieden,

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