THE MOLECULAR WEIGHT OF THE UNDEGRADED POLYPEPTIDE CHAIN OF YEAST HEXOKINASE

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Summary

Commercial preparations of yeast hexokinase are contaminated with a trace of at least one proteolytic enzyme. This contaminant will digest the hexokinase in the presence of sodium dodecyl sulfate (SDS), and a reliable molecular weight can be obtained by SDS-polyacrylamide gel electrophoresis only when specific steps are taken to prevent this proteolysis. When appropriate precautions are taken, this technique yields a value of about 51,000 for the polypeptide chain molecular weight of yeast hexokinase. A similar value is obtained by gel filtration in 6M guanidine-HCl.

Introduction

A species with a molecular weight of about 25,000 seems generally accepted as the ultimate polypeptide subunit of yeast hexokinase (1,2,3,4,5). However, two laboratories have reported evidence indicating that the native enzyme possesses but a single binding site for glucose per 50,000 daltons (6,7). Since, in addition, attack by yeast proteases is known to be a problem in the isolation and characterization of this enzyme (8,9), it seemed possible that in fact the ultimate subunit of yeast hexokinase is a polypeptide chain with a molecular weight of about 50,000, and that the smaller components observed were proteolytic breakdown products, in spite of efforts (4,8,9) to circumvent this problem. The experiments presented here seem to confirm this possibility, and also illustrate a significant

danger attending the use of SDS-polyacrylamide[a] gel electrophoresis.

Materials and Methods

Yeast hexokinase was purchased from Boehringer (lots 6309395 and 6359296), from Sigma (lot 67B7660), and from Calbiochem (lot 855021), and was used without further purification. Other proteins used as molecular weight standards were as described by Weber and Osborn (10), and were kindly supplied by Dr. Klaus Weber. Reagents and procedures for SDS-polyacrylamide gel electrophoresis were as described by Shapiro, et al (11), incorporating the modifications of Weber and Osborn (10).

Guanidine-HCl was purchased as "Ultrapure" from Mann, and used without further purification. Gel filtration was performed on a .9 x 80 cm column of Agarose (Bio Gel A5M), eluting with 6M guanidine-HCl (without 2-mercaptoethanol), essentially as described by Fish, et al (12). All protein samples were reduced and alkylated with ICH_2COOH before application to the column. Preparatory to alkylation, protein samples were dissolved in .09M tris-HCl, .003M EDTA, pH8.0, containing 5.5M guanidine-HCl at 100°; 2-mercaptoethanol was added to .14M, and incubation conducted at 37° overnight.

Results and Discussion

As expected (8,9), all commercial preparations of yeast hexokinase tested are contaminated with proteolytic activity. If, to a solution of hexokinase in buffer (.4mg/ml in .03M tris-HCl, .001M EDTA, .2M NaCl, pH 7.6) at 25°, SDS and 2-mercaptoethanol are added to 1% each, a rapid and extensive proteolysis

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[a] The abbreviation used is SDS (sodium dodecyl sulfate).

ensues unless specific steps are taken to prevent this. The proteolysis is revealed by a replacement of higher molecular weight forms by smaller components in the subsequent gel electrophoretic patterns. Treatments found effective in minimizing this proteolysis are: (a) subjection of the sample to a 3min exposure to 100° immediately following addition of the SDS and mercaptoethanol; (b) treatment of the enzyme solution with the proteolytic inhibitor phenylmethanesulfonyl fluoride (13; used as described in 9) for several minutes immediately prior to the addition of SDS and mercaptoethanol; (c) initial solution of the hexokinase not in buffer, but in hot 5.5M guanidine-HCl, followed by reduction and alkylation as described in Methods, with dialysis then through 9.2M urea into an SDS solution. Each of these treatments presumably works by inactivating the contaminating protease sufficiently rapidly that little digestion of hexokinase can occur; if any of them is applied, an electrophoretic pattern similar to that of gel a, Figure 1, is obtained. In this pattern, a major band containing at least 80% of the protein (by densitometry on the stained gel) is observed in a position corresponding to a molecular weight (10,11,14) of about 52,000. Minor bands are observed with molecular weights of about 32,000 and about 20,500. No material is observed in the region corresponding to a molecular weight of 25,000. Since both non-covalent and disulfide links between chains should be destroyed under the conditions used, it seems very unlikely that the material of molecular weight 52,000 can be other than a single polypeptide chain. I presume this represents the undegraded monomer of yeast hexokinase.

If none of the treatments (a)-(c) is applied, but incubation just continued at 25° after the addition of SDS and mercap-

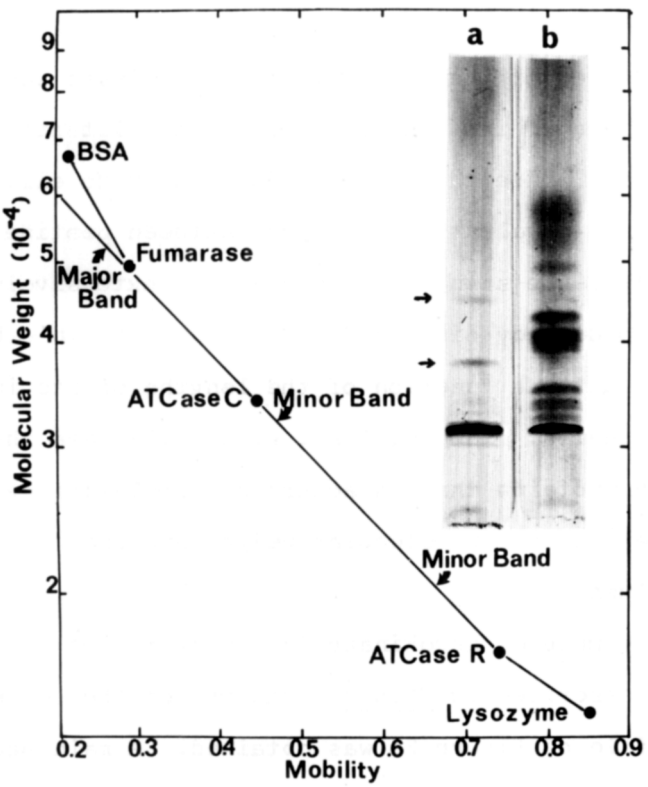

Figure 1. SDS-polyacrylamide gel electrophoresis of yeast hexokinase protected (gel a) and unprotected (gel b) against proteolysis in SDS solution. Electrophoresis was from bottom to top of the gels as pictured. The "Minor Bands" in gel a are indicated by arrows. The exact form of the standard curve to the left of fumarase is not clear from these data; the two lines indicate the limits. BSA is bovine serum albumin; ATCase C and R the catalytic and regulatory chains of E. coli aspartate transcarbamylase.

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toethanol to the hexokinase solution in buffer, a pattern similar to gel b, Figure 1 (Boehringer, lot 6309395), or with a complete disappearance of the higher molecular weight components (Sigma and Calbiochem enzymes) is observed. This wholesale degradation apparently occurs only after the addition of the SDS, as a 13hr incubation of such a hexokinase solution in buffer at 0°, fol-

lowed by treatment (a) or (b), still yields a pattern similar to that of gel a. Further information on this degradation process will be presented elsewhere.

Confirmation of these results for hexokinase was obtained by investigation of its behavior during gel filtration in 6M guanidine-HCl. Since this solvent is thought to eliminate completely all non-covalent interactions between peptide chains (15), and since the sample was reduced and extensively carboxymethylated to destroy disulfide bonds, the protein's behavior should be strictly a function of the lengths of its individual polypeptide chains. Indeed, Fish, et al (12), have shown for a series of 19 proteins that an accurate correlation exists between polypeptide chain molecular weight and position of elution from the column.

When a sample of hexokinase (Boehringer, lot 6359296), prepared as described in Methods, was run on the column, the elution profile of Figure 2a was obtained. A main peak was observed with an elution position corresponding to a molecular weight of about 51,000 (Figure 2b). Some smaller material was observed as well. SDS-polyacrylamide gel electrophoreses run on samples of material taken at the arrows in Figure 2a reveal the same bands seen in Figure 1, gel a, with a strict correspondence between their elution position from the column and their rate of migration on electrophoresis. Again the hypothesis that the polypeptide chain of yeast hexokinase has a molecular weight of about 50,000 seems strongly supported.

The earlier misleading results indicating a smaller fundamental polypeptide unit for this enzyme must surely have been due to proteolysis of the chain of molecular weight about 50,000. I suggest that the fact that these results were obtained by in-

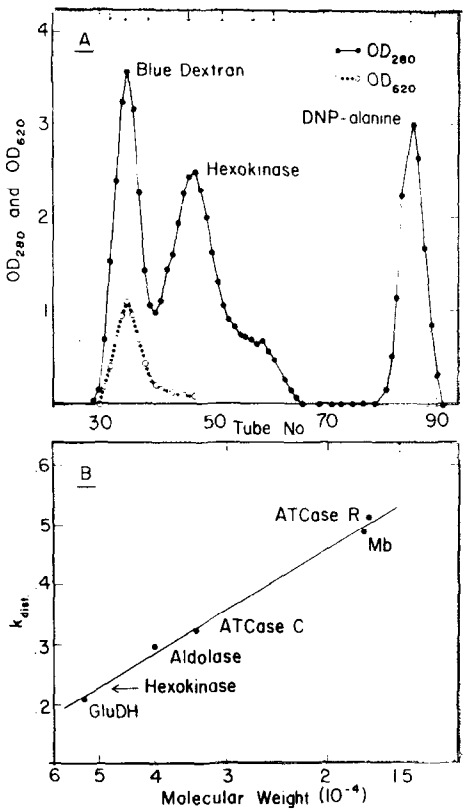

Figure 2. Gel filtration of hexokinase in 6M guanidine-HCl. A. Elution profile. Blue Dextran and DNP-alanine were included as internal standards in each run. Small arrows across the top indicate sites from which samples for SDS-gels were taken. B. Standard Curve (from Rosenbusch & Weber, in preparation). k_{dist} is the distribution coefficient (see reference 12); that for hexokinase is shown by the arrow. GluDH, glutamate dehydrogenase; Mb, myoglobin; ATCase, see Figure 1.

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vestigators who were aware of the problem of proteolytic modification of hexokinase, and who were taking considerable pains to avoid this, indicates the extreme difficulty which may be encountered in attempting to avoid completely such proteolytic effects. I suggest also that the enhancement of proteolysis by SDS, as noted above, obligates one who uses the SDS-gel electrophoretic technique on a previously untested protein to perform controls such as (a)-(c), above. This also minimizes the pos-

sibility that some rare protein will not be denatured by the incubation usually employed, as already noted by Weber and Osborn (10). These results do not, of course, negate the reliability of the technique for molecular weight determinations, as reported by others (10,11,14).

Acknowledgements

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