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Chemical and Physical Studies of *Neurospora crassa* Invertase. Molecular Weight, Amino Acid and Carbohydrate Composition, and Quaternary Structure*

Z. D. Meachum, Jr., H. J. Colvin, Jr., and H. D. Braymer†

ABSTRACT: *Neurospora crassa* invertase was found to be a glycoprotein containing 11% mannose and 3% glucosamine. Its molecular weight was found to be 210,000 ± 15,600 in pH 5.0 buffer and 51,500 in 6 M guanidine hydrochloride under reducing conditions. A tetrameric structure is thus indicated. At pH 5.0 the enzyme had an $s^0_{20,w}$ = 10.5 S. Under alkaline conditions it dissociated to give a molecule with $s_{20,w}$ = 5.2 S, and as alkali concentration was increased, it was converted into a 3.8S species. Both the 10.5S and 5.2S forms occur as such in crude extracts of the organism and both are enzymatically active. The amino acid composition of the enzyme has been determined and tryptic peptide fingerprints have been prepared. These studies suggest the possibility that the enzyme may be composed of more than one type of subunit. The native enzyme was found to contain four disulfide bonds per mole.

The *Neurospora crassa* invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) catalyzes the hydrolysis of β-fructoside bonds in a variety of substrates ranging in molecular size from β-methylfructose to inulin. The chemical characterization of this enzyme is of interest because of its localization in the area between the cell wall and the cell membrane of the fungus. In addition, once fully characterized, the wild-type enzyme will serve as a standard with which to compare cross reacting proteins purified from *Neurospora* mutants lacking invertase activity (Sargent and Woodward, 1969).

Metzenberg (1963a) purified invertase from *Neurospora* and partially characterized the enzyme. His results indicated that the enzyme exists in two active multimeric forms, the larger species having an $s_{20,w}$ of 10.3 S and the smaller one an $s_{20,w}$ of 5.2 S (Metzenberg, 1964). His studies showed that the 10.3S form could be dissociated into active subunits (5.2 S) by heat or by formic acid treatment in the presence of 1 M sodium chloride.

The studies described here were undertaken with the goal of elucidating the quaternary structure of the enzyme using chemical and physical evidence. This communication reports the homogeneity of the purified enzyme, its molecular weight, amino acid composition, and disulfide content, and evidence for subunits smaller than the active subunit. Preliminary studies on the carbohydrate moiety of the enzyme are also reported.

Experimental Section

Materials. Diethylaminoethylcellulose (DEAE) and carboxymethylcellulose (CM) were obtained from Schleicher & Schuell. Glucostat reagents and L-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone trypsin were obtained from Worthington Biochemical Corp., and glucose Tes-Tape from Eli Lilly. Anti-invertase antiserum was prepared by Antibodies Inc., Davis, Calif., using invertase preparations demonstrated to be homogeneous by several criteria. Sodium *p*-hydroxymercuribenzoate and Tris(hydroxymethyl)aminomethane were purchased from Sigma Chemical Co., Sephadex G-100 was obtained from Pharmacia Fine Chemicals, and guanidine hydrochloride (Sequanal grade) from Pierce Chemical Co. All other chemicals were reagent grade.

Growth Conditions. N. crassa wild-type strain SF 26 (Gratzner and Sheehan, 1969) was used as the source of invertase. This strain, a derivative of the standard wild-type STA 4, was selected for increased levels of the carbohydrases amylase and invertase. Conidia from 4- to 5-day-old cultures were used to inoculate 500-ml florence flasks containing 250 ml of Vogel's (1964) citrate minimal medium to which had been added sufficient sucrose (autoclaved separately in H_2O) to achieve a final concentration of 1.5% carbon source. After growth on a shaker at 34° for 4 days, one of these cultures was then used

* From the Department of Microbiology, Louisiana State University, Baton Rouge, Louisiana 70803. *Received August 10, 1970.* Supported by National Science Foundation Grant GB-6382. H. J. C. was the recipient of a predoctoral traineeship under National Institutes of Health Training Grant 5T1-GM-00692. Taken in part from a dissertation submitted by Z. D. M. in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Louisiana State University, Baton Rouge, La., 1970. Presented in part before the 158th National Meeting of the American Chemical Society, New York, N. Y., Sept 1969.

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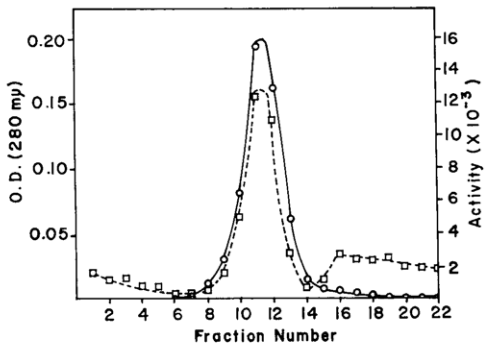

FIGURE 1: Glycerol (8–33%) density gradient sedimentation of purified invertase. The gradient was prepared in 0.05 M sodium acetate buffer, pH 5.0; (○) activity (units per fraction); (□) optical density at 280 nm.

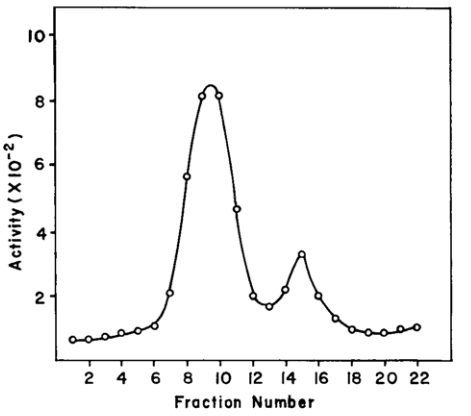

FIGURE 2: Glycerol (8–33%) density gradient sedimentation of a crude extract of mycelial powder. The gradient was prepared in 0.05 M sodium acetate buffer (pH 5.0) invertase activity expressed as units per fraction.

to inoculate a carboy containing 15 l. of medium identical with that described above. Usually four 15-l. cultures were grown for 60 hr with vigorous aeration. At the end of this growth period, mycelia were harvested by pouring the culture through cheesecloth on a large sieve. The mycelial mat was washed thoroughly with chilled deionized water and then pressed dry using a Carver laboratory press. The partially dry pads were then broken into small pieces and lyophilized. The lyophilized mycelia were ground to a fine powder in a Wiley mill and stored at −20° until extracted.

Assays. The invertase assay used was similar to one described previously (Metzenberg, 1962), but modified as follows. Sucrose hydrolysis was measured by determining the release of glucose using Glucostat. The reaction volume was 0.5 ml and contained 25 μmoles of sucrose in 0.04 M sodium acetate buffer (pH 5.0). Incubation time was 15 min at 37° and the reaction was stopped by heating for 1 min in a boiling-water bath. The reaction mixture was then cooled to room temperature and 9.5 ml of Glucostat reagent was added. The reaction was allowed to proceed for 10 min at 23° and then stopped by the addition of 1 drop of 4 N HCl. Color production was measured in a Klett photometer using the 420–450-nm filter. The proper controls and a standard glucose curve were determined along with each assay. These results were then converted into invertase units (micromoles of sucrose hydrolyzed per minute at 37°).

Protein was assayed by the method of Lowry *et al.* (1951), or by measuring the optical density at 280 nm and calculating the protein concentration using the experimentally determined extinction coefficient of invertase ($E^{1\%}_{1\text{ cm}}$) of 18.6. The extinction coefficient was determined by the refractometric method of Babul and Stellwagen (1969). The value obtained by this method agrees with the one determined by Metzenberg (1963a) on the basis of dry weight.

Enzyme Purification. The purification of invertase was done according to the procedure of Metzenberg (1963a) with the following exception. The enzyme was extracted from lyophilized powder (200 g) with 0.075 M sodium acetate buffer, pH 4.2 (15 ml/g of powder). This modification resulted in a much higher initial specific activity (18.5) than extraction at pH 5.0 (12.5) with an equal recovery of total enzyme units. The maximum specific activity of the purified enzyme obtained from the lyophilized powder was 1820, which compares favorably to the value of 1890 reported by Metzenberg (1963a) for invertase prepared from fresh mycelia.

Density Gradient Sedimentation. Gradients of 8 to 33% (0.88–3.62 M) glycerol in 0.05 M sodium acetate buffer (pH 5.0) were used in density gradient studies of the enzyme. The methodology of Martin and Ames (1960) was used, and a Spinco Model L-2 preparative ultracentrifuge equipped with an SW39 rotor was employed. The 4.6-ml gradients were fractionated into 21 or 22 fractions. The activity and protein profiles of the purified invertase are shown in Figure 1. The purified protein evidences only one activity peak and one protein peak, and these are superimposed, indicating the purity of the invertase preparation with respect to molecular size. The $s_{20,w}$, calculated using catalase as a standard, is 10 S. A typical activity profile of a crude cell extract is seen in Figure 2. Two activity peaks are seen here, one with an $s_{20,w}$ of 10 S and the other with an $s_{20,w}$ of 5.2 S. The heavier peak represented 88% of the total activity and the lighter peak, 12%, as measured by the areas under the peaks.

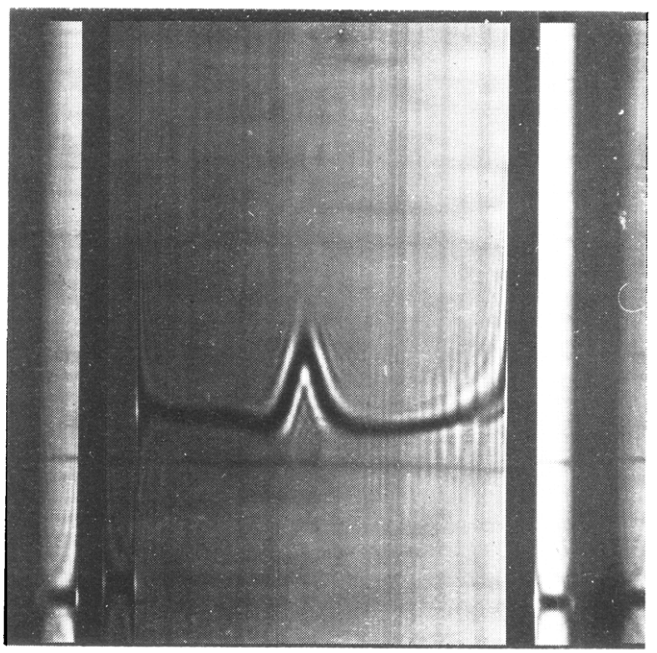

FIGURE 3: Sedimentation velocity ultracentrifugation of invertase at pH 5.0. The centrifugation was performed at 20°, with a rotor speed of 56,000 rpm. The photograph was taken 45 min after reaching two-thirds speed. $s_{20,w}$ = 10.0 S.

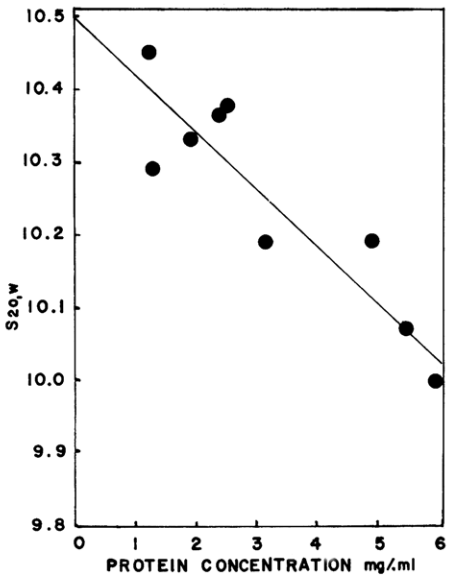

FIGURE 4: Determination of $s_{20,w}$ of invertase. All runs were performed at pH 5.0, 20°, and 56,000 rpm.

Sedimentation Analysis. The purified invertase was analyzed in a Spinco Model E analytical ultracentrifuge using the schlieren optical system. The enzyme was dialyzed against 0.05 M sodium acetate buffer (pH 5.0) containing 0.1 M NaCl, and sedimented in a 2.5-deg double-sector cell at 20° and 56,000 rpm. Under these conditions the enzyme sedimented as a single peak with an $s^0_{20,w}$ of 10.5 S (Figure 3). The $s^0_{20,w}$ was determined by calculating the $s_{20,w}$ of invertase solutions ranging in concentration from 1 to 6 mg per ml and extrapolating to zero concentration (Figure 4). In some experiments, the pH of the above enzyme solutions was adjusted to 8.9 with 1 N NaOH and the solution was immediately resedimented under the same conditions. This resulted in the appearance of a peak with an $s_{20,w}$ of 5.6 S and another with an $s_{20,w}$ of 9.9 S (Figure 5). When the pH of the protein solution was adjusted to 11.9 with 1 N NaOH, only one peak, with an $s_{20,w}$ of 3.8 S, was observed (Figure 6). When the original preparation was dialyzed against 0.05 M acetic acid (pH 3.0) containing 0.1 M NaCl and then sedimented at 20° and 56,000 rpm, one peak was observed with an $s_{20,w}$ of 3.8 S (Figure 7).

Electrophoresis. The purified protein was subjected to electrophoresis on polyacrylamide gels using the discontinu-

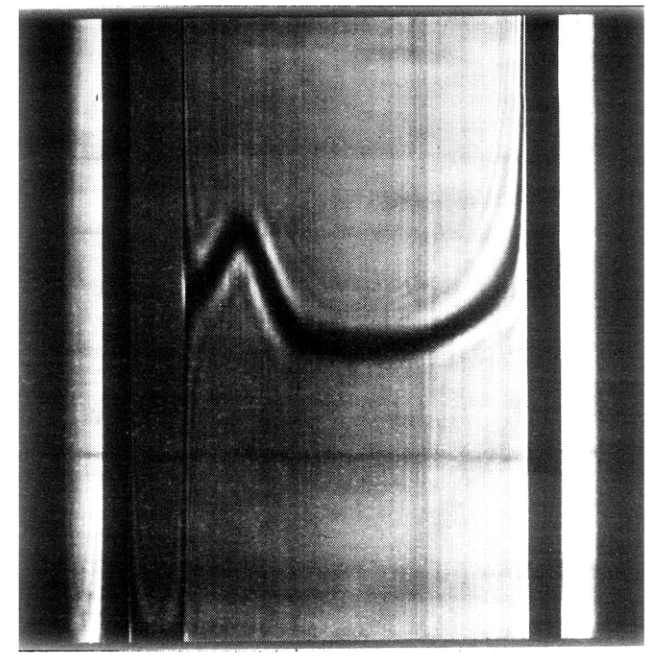

FIGURE 6: Sedimentation velocity ultracentrifugation of invertase at pH 11.9. Physical conditions as in Figure 3. The photograph was taken 52 min after reaching two-thirds speed. $s_{20,w}$ = 3.8 S.

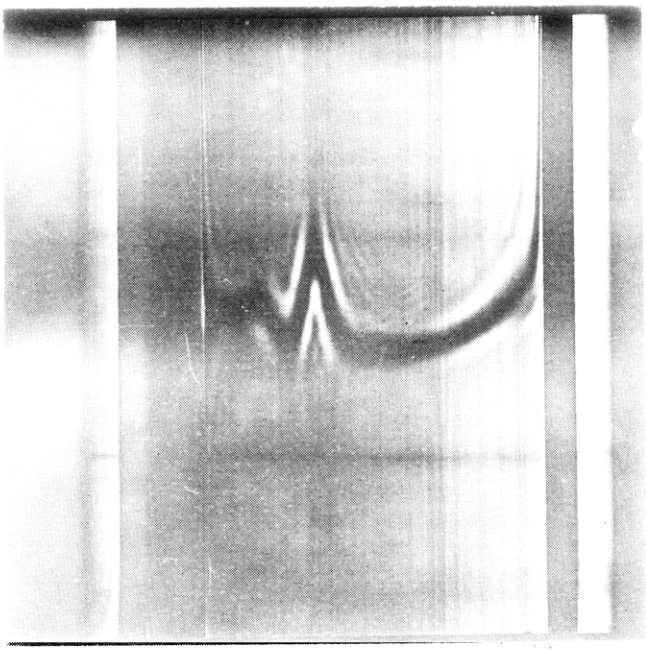

FIGURE 5: Sedimentation velocity ultracentrifugation of invertase at pH 8.9. Physical conditions as in Figure 3. The photograph was taken 30 min after reaching two-thirds speed. The major peak had an $s_{20,w}$ of 9.9 S and the minor peak, 5.6 S.

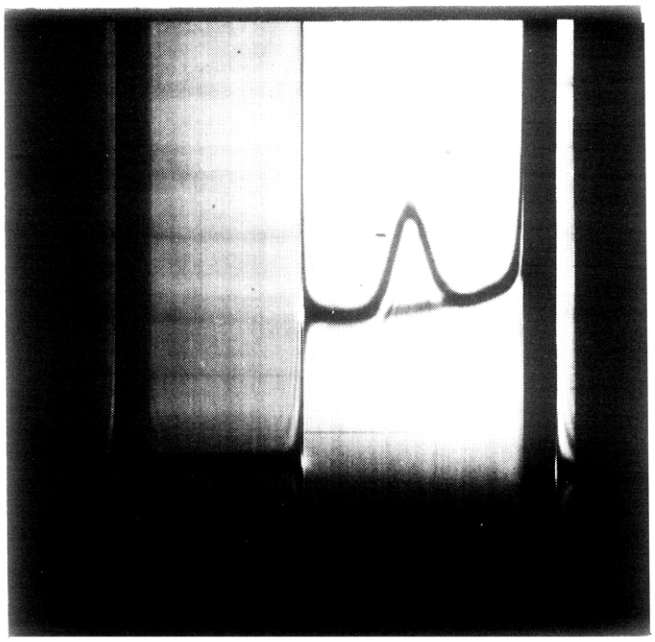

FIGURE 7: Sedimentation velocity ultracentrifugation of invertase at pH 3.0. Physical conditions as in Figure 3. The photograph was taken 10 min after reaching two-thirds speed. $s_{20,w}$ = 3.8 S.

TABLE I: Amino Acid Composition of *Neurospora* Invertase.

Amino Acid	g/100 g of Protein	Residues/Mole[a]	Integral No. of Residues[f]
Lysine	3.95	54.1 ± 0.5	55
Histidine	2.04	25.8 ± 0.6	26
Arginine	4.65	52.6 ± 0.6	54
Tryptophan[b]	4.35	44.3	44
Glucosamine (Nac)[c]	2.92	25.2	26
Aspartic acid	12.11	185.1 ± 0.6	189
Threonine[c]	7.74	134.6	137
Serine[c]	6.80	138.2	141
Glutamic acid	9.33	127.6 ± 0.4	130
Proline	5.01	90.9 ± 1.7	93
Glycine	5.00	154.2 ± 0.6	158
Alanine	4.87	121.3 ± 0.8	124
Half-cystine[d]	0.49	8.8	9
Valine[e]	6.69	119.0 ± 1.4	122
Methionine	1.46	19.3 ± 0.3	20
Isoleucine	3.61	55.8 ± 0.6	57
Leucine	7.16	111.5 ± 0.4	114
Tyrosine	5.72	61.8 ± 0.5	63
Phenylalanine	5.48	65.7 ± 0.3	67

[a] Based on a molecular weight of 187,200 for the protein portion of the glycoprotein. The variability indicated is the standard error of the mean. Results are from a minimum of six separate hydrolyses. [b] Determined spectrophotometrically by the method of Goodwin and Morton (1946). [c] Values extrapolated to zero time of hydrolysis. [d] Determined after performic oxidation. [e] Average value of three 72-hr hydrolysates. [f] Corrected for an estimated 97.9% recovery.

ous buffer method of Davis (1964) and a running pH of 9.3. Under these conditions the enzyme appeared as one major protein band and one barely detectable band when stained with Amido Black 10B (Figure 8). Gels run under the same conditions and stained for enzymatic activity using the method of Eilers *et al.* (1964) demonstrated that both bands were enzymatically active.

Amino Acid Composition. Amino acid analyses were performed on a Spinco 120C instrument using the 4-hr accelerated procedure based on the methods of Spackman *et al.* (1958). Samples containing 1 mg of invertase were hydrolyzed with 1 ml of constant-boiling HCl at 110° in sealed evacuated tubes for 24, 48, and 72 hr. Triplicate hydrolysates were prepared for each hydrolysis time.

The total amino acid composition is given in Table I. Glucosamine, serine, and threonine content were determined by extrapolation to zero hydrolysis time. The value for valine represents the average of the 72-hr values. The rest of the amino acids were determined by averaging the 24-, 48-, and 72-hr values. Tryptophan was determined by the method of Goodwin and Morton (1946). Cysteic acid was determined after performic acid oxidation using the method of Moore (1963). The number of residues indicated in the table is based on the molecular weight as determined by sedimentation equilibrium ultracentrifugation and corrected for carbohydrate content.

Electrofocusing. The protein was subjected to isoelectric

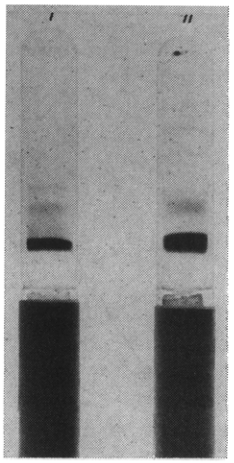

FIGURE 8: Polyacrylamide gel electrophoresis of purified invertase at pH 9.3. Sample size: gel I, 50 μg; gel II, 200 μg.

focusing utilizing the technique of Vesterberg and Svensson (1966). In this method the protein migrates to its isoelectric point in a pH gradient stabilized by a glycerol density gradient (0–60%). A 110-ml column was employed using the pH 4–6 ampholyte. A 2.8-mg sample of the enzyme was subjected to isoelectric focusing for 48 hr at 4°. There was one peak of protein and the enzymatic activity paralleled the protein distribution. The p*I* of the peak was 4.78.

Carbohydrate Composition. The carbohydrate content of the enzyme was determined by the phenol–H_2SO_4 method (Dubois *et al.*, 1956) and by the tryptophan–borosulfuric acid method (Badin *et al.*, 1953). The two colorimetric methods were in excellent quantitative agreement and indicated a hexose content of 11%. The pure enzyme was dissolved in 0.05 M acetic acid and applied to a Sephadex G-100 column (2.5 × 53 cm). The gel had been previously swollen in 0.05 M acetic acid. This solvent was chosen because it dissociates the enzyme (Metzenberg, 1964) to the 5.2S subunit. The protein was eluted with the same solvent at a flow rate of 18.6 ml/hr and 3.1-ml fractions were collected. The pH of each fraction was adjusted to pH 5.0 with 3 M sodium acetate and the optical density at 280 nm was measured. The hexose content of an aliquot of each fraction was determined by the phenol–H_2SO_4 method. The carbohydrate and protein profiles were symmetrical and could be superimposed (Figure 9). Qualitative studies of the carbohydrate moiety were performed by the gas–liquid chromatographic procedure of Lehnhardt and Winzler (1968). These studies revealed that mannose was the major hexose component of invertase, accounting for about 93% of the

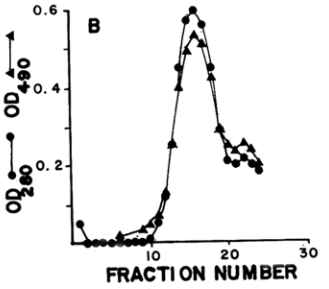

FIGURE 9: Gel filtration on Sephadex G-100 of 10 mg of the pure enzyme. (●) Optical density at 280 nm; (▲) optical density at 490 nm after reaction of the aliquot with the phenol–H_2SO_4 reagent.

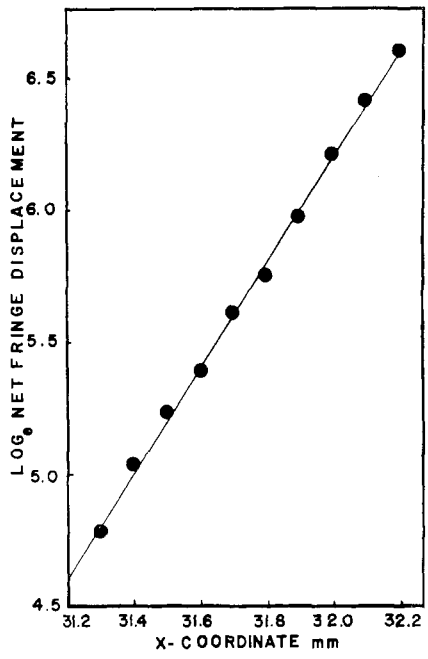

FIGURE 10: Plot of equilibrium ultracentrifugation of invertase at pH 5.0. A solution of invertase (0.098 mg/ml) was dialyzed against 0.05 M sodium acetate buffer (pH 5.0), containing 0.1 M NaCl, and subjected to centrifugation at 16,000 rpm in the An-D rotor in a 12-mm double-sector cell with sapphire windows for 24 hr at 20°.

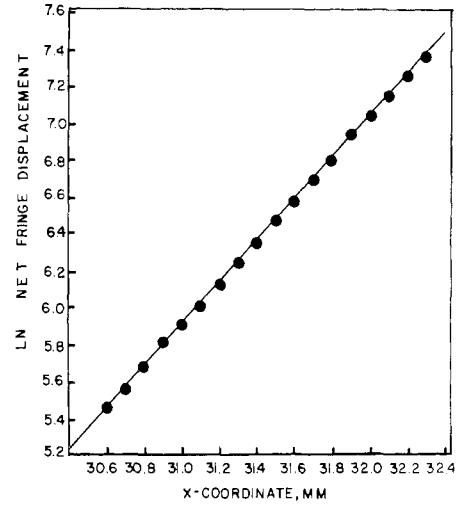

FIGURE 11: Plot of equilibrium ultracentrifugation of invertase under dissociating conditons. The protein (final concentration ~0.8 mg/ml) was dialyzed against 5.889 M guanidine hydrochloride containing 0.1 M 2-mercaptoethanol. Other conditions as in Figure 10, except that the rotor speed was 28,000 rpm and the temperature was 25°.

neutral sugar content. Galactose and a small amount of fucose were also present.

Sedimentation Equilibirum Centrifugation. The molecular weight of the protein at pH 5.0 was determined by the sedimentation equilibrium method of Yphantis (1964) using interference optics. The fluorochemical FC-43 was used as a base for a solution column 3 mm in height. The solvent was 0.05 M sodium acetate buffer (pH 5.0) containing 0.1 M NaCl. Initial protein concentrations ranged from 0.098 to 0.78 mg per ml. Photographs were taken on Eastman Spectroscopic plates, type II G. Equilibrium was attained employing a rotor speed of 16,000 rpm for 24 hr at 20°. Plotting the natural log of the fringe displacement as a function of comparator X coordinate yielded a straight line (Figure 10), indicating the homogeneity of the preparation. The molecular weight calculated for the native enzyme in a preparation containing 11% carbohydrate was 210,000 ± 15,600.

The molecular weight of invertase was also determined in a solvent containing 5.889 M guanidine hydrochloride and 0.1 M 2-mercaptoethanol (Figure 11). The rotor speed was 28,000 rpm and the temperature, 25°. Calculations were made using the solvent density given by Kawahara and Tanford (1966). Under these conditions the enzyme dissociated to give a subunit with a molecular weight of 51,500.

Calculation of $\bar{v}$. The $\bar{v}$ of invertase was calculated from its composition by the method of Cohn and Edsall (1943) using figures supplied by them for the $\bar{v}$ of amino acid residues and figures supplied by Gibbons (1966) for the $\bar{v}$ of hexoses and *N*-acetylhexosamines. The $\bar{v}$ of the native enzyme (11% neutral sugar) was 0.711 cm^3/g. The $\bar{v}$ of the protein moiety alone (including *N*-acetylglucosamine) was 0.723 cm^3/g.

Determination of Disulfide Content. The number of free sulfhydryl groups in the enzyme was estimated by titration with sodium *p*-hydroxymercuribenzoate in the presence of 4.0 M urea using the method of Boyer (1954). The titration indicated that the protein contained at the most 2 moles of cysteine/mole. The number of free sulfhydryl groups was also determined by the procedure of Ellman (1959) in the presence of 6 M urea. This method gave an estimate of one free sulfhydryl group per mole of enzyme. The amino acid analysis of performic acid oxidized protein indicated that the protein contained 9 moles of cysteine/mole. This would indicate the presence of four disulfide bonds per mole of enzyme.

Tryptic Peptide Map. The procedure used for tryptic digestion and separation of the peptides has been described by Helinski and Yanofsky (1962). The invertase was reduced and S carboxymethylated according to the method of Hirs (1967) prior to being digested with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone trypsin. Peptide maps were prepared on aliquots taken from the digestion mixture after 15 min, and 1-, 2-, 4-, and 6-hr digestion. About 43 peptides were detected on these maps by spraying with ninhydrin (0.3% in acetone).

Immunochemical Examination. Rabbit anti-invertase antiserum was prepared by footpad injection using Freund's adjuvant and the pure protein. The purity of the protein was then examined by double diffusion using the Ouchterlony plate method (Ouchterlony, 1949). The agar plates contained 0.01 M Tris buffer (pH 8.0). These plates showed one precipitin band with the purified enzyme and two bands with the crude extract. This agrees with the density gradient experiments on the crude extract which indicated that there are two active multimeric forms of the enzyme. The precipitin band of the purified enzyme was stained for enzymatic activity using the glucose oxidase reagent described by Comer (1956) containing 0.5 M sucrose. The precipitin band stained very dark green, indicating that the enzyme–antibody complex is active. The antiserum was found to lack neutralizing activity.

Discussion

The evidence presented in this paper indicates that *Neurospora* invertase is composed of four subunits and that the native form of the enzyme has a molecular weight of 210,000. The purity of the enzyme preparation is indicated by the agreement of protein and activity profiles in the glycerol density

gradient, and by the sedimentation of the enzyme in the ultracentrifuge as a single symmetrical peak at pH 5.0. The invertase described here was isolated from a *Neurospora* strain (Gratzner and Sheehan, 1969) which produces levels of enzyme ten fold higher than the wild-type organism and would not necessarily be identical with the invertase characterized by Metzenberg (1963a). However, the sedimentation coefficients of the two invertase preparations indicated that the enzymes are similar.

The sedimentation behavior of the enzyme as the pH is increased to 8.9 shows that the enzyme is capable of dissociation into subunits with an $s_{20,w}$ of 5.2 S. The fact that gel electrophoresis at pH 9.3 gave two protein bands, each of which was active, indicates that this 5.2S subunit is enzymatically active. This is also substantiated by the presence of the active subunits in the crude extract. The purification procedure of Metzenberg (1963a) was used in purifying the enzyme, hence it was not surprising that the purified material contained only the multimeric enzyme. These results are consistent with the findings of Metzenberg (1964) that the enzyme can be dissociated into active subunits by heat or by high salt concentration at low or high pH. Further dissociation of the enzyme at pH 3.0 or 11.9 to an entity with an $s_{20,w}$ of 3.8 S indicates either that the 5.2S active form is composed of smaller subunits or that the 5.2S subunit unfolds under these conditions to give a less compact structure. The boundary seen at pH 3.0 is symmetrical, whereas the one seen at pH 11.9 is asymmetrical and apparently contains material smaller than 3.8 S. The conversion of the 10.5S form into the 5.2S form is reversible, while conversion into the 3.8S form is only partially so.

Equilibrium centrifugation in the presence of guanidine hydrochloride and 2-mercaptoethanol indicated that the subunit molecular weight of invertase is 51,500. No evidence of heterogeneity was seen under these conditions. The number of lysine plus arginine residues per 210,000 molecular weight is 109. Thus, if the subunits are identical in primary structure as well as molecular weight, one would expect to obtain 27–28 peptides by tryptic digestion. In fact, 43 peptides were obtained, suggesting the possibility that more than one type of subunit is present. However, the number of tryptic peptides of unique primary structure may well be smaller than 43, since the protein contains hexose, glucosamine, and disulfide bonds. Variation in the number or position of attachment of the carbohydrate residues may result in tryptic peptides that are identical in primary structure but different in the amount of covalently bound carbohydrate. The question of the identity of the subunits is presently being investigated by determining the amino- and carboxy-terminal amino acids of the protein.

The small number of free sulfhydryl groups as determined by sodium *p*-hydroxymercuribenzoate titration in urea, coupled with the number of cysteic acid residues found after performic acid oxidation, indicates that the protein contains 4 disulfide bonds/210,000-dalton multimer. If S carboxymethylation was not complete, which would permit disulfide interchange, or if the S-carboxymethylation procedure affected other residues, extra peptides could appear on the peptide maps.

Neuman and Lampen (1967) have shown that yeast invertase is a glycoprotein, containing 50% mannan and 2–3% glucosamine. Our results suggest that *Neurospora* invertase is also a mannan protein. The enzyme contains 3% glucosamine and 11% mannose. Gel filtration of the dissociated enzyme on Sephadex G-100 and measurement of the carbohydrate content indicated that the amount of carbohydrate is constant over the eluted protein peak. This is in contrast to the purified yeast invertase (Neuman and Lampen, 1967) which appears to consist of a mixture of molecules with small variations in the amount of attached carbohydrate.

The cell wall of *Neurospora* can be separated into four major fractions: β-1,3-glucan, chitin, a glucan–peptide–galactosamine complex, and a heterogeneous fourth fraction containing simple sugars and sugar derivatives (Mahadevan and Tatum, 1965). Invertase is known to be localized in the intramural space (Metzenberg, 1963b); therefore the presence of glucosamine and mannan in the enzyme suggests the possibility that it may be covalently attached to a cell wall component. This idea has been supported by the finding that chitinase will release cell wall bound invertase (Meachum and Braymer, 1969). Lampen (1968) has postulated that the yeast enzyme is localized in the cell wall by the mannan moiety. Therefore it seems that both yeast invertase and *Neurospora* invertase are localized in the cell wall by a carbohydrate component attached to the protein. Further studies on the structure of invertase and its relationship to the cell wall should clarify the role of this enzyme in the structure and function of the fungal cell.

Acknowledgment

The authors wish to express their gratitude to Miss Jane Duckenfield and Miss Sylvia Schreiner for their technical assistance. The authors are indebted to Dr. R. J. Winzler, Department of Chemistry, Florida State University, Tallahassee, Fla., for performing the gas–liquid chromatographic analysis on the neutral sugars of invertase.

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Reassociation of Soluble Monoamine Oxidase with Lipid-Depleted Mitochondria in the Presence of Phospholipids*

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ABSTRACT: It has previously been shown that the monoamine oxidase of pig liver mitochondria can be rendered buffer soluble by extraction of the mitochondria with ethyl methyl ketone in the presence of ammonium sulfate and that the crucial point in the extraction procedure is to remove the highly acidic phospholipids from the mitochondria. By sucrose density gradient centrifugation and by gel filtration it was found that the enzyme forms soluble complexes with some highly acidic phospholipids but not with phosphatidylcholine.

When enzyme solution was mixed with a phospholipid dispersion and lipid-depleted mitochondria were then added, the enzyme bound to the mitochondrial residues. Such binding was observed with all highly acidic phospholipids tested, whereas phosphatidylcholine and phosphatidylethanolamine caused no binding. The effect on the binding of various amounts of cardiolipin, enzyme and lipid-depleted mitochondria was studied, but no simple stoichiometry was found. The lowest ratio of cardiolipin to rebound enzyme observed was about eight by weight, indicating that relatively large amounts of phospholipid were necessary for the binding. Binding occurred also with lipid-depleted red cell membranes and with lipid-depleted milk fat globule membranes. The prior formation of soluble enzyme–phospholipid complexes was not necessary for the binding since binding also occurred when lipid-depleted mitochondria were first relipidated and then added to enzyme solution. The extent of binding was higher at 37° than at 4° and was slightly decreased by high ionic strength. The rebound enzyme could not be extracted from the mitochondrial residues by buffer. It is concluded that this monoamine oxidase can interact with highly acidic phospholipids either in solution, forming soluble complexes, or with the same phospholipids bound to insoluble membrane residues, then forming insoluble complexes.

The major constituents of biological membranes are proteins and polar lipids. How these constituents are arranged to form the membrane is not known, although several detailed models have been proposed. One approach to this problem is the study of interactions between membrane proteins and polar lipids. Hollunger and Oreland (1970) have recently described a method for obtaining monoamine oxidase from pig liver mitochondria in water-soluble form by a two-step extraction with ethyl methyl ketone. L. Oreland and T. Olivecrona (to be published) have subsequently shown that extraction of the acidic phospholipids from the mitochondria is the crucial point in the extraction procedure and have proposed that the enzyme is bound to the mitochondrial structure *in vivo* by interaction with such phospholipids. In the present paper we show that the soluble enzyme rebinds to lipid-depleted mitochondria when anionic phospholipids are added. Since the enzyme activity can be measured by a simple spectrophotometric assay and is the same in the presence or absence of added phospholipids, we consider this a useful system in which to study the interaction between a membrane enzyme and phospholipids.

Materials

Phosphatidylcholine and Phosphatidylethanolamine. Two egg yolks were homogenized in chloroform–methanol (2:1, v/v) and the final volume of solvent adjusted to 250 ml. The homogenate was filtered and 50 ml of aqueous 0.01 M $CaCl_2$ was added. The mixture was allowed to separate into two layers. To remove nonlipid contaminants, the lower, chloroform, solution of lipid was passed through a column of cellulose powder and eluted with a further 50 ml of chloroform. The combined eluates were concentrated to 10 ml and poured into 100 ml of acetone (4°). The precipitate

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