References

Collins, K. D., and Stark, G. R. (1969), *J. Biol. Chem. 244*, 1869.
Collins, K. D., and Stark, G. R. (1971), *J. Biol. Chem. 246*, 6599.
Davies, G. E., and Stark, G. R. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 651.
Davies, G. E., Vanaman, T. C., and Stark, G. R. (1970), *J. Biol. Chem. 245*, 1175.
Gerhart, J. C., and Holoubek, H. (1967), *J. Biol. Chem. 242*, 2886.
Hammes, G. G., Porter, R. W., and Stark, G. R. (1971), *Biochemistry 10*, 1046.
Hammes, G. G., Porter, R. W., and Wu, C. W. (1970), *Biochemistry 9*, 2992.
Jacobson, G. R., and Stark, G. R. (1973), *Enzymes, 3rd ed* (in press).
Kirschner, M. W. (1971), Ph.D. Thesis, University of California at Berkeley, Berkeley, Calif.
London, R. E. (1973), Ph.D. Thesis, University of Illinois, Urbana, Ill.
Marshall, A., Schmidt, P. G., and Sykes, B. D. (1972), *Biochemistry 11*, 387.
Meighen, E. A., Pigiet, V., and Schachman, H. K. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 234.
Parsons, S. M., and Raftery, M. A. (1972a), *Biochemistry 11*, 1623.
Parsons, S. M., and Raftery, M. A. (1972b), *Biochemistry 11*, 1630.
Porter, R. W., Modebe, M. O., and Stark, G. R. (1969), *J. Biol. Chem. 244*, 1846.
Sachs, D. H., Schechter, A. N., and Cohen, J. S. (1971), *J. Biol. Chem. 246*, 6576.
Schmidt, P. G., Stark, G. R., and Baldeschwieler, J. D. (1969), *J. Biol. Chem. 244*, 1860.
Stark, G. R. (1971), *J. Biol. Chem. 246*, 3064.
Swift, T. J., and Connick, R. E. (1962), *J. Chem. Phys. 37*, 307.
Sykes, B. D. (1969), *J. Amer. Chem. Soc. 91*, 949.
Sykes, B. D., Schmidt, P. G., and Stark, G. R. (1970), *J. Biol. Chem. 245*, 1180.
Tanford, C. (1962), *Advan. Protein Chem. 17*, 69.
Weber, K. (1968), *Nature (London) 218*, 1116.

Purification and Characterization of Aspartic β-Semialdehyde Dehydrogenase from Yeast and Purification of an Isozyme of Glyceraldehyde-3-Phosphate Dehydrogenase†

Michael J. Holland‡ and E. W. Westhead*

ABSTRACT: Aspartic β-semialdehyde dehydrogenase has been isolated from commercial baker's yeast in high yield. Purified aspartic β-semialdehyde dehydrogenase was judged homogeneous by sedimentation equilibrium and by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate. Optimum conditions for purification include the use of deionized water, 5–10 mM EDTA, reducing agents, and low metal content ammonium sulfate, with all operations at 20–25°. The molecular weight of the enzyme is 156,000, and it appears to have four identical subunits of 41,000 ± 4,000. The 280:260 nm absorbance ratio is 1.9 without charcoal treatment. The copurification of aspartic β-semialdehyde dehydrogenase and yeast glyceraldehyde-3-phosphate dehydrogenase through six major purification procedures, as well as methods for the separation of five major isozymes of glyceraldehyde-3-phosphate dehydrogenase by hydroxylapatite chromatography, are also described.

During the past several years a number of laboratories have been engaged in comparative studies of dehydrogenases in an effort to establish general principles of enzyme catalysis and structure (*e.g.*, Sund, 1970). Among other things these studies have shown that the subunit molecular weights of dehydrogenases are quite variable and, more importantly, that there do not seem to be common critical residues at the active sites of these different dehydrogenases.

Recent analysis of the primary structures of alcohol dehydrogenase (Jörnvall and Harris, 1970; Jörnvall, 1970a,b), glutamate dehydrogenase (Smith *et al.*, 1970), and glyceraldehyde-3-phosphate dehydrogenase (Davidson *et al.*, 1967; Harris and Perham, 1968) have produced more puzzling results since the limited amount of sequence homology among these three enzymes does not provide any easily interpreted clues to their evolutionary history or to a generalized mechanism of action of these dehydrogenases. It appears that if these enzymes were in fact evolved from a common precursor, then the degree of divergence demanded by their respective functions was quite substantial. Since the reaction catalyzed by aspartic β-semialdehyde dehydrogenase is so similar to that catalyzed by glyceraldehyde-3-phosphate dehydrogenase, we decided that an investigation of its structure and properties would be valuable. The complexity of these two reactions is

† From the Department of Biochemistry, University of Massachusetts, Amherst, Massachusetts 01002. *Received July 11, 1972.* Supported by National Institutes of Health Grant AM11157. A preliminary account of this work has been given (Holland and Westhead, 1969). This work is part of the Ph.D. Thesis (1971) of M. J. Holland, which is available from University Microfilms, Ann Arbor, Mich. 48106.

‡ Present address: Department of Biochemistry and Biophysics, University of California at San Francisco, San Francisco, Calif. 94122.

relatively great, suggesting that the probability of the two enzymes evolving from a common precursor might be *a priori* relatively high. The fact that the enzymes have such biologically different roles and use different coenzymes would indicate that much of their protein structure might have undergone extensive divergence; however, the similarity of their reaction also suggests the possibility of some remaining critical structural and functional aspects, which could be easily studied.

Physiologically, aspartic β-semialdehyde dehydrogenase catalyzes the reduction of β-aspartyl phosphate to aspartic β-semialdehyde and inorganic phosphate, using NADPH. In yeast, this reaction is on the pathway of threonine biosynthesis.

Two partial purifications of yeast aspartic β-seimialdehyde dehydrogenase have been reported. Black and Wright (1955) reported a 30-fold purification from commercial baker's yeast and Surdin (1967), extending the Black and Wright purification to include fractionations on Sephadex G-200, DEAE-cellulose, and hydroxylapatite, obtained a 250-fold purification from a mutant of *Saccharomyces cerevisiae*. We present here a scheme for the complete purification of this enzyme in excellent yield from the very low quantity of the enzyme present in commercial yeast.

Yeast glyceraldehyde-3-phosphate dehydrogenase, present to the extent of about 5% of the dry weight of yeast (Krebs *et al.*, 1953), has been purified and studied extensively. Because of the current interest in this enzyme and the puzzling nature of its subunit interactions (Kirschner and Voigt, 1968; Kirschner *et al.*, 1966; Trentham, 1968; Stancel and Deal, 1969), we also give a purification procedure for one isozyme which we obtained as a by-product of our efforts to purify the aspartic β-semialdehyde dehydrogenase.

Material and Methods

Deionized water used throughout all experiments was supplied by a Continental Deionized Water System.

Oil-free baker's yeast was donated by Anheuser-Busch, Inc., Rahway, N. J., through the courtesy of Mr. E. T. Palomba, Plant Manager.

Aspartic β-semialdehyde was prepared as described by Black and Wright (1955) by the ozonolysis of L-allylglycine, using a Model T408 Welsback ozonator. After purification on a column by Dowex 50, the substrate was stored as eluted in 4 N HCl, at −10°. Preparations were assayed with aspartic β-semialdehyde dehydrogenase and found to be stable at least 1 year this way. Before use, samples of the substrate were titrated to pH 2–3 with 5 N KOH and used directly in the assay solutions. No pH shift of the assay solution occurs if the aspartic β-semialdehyde is sufficiently concentrated to permit use of small volumes (*e.g.*, 20 μl/ml of assay solution). The titrated aliquots were found to be stable at −10° for at least 2 months. The aldehyde is very unstable at pH values above 5, however.

DL-Glyceraldehyde-3-phosphoric acid was prepared from DL-glyceraldehyde-3-phosphate diethyl acetal, barium salt, according to the instructions of the supplier (Sigma Chemical Co.), except that hydrolysis was carried out for 10 min instead of 3 to increase the yield of substrate. After assay, the preparation was diluted with water to give a final concentration of 25 mg of D-glyceraldehyde 3-phosphate/ml. This solution was stored at −10° for up to 6 months with only a 10% loss of substrate.

Sources of other materials were: Sephadex G-25, Sephadex G-200, and DEAE-Sephadex A-50 from Pharmacia Fine Chemicals, Inc.; Bio-Gel hydroxylapatite and Dowex-50 (hydrogen form) from Bio-Rad Laboratories; Tris base (trizma base, reagent grade), protamine sulfate (grade II), $NADP^+$ (monosodium salt), NADPH (type I, tetrasodium salt), NAD^+ (grade III), NADH (grade III, disodium salt), sodium dodecyl sulfate, catalase, aldolase (crystalline suspension in $(NH_4)_2SO_4$), β-mercaptoethanol and Coomassie Brilliant Blue R (special enzyme grade), and Bromophenol Blue from Mann Research Labs., Orangeburg, N. Y.; Amido Black 10B from Hartman-Leddon Co., Philadelphia, Pa.; ammonium persulfate and *N,N,N,'N'*-tetramethylenediamine from E-C Apparatus Corp., Philadelphia, Pa.; acrylamide and bisacrylamide from Canal Industrial Corp., Rockville, Md.; dithiothreitol (A grade) from Calbiochem, Los Angeles, Calif.; trypsin (L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone) from Worthington Biochemical Corp., Freehold, N. J. All unspecified reagents were analytical reagent grade and were used without further purification.

Conductivity measurements were made with a Radiometer Type CDM2d conductivity meter and pH was determined with a Radiometer Type PHM 26 expanded-scale pH meter. A Gilford Model 2000 spectrophotometer equipped with a thermostated cell compartment was used for activity measurements.

Protein was concentrated in an Amicon Model 200 ultrafiltration cell with UM-10 Diaflo membrane. Protein concentration was determined by the method of Lowry *et al.* (1951), using crystallized bovine serum albumin for standard curve determinations. Enzyme samples were desalted on Sephadex G-25 before protein determinations in order to remove dithiothreitol which causes abnormally high Lowry determinations. The concentration of purified glyceraldehyde-3-phosphate dehydrogenase was measured by the absorbance at 280 nm, using $\epsilon_{280}^{0.1\%}$ 0.89 (Kirschner and Voigt, 1968).

Assay Procedures. Aspartic β-semialdehyde dehydrogenase activity measurements were made in the direction of β-aspartyl phosphate formation as described by Black and Wright (1955). The assay is based on absorption by reduced triphosphopyridine nucleotide (NADPH) at 340 nm. Assays were routinely performed at 30° in a total volume of 1 ml under the following conditions: 0.1 M potassium phosphate buffer, pH 7.5, 10 mM EDTA, 0.1 mM dithiothreitol, 2 mM aspartic β-semialdehyde, 0.5 mM $NADP^+$, and 30 mM K_2CO_3. A unit of enzyme activity is defined as the quantity of enzyme which causes the formation of 1 μmol of NADPH/min under these conditions.

Glyceraldehyde-3-phosphate dehydrogenase was assayed in the direction of 1,3-diphosphoglyceric acid formation by following the absorption of reduced diphosphopyridine nucleotide (NADH) at 340 nm. Assays were routinely performed at 30° in a total volume of 1 ml under the following conditions: 0.1 M potassium phosphate buffer, pH 7.5, 10 mM EDTA, 0.1 mM dithiothreitol, 1 mM NAD^+, and 4.2 mM glyceraldehyde 3-phosphate. A unit of enzyme activity is defined as that quantity of enzyme which forms 1 μmol of NADH/min under these conditions.

Gel Electrophoresis. Polyacrylamide disc gel electrophoresis was carried out as described by Brewer (1967), using a Buchler Instruments temperature-regulated disc electrophoresis apparatus; 7.5% acrylamide lower gels at pH 8.9, 2.5% acrylamide upper gels at pH 6.9, and Tris-glycine buffer, pH 8.3, were used.

Electrophoresis was run at a constant current of 4–5 mA/

tube for 2 hr. Gels were stained with 1% Amido Black (in 7.5% acetic acid) for 30 min and destained electrophoretically in 7% acetic acid.

Identification of bands by enzyme activity measurement was accomplished by slicing unstained gels into 1-mm sections. The sections were placed in 0.3 ml of assay buffer, broken into small pieces, and allowed to stand for 15 min, and then aliquots were assayed as described above.

Molecular Weight Determinations. Sedimentation equilibrium ultracentrifugation was done using the multichannel cell described by Yphantis (1964), with the scanner attachment for the Beckman Model E ultracentrifuge operated at the slowest scanning speed. Protein solutions were in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA. Since the specific volume of aspartic β-semialdehyde dehydrogenase was not determined, a value of 0.75 was used for molecular weight calculations.

Subunit molecular weight determinations by sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out by the procedure of Weber and Osborn (1969). Catalase, aldolase, yeast glyceraldehyde-3-phosphate dehydrogenase, and trypsin were used as standards at a concentration of 0.4 mg/ml. Acrylamide gels (10%) were used. After electrophoresis at 8 mA/tube for 4 hr, the gels were stained for 10 hr with Coomassie Brilliant Blue. The gels were then destained electrophoretically for 2 hr. The mobility of each enzyme was determined and plotted against the logarithm of its subunit molecular weight to obtain a standard curve from which the aspartic β-semialdehyde dehydrogenase subunit molecular weight could be calculated.

Results

Purification of Aspartic β-Semialdehyde Dehydrogenase

General Conditions. The purification was carried out at room temperature since both aspartic β-semialdehyde dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase (Stancel and Deal, 1969) undergo cold-induced irreversible inactivations. Enzyme fractions were stored at room temperature in an air-tight glass chamber saturated with toluene vapor. We found that this procedure completely prevented the growth of microorganisms in solutions stored at room temperature.

Crude Extract. Baker's yeast (2500 g) is suspended in 2.5 l. of 1 M Tris buffer, pH 8.5, containing 10 mM EDTA and 0.1 mM β-mercaptoethanol. This buffer concentration is needed for pH control and high specific activity. The cell suspension is homogenized to eliminate clumps and the cells lysed with a French press. Optimum cell lysis was obtained at a pressure of 15,000 psi. Upon lysis the pH drops to 5.5 and is readjusted to 7.5 by addition of solid Tris base. The extract is then centrifuged at 10,000*g* for 20 min at 25° and the supernatant dialyzed overnight against 15 l. of 0.1 M phosphate buffer, pH 7.5, containing 10 mM EDTA and 0.1 mM β-mercaptoethanol. Thorough dialysis is essential to a successful heat treatment.

Heat Treatment. The crude extract, pH 7.5, is heat treated at 55° for 6 min according to Surdin (1967). It should be noted that below pH 7.5 aspartic β-semialdehyde dehydrogenase is unstable to this heat treatment and poor yields result. The extract is quickly brought to 55° by swirling 400 ml of extract in a 2-l. Erlenmeyer flask in a boiling water bath. The extract is maintained at 55° in a constant temperature bath for 6 min with swirling. Optimum purification is achieved by then bringing the extract to 20° very quickly in a salt–ice bath. The extract is then centrifuged for 20 min at 10,000*g* and the inactive precipitate discarded.

Protamine Sulfate Precipitation. The heat-treated extract, pH 7.5, is made 0.25% in protamine sulfate by the addition of 15 ml of 2% protamine sulfate solution/105 ml of extract. After 30 min of stirring, the mixture is centrifuged at 10,000*g* for 20 min to eliminate the inactive precipitate. (We have found no purification by streptomycin sulfate up to 2%.)

Ammonium Sulfate Fractionation. 0–50% FRACTION. Solid ammonium sulfate (37.7 g) is added per 120 ml of protamine sulfate extract. The solution is stirred for 1 hr and then centrifuged at 10,000*g* for 20 min to remove the inactive precipitate.

50–70% FRACTION. An additional 18.5 g of solid ammonium sulfate was added per 132 ml of supernatant from the 0–50% fraction. After 2 hr of stirring, the solution is centrifuged at 10,000*g* for 20 min. The active precipitate is redissolved in 0.1 M potassium phosphate buffer, pH 7.5, containing 10 mM EDTA, 10% glycerol, and 0.1 mM dithiothreitol.

It is essential that low metal content ammonium sulfate be used to maintain enzyme activity. Sulfate is a strong reversible inhibitor of aspartic β-semialdehyde dehydrogenase and assays at this stage of purification are quite irreproducible.

Chromatography on DEAE-Sephadex A-50. DEAE-Sephadex was treated with 0.5 M HCl for 30 min followed by 0.5 M KOH for 30 min, neutralized, and suspended in equilibrium buffer, 1 mM potassium phosphate, pH 6.8–0.01 M KCl–0.1 mM dithiothreitol–10% glycerol (conductivity 0.7 mg/cm³). The ion exchanger is siphoned into a column (27 × 9 cm) and further equilibrated with 4 l. of the above buffer. The redissolved protein sample from the previous step is exchanged into DEAE-Sephadex equilibration buffer on Sephadex G-25 and then applied to the DEAE-Sephadex column. The protein is eluted with a 6-l. KCl gradient (0.01–0.2 M KCl) made up in the above buffer.

Fractions containing aspartic β-semialdehyde dehydrogenase activity are pooled and solid $(NH_4)_2SO_4$ (480 g/l.) is added to obtain 90% of saturation at room temperature. This solution must be stirred slowly overnight to obtain a quantitative recovery of enzyme. The active precipitate is collected by centrifugation at 10,000*g* for 20 min and resuspended in 0.1 M potassium phosphate buffer, pH 7.5, containing 10 mM EDTA, 10% glycerol, and 0.1 mM dithiothreitol.

It must be emphasized that we have observed 50–80% loss of activity at this point when the DEAE-Sephadex was washed less extensively than described above. With these precautions, 90% yields are regularly obtained. A typical elution pattern is shown in Figure 1, which also shows the position of glyceraldehyde-3-phosphate dehydrogenase.

Chromatography on Bio-Gel Hydroxylapatite with a Phosphate Gradient. Hydroxylapatite is suspended in 1 mM sodium phosphate buffer, pH 6.8, with careful stirring and then poured into a 25 × 5 cm column. Careful stirring of the gel is most critical to maintenance of the $CaPO_4$ crystals. Disruption of this crystal structure causes a marked decrease in flow rate, which affords poor resolution. The column is first washed with 150 ml of 2 M potassium phosphate buffer, pH 6.8, then equilibrated with 4 l. of 2 mM potassium phosphate buffer, pH 6.8, containing 0.2 mM dithiothreitol and 10% glycerol. A flow rate of 1.0–1.5 ml/min is obtained by gravity, provided the gel has been handled carefully.

The concentrated protein solution from the last step is exchanged into the buffer used for final equilibration of the hydroxylapatite column on a 25 × 5 cm column of Sephadex G-25. This protein is applied to the hydroxylapatite column and eluted with a 4-l. potassium phosphate gradient (0.1–0.2 M), pH 6.8, made up in hydroxylapatite equilibration buffer. A typical elution profile is shown in Figure 2. A measure of

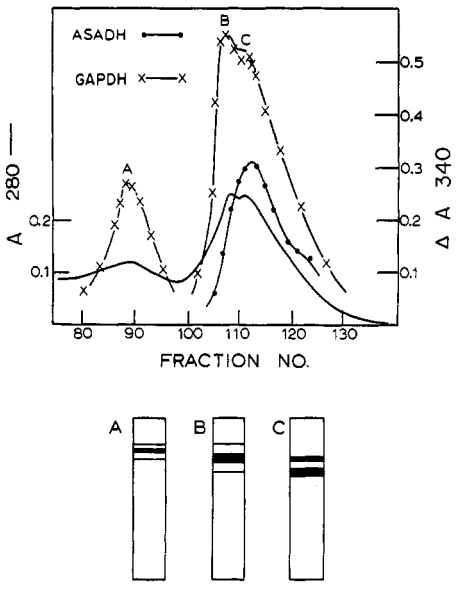

FIGURE 1: The elution pattern of aspartic β-semialdehyde dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase from a DEAE-Sephadex A-50 column (25 × 5 cm) eluted with a 4-l. (0.01–0.2 M KCl) linear gradient. The elution buffer contained 1 mM potassium phosphate buffer, pH 6.8, 0.1 mM dithiothreitol, and 10% glycerol. Fractions (10 ml) were collected: (×) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity; (●) aspartic β-semialdehyde dehydrogenase (ASADH) activity; (——) A_{280} nm. The pH 8.3 disc gel electrophoresis glyceraldehyde-3-phosphate dehydrogenase patterns for the three major protein peaks are shown below the column elution pattern.

purity of the preparation at this point is shown by the elution pattern of the preparation on Sephadex G-200 in Figure 3.

Chromatography on Hydroxylapatite with a Sulfate Gradient. The Bio-Gel hydroxylapatite column used in the previous step is regenerated with 150 ml of 2 M potassium phosphate buffer, pH 6.8, followed by 2 l. of 2 mM potassium phosphate buffer, pH 6.8, containing 0.2 mM dithiothreitol and 10% glycerol. Active fractions from the previous Bio-Gel hydroxylapatite column are pooled and concentrated by ultrafiltration. The concentrated protein is again equilibrated with the hydroxylapatite equilibration buffer on the Sephadex G-25 column and applied to the hydroxylapatite column and eluted this time with a 4-l. $(NH_4)_2SO_4$ gradient (0.2–0.6 M) made up in hydroxylapatite equilibration buffer. Results of this step are shown in Figure 4.

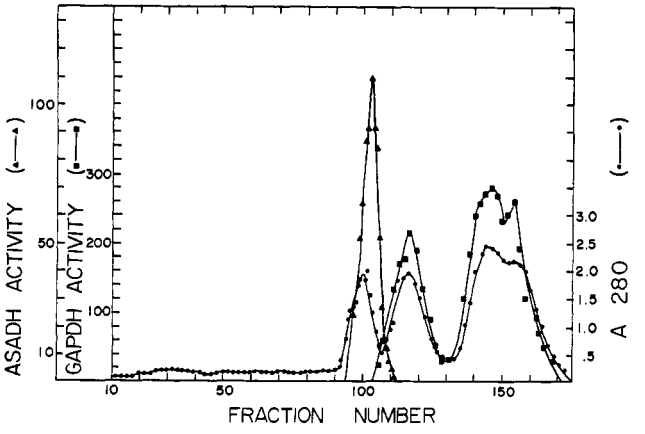

FIGURE 2: The elution pattern of aspartic β-semialdehyde dehydrogenase from hydroxylapatite with a phosphate gradient. The elution buffer contained 0.1 mM dithiothreitol, 10% glycerol, and variable potassium phosphate, pH 6.8. Fractions (10 ml) were collected: (■) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity; (▲) aspartic β-semialdehyde dehydrogenase (ASADH) activity; (●) A_{280nm}.

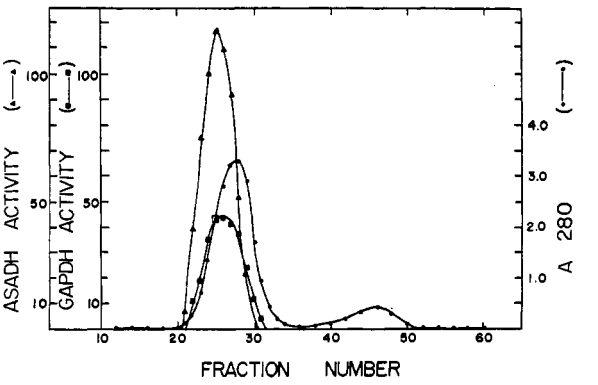

FIGURE 3: The elution pattern of aspartic β-semialdehyde dehydrogenase from Sephadex G-200 after hydroxylapatite chromatography with a phosphate gradient. The elution buffer contained 1 mM potassium phosphate buffer, pH 6.8, 0.1 mM dithiothreitol, 10 mM EDTA, and 10% glycerol. Fractions (5 ml) were collected: (■) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity; (▲) aspartic β-semialdehyde dehydrogenase (ASADH) activity; (●) A_{280nm}.

Acid Treatment. Active fractions from the last step are pooled and concentrated by ultrafiltration to between 4 and 5 mg/ml. This solution is put into a 12-ml conical centrifuge tube and the pH adjusted to 4.3 by dropwise addition, with stirring, of 2 M formic acid as described by Black and Wright (1955). The solution is allowed to stand at pH 4.3 (room temperature, *ca.* 25°) for 30 min with no loss in enzyme activity. At this stage of purification, aspartic β-semialdehyde dehydrogenase *does not* precipitate under these conditions. The inactive precipitate is removed by centrifugation and the supernatant brought to pH 7.5 by slow addition, with stirring, of 1 M Tris buffer, pH 8.5, containing 1 mM dithiothreitol.

Disc gel electrophoresis patterns after the last two purification steps are shown in Figure 5a and b. Duplicate polyacrylamide gels of the fractions were cut into 1-mm sections and assayed for aspartic β-semialdehyde dehydrogenase and glyc-

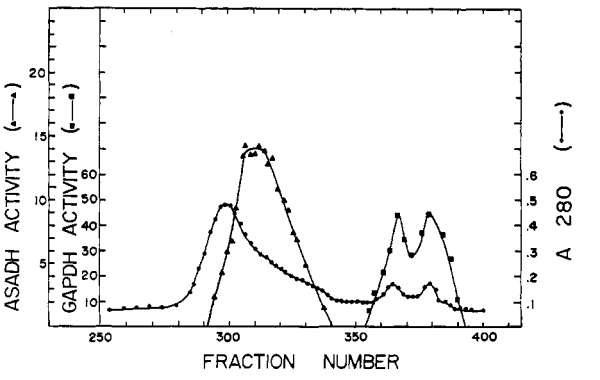

FIGURE 4: The elution pattern of aspartic β-semialdehyde dehydrogenase from hydroxylapatite with a sulfate gradient. The elution buffer contained 1 mM potassium phosphate buffer, pH 6.8, 0.1 mM dithiothreitol, 10% glycerol, and variable ammonium sulfate. Fractions (10 ml) were collected: (■) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity; (▲) aspartic β-semialdehyde dehydrogenase (ASADH) activity; (●) A_{280nm}.

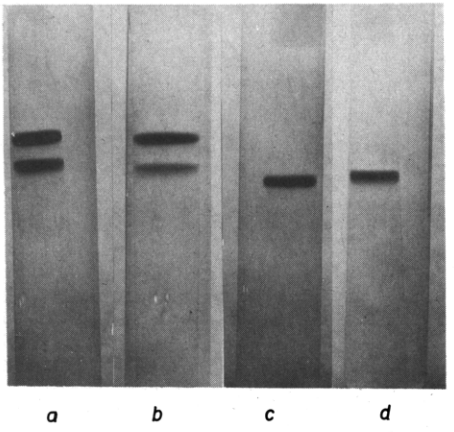

FIGURE 5: Disc gel electrophoresis patterns at pH 8.3. The upper band in gels a and b contains aspartic β-semialdehyde dehydrogenase (determined by cutting each gel into 1-mm sections and assaying each section for activity); (a) aspartic β-semialdehyde dehydrogenase after chromatography on hydroxylapatite with a sulfate gradient; (b) aspartic β-semialdehyde dehydrogenase after formic acid treatment; (c) aspartic β-semialdehyde dehydrogenase after chromatography on Sephadex G-200, specific activity 73; (d) glyceraldehyde-3-phosphate dehydrogenase species which eluted first from hydroxylapatite with a phosphate gradient.

TABLE I: Purification Scheme for Aspartic β-Semialdehyde Dehydrogenase and Glyceraldehyde-3-Phosphate Dehydrogenase from 400 g of Baker's Yeast.

	(Units × 10^3)	Protein (mg)	Sp Act.
Purification of Aspartic β-Semialdehyde Dehydrogenase			
Protamine SO_4^{2-}	467	3900	0.12
$(NH_4)_2SO_4$ (50–70%)		960	
DEAE-Sephadex A-50	413	228	1.8
Bio-Gel hydroxylapatite (PO_4^{3-})	374	48.0	7.8
Bio-Gel hydroxylapatite (SO_4^{2-})	330	25.4	13
Acid treatment	348	10.2	34
Sephadex G-200	289	3.98	73
Purification of a Single Isozyme of Glyceraldehyde-3-Phosphate Dehydrogenase			
DEAE-Sephadex A-50	21,286	197	108
Bio-Gel hydroxylapatite (PO_4^{3-})	6,692	57.2	117

eraldehyde-3-phosphate dehydrogenase activities as described under Methods. The upper band of both gels was identified as aspartic β-semialdehyde dehydrogenase on the basis of these activity measurements. Neither gel contained glyceraldehyde-3-phosphate dehydrogenase activity. The lower band is an unidentified impurity which is removed by the subsequent Sephadex G-200 chromatography step described below.

Chromatography on Sephadex G-200. A 100 × 5 cm column of Sephadex G-200 is equilibrated with 1 l. of deaerated 2 mM potassium phosphate buffer, pH 6.8, containing 10 mM EDTA, 0.2 mM dithiothreitol and 10% glycerol. The acid-treated protein sample (15 ml) is applied to the column and eluted by upward flow. Figure 6 shows a typical elution profile. The complete purification scheme is outlined in Table I which also shows, in the first column, the excellent overall recovery of enzymatic activity. Figure 5c shows a gel electrophoresis pattern of the enzyme after Sephadex G-200 chromatography, with only a single visible component.

Physical Properties of the Enzyme. Since glyceraldehyde-3-phosphate dehydrogenase from some species contains tightly bound pyridine nucleotide, the ratio of absorbancy at 280 nm to that at 260 nm was determined for pure aspartic β-semialdehyde dehydrogenase and found to be 1.9. This is a good indication that this enzyme is substantially free of bound $NADP^+$ (*cf.* Kirschner and Voigt, 1968).

The molecular weight of the purified enzyme (sp act. 73) was obtained by sedimentation equilibrium using the meniscus depletion technique of Yphantis (1964). An enzyme solution, 0.8 mg/ml, was centrifuged for 72 hr at 14,050 rpm. The plot of $\ln c_i - c_0$ *vs.* r^2 was linear over a sixfold range of protein concentration, giving a mol wt of 156,000 for aspartic β-semialdehyde dehydrogenase, *assuming* a specific volume of 0.75. Equilibrium sedimentation experiments at lower protein concentrations showed curvature near the meniscus with the best-fit linear portions yielding a mol wt from 140,000 to 147,000. In solutions containing 0.1 M KCl no satisfactory linear plots were obtained. The enzyme apparently readily dissociates at low concentrations under some conditions.

Subunit molecular weight determinations on gels containing sodium dodecyl sulfate were carried out as described under Methods. The calculated subunit molecular weight was 41,000 ± 4,000, a value found to be readily reproducible. This calculated subunit molecular weight indicates that there are four subunits in the native molecule of 156,000. Only a single clear band was found in the absence of marker enzymes indicating that all four subunits have the same molecular weight.

Kinetic constants obtained by us differ in only one respect from a previously published value (Black and Wright, 1955; Surdin, 1967). We obtained a K_m value for $NADP^+$ at pH 8.5, 30°, with excess carbonate, of about 3.6×10^{-5} M, in agreement with data of Black and Wright, but much less than the value of 5.4×10^{-4} M reported by Surdin.

The specific activity of this preparation is 73 μmol of NADPH oxidized per minute per milligram of protein and our overall yield of activity from crude extract to apparently pure enzyme is nearly 60% with a 600-fold purification.

Purification of One Isozyme of Glyceraldehyde-3-Phosphate Dehydrogenase. It may be seen from Figure 1 that as early as the DEAE-Sephadex step, glyceraldehyde-3-phosphate dehydrogenase partially separates into three groups of iso-

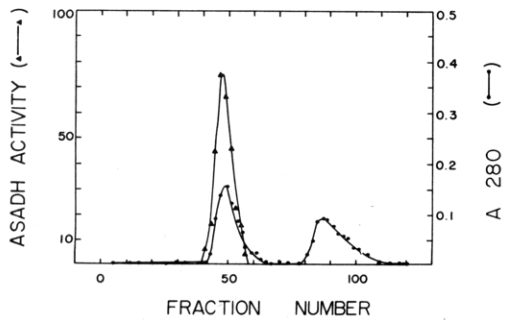

FIGURE 6: The elution pattern of aspartic β-semialdehyde dehydrogenase from Sephadex G-200 The elution buffer contained 1 mM potassium phosphate buffer, pH 6.8, 0.1 mM dithiothreitol, 10 mM EDTA, and 10% glycerol. Fractions (5 ml) were collected: (▲) aspartic β-semialdehyde dehydrogenase (ASADH) activity; (●) A_{280nm}.

zymes. Only the material present in the aspartic β-semialdehyde dehydrogenase fractions of Figure 1 was further purified. In Figure 2, the separation of this material into at least three types of glyceraldehyde-3-phosphate dehydrogenase occurs. The first peak of the glyceraldehyde phosphate enzyme is well separated from subsequent isozymes and, as shown in Figure 5d, this material migrates as a single band on gel electrophoresis. Nevertheless, this material is clearly composed of isozymes separable by sulfate elution from hydroxylapatite as shown in Figure 4. After DEAE-Sephadex, the specific activity of the glyceraldehyde-3-phosphate dehydrogenase was 108, in this particular preparation. Further chromatography on hydroxylapatite with phosphate and sulfate gradients did not appreciably change this value, indicating that the different species resolved by hydroxylapatite chromatography were not partially inactivated forms of glyceraldehyde-3-phosphate dehydrogenase.

Discussion

The data in Table I are taken from a single purification. The success of this preparation depends critically on efficient elimination of heavy metals from the preparation and preventing the oxidation of sulfhydryl groups. Furthermore, the enzyme is markedly cold inactivated and this loss of activity can become irreversible. If, for example, the temperature is 3° rather than room temperature during dialysis to remove ammonium sulfate, almost 90% of the activity is lost. Use of special low-metal ammonium sulfate and careful washing of all columns with buffer containing 0.1 mM dithiothreitol greatly improved our yields. The specific activities of our crude extracts approximated those of Black and Wright (1955) while the activity of the crude extract reported by Surdin was nine times lower. Our overall yields of 60% or better are an order of magnitude better than those reported by Surdin but can only be achieved if the preparation is carried out carefully and the final steps done rapidly. The pure enzyme loses as much as 50% of its activity in 2 weeks. In contrast, after the second hydroxylapatite step, the enzyme can be stored with little loss of activity for as long as 3 months.

The acid precipitation used by us is taken from the work of Black and Wright (1955), but functions in reverse here. That is, at the stage of purification in which we use it, aspartic β-semialdehyde dehydrogenase *does not* precipitate. The aspartic β-semialdehyde dehydrogenase is completely stable at pH 4.3 for more than 2 hr.

Surdin (1967) estimated the molecular weight of native aspartic β-semialdehyde dehydrogenase to be 140,000 on Sephadex G-200 and reported sedimentation coefficients at a number of different pH values using density gradient centrifugation. Employing catalase and alcohol dehydrogenase as markers, she estimated the molecular weight of aspartic β-semialdehyde dehydrogenase to be 165,000 at pH 8.4 and 109,000 at pH 3.0. These results, together with her finding that the enzyme is rapidly inactivated at 55° when the pH is lowered below 6.5, suggest that the aspartic β-semialdehyde dehydrogenase undergoes dissociation into subunits at lower pH values.

Our sedimentation equilibrium data show that the molecular weight of aspartic β-semialdehyde dehydrogenase is greater than reported values for glyceraldehyde-3-phosphate dehydrogenase. This observation is further substantiated by gel filtration data such as that in Figure 3. Such elution patterns consistently show aspartic β-semialdehyde dehydrogenase moving slightly ahead of glyceraldehyde-3-phosphate dehydrogenase (mol wt 144,000). The sharp activity peak for aspartic β-semialdehyde dehydrogenase on G-200 indicates that significant dissociation is not occurring on this column.

Our electrophoresis in sodium dodecyl sulfate solution gives subunit molecular weights in agreement with the sedimentation equilibrium data (41,000 ± 4,000), shows lack of different size subunits, and again supports the previous evidence that this protein has a molecular weight between 15 and 20% greater than that of glyceraldehyde-3-phosphate dehydrogenase.

Glyceraldehyde-3-Phosphate Dehydrogenase Isozyme. The highest activity reported for this enzyme is 145–158 μmol of NADH/minute per milligram at pH 8.5 (Kirschner and Voigt, 1968), while the values more usually reported for pure enzyme range from 60 to 90 μmol of NADH per minute per milligram at 30°. Our preparations routinely show a specific activity of about 120 μmol per minute per milligram at pH 7.5. Kirschner and Voigt also report an absorbance ratio A_{280}/A_{260} for pure enzyme, charcoal treated to remove bound NAD^+, of 2.13–2.18; our preparations had a ratio of 2.1 without prior treatment with charcoal.

Both the above authors and Lebherz and Rutter (1967) reported five electrophoretically distinct forms of the enzyme. Kirschner and Voigt (1968) purified the most electrophoretically mobile band on DEAE-Sephadex A-50. Further electrophoretic studies in their laboratory on the five major isozymes of glyceraldehyde-3-phosphate dehydrogenase revealed that each major band is composed of at least two subbands. Activity staining of the gels with nitroblue tetrazolium (3,3′-dimethoxybiphenylene-(4,4′)]-bis[2-(*p*-nitrophenyl)-5-phenylditetrazolium chloride]) revealed a total of 13–14 bands containing glyceraldehyde-3-phosphate dehydrogenase activity. The authors suggest the possibility of three homologous subunits randomly combining to form a total of 15 isozymes. Our results shown in Figures 1, 2, and 4 show that chromatography on hydroxylapatite is able to resolve these isozymes much better than DEAE-Sephadex can. In addition to improved resolution of these isozymes on hydroxylapatite, it should be emphasized that the specific activities of these resolvable forms of glyceraldehyde-3-phosphate dehydrogenase are very similar. The data suggest strongly that these are in fact different isozymes of glyceraldehyde-3-phosphate dehydrogenase and not partially inactivated forms of the enzyme found *in vivo*. We hope that this information may be useful to those interested in this particular enzyme.

The extensive copurification of these two aldehyde dehydrogenases is a preliminary indication of molecular similarity which will be further substantiated in the two succeeding publications.

Acknowledgment

We wish to thank Professor C. D. Cox, head of our Microbiology Department, for unrestricted use of that department's analytical ultracentrifuge.

References

Black, S., and Wright, N. G. (1955), *J. Biol. Chem. 213*, 39.
Brewer, J. M. (1967), *Science 156*, 256.
Davidson, B. E., Sajgo, M., Noller, H. F., and Harris, J. I. (1967), *Nature (London) 216*, 1181.
Harris, J. I., and Perham, R. N. (1968), *Nature (London) 219*, 1025.

Holland, M. J., and Westhead, E. W. (1969), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 28*, 2627 Abstr.
Jörnvall, H. (1970a), *Eur. J. Biochem. 14*, 521.
Jörnvall, H. (1970b), *Nature (London) 225*, 1133.
Jörnvall, H., and Harris, J. I. (1970), *Eur. J. Biochem. 13*, 565.
Kirschner, K., Eigen, M., Bittman, R., and Voigt, B. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1661.
Kirschner, K., and Voigt, B. (1968), *Z. Phys. Chem. 349*, 632.
Krebs, E. G., Rafter, G. W., and Junge, J. M. (1953), *J. Biol. Chem. 200*, 479.
Lebherz, H. G., and Rutter, W. J. (1967), *Science 157*, 1198.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Langley, T. J., and Melamed, M. D. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 724.
Stancel, G. M., and Deal, W. C., Jr. (1969), *Biochemistry 8*, 4005.
Sund, H., Ed. (1970), Pyridine Nucleotide-Dependent Dehydrogenases, New York, N. Y., Springer-Verlag.
Surdin, Y. (1967), *Eur. J. Biochem. 2*, 341.
Trentham, D. R. (1968), *Biochem. J. 109*, 603.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Adenosine 5′-Triphosphate Induced Cold Inactivation of Yeast Aspartic β-Semialdehyde Dehydrogenase†

Michael J. Holland‡ and E. W. Westhead*

ABSTRACT: Yeast aspartic β-semialdehyde dehydrogenase undergoes a time-dependent inactivation in the presence of adenosine 5′-triphosphate at 0°. Equilibrium studies of this inactivation at different protein concentrations show that tetrameric aspartic β-semialdehyde dehydrogenase dissociates under these conditions into inactive dimers. Optimum conditions for inactivation are 3.5 mM ATP, 0°, pH 6.5, and 2 mM dithiothreitol. Inactivation can be completely reversed by warming the reaction mixture to 25° in the presence or absence of ATP or by removing ATP at 0°. The reaction can be described as a reversible tetramer–dimer equilibrium with a dissociation constant of 5.2×10^{-8} mol/l. at pH 7.0 in the presence of 20 mM ATP at 0°. Inactivation by ATP under these conditions is cooperative and the data indicate that at least 2.5 mol of ATP/mol of native aspartic β-semialdehyde dehydrogenase is involved in the inactivation. The inactivation of aspartic β-semialdehyde dehydrogenase at 0° is also promoted, but more weakly, by NADPH–Tris-chloride buffer and potassium phosphate. NADH protects against inactivation while $NADP^+$ and NAD^+ appear to have no measurable effect on the enzyme at 0°. These data are discussed and compared with the published results for yeast glyceraldehyde-3-phosphate dehydrogenase.

The phenomenon of cold inactivation is widespread among different classes of enzymes, especially those with multiple subunits. The factors that influence cold inactivation and the structural changes accompanying it vary markedly (see, for example, the discussion in Irias *et al.*, 1969). Glyceraldehyde-3-phosphate dehydrogenase from both rabbit muscle and yeast have been shown by Deal and coworkers to undergo reversible cold inactivation specifically induced by the presence of ATP (Constantinides and Deal, 1969, 1970; Yang and Deal, 1969a,b; Stancel and Deal, 1969). Both of these tetrameric enzymes undergo dissociation during cold inactivation. The rabbit muscle enzyme dissociates into dimers or monomers depending on conditions, while the yeast enzyme was observed to form only monomers.

In our study of aspartic β-semialdehyde dehydrogenase we have been particularly interested in the question of homology between this enzyme and glyceraldehyde-3-phosphate dehydrogenase. The specific cold-induced changes of glyceraldehyde-3-phosphate dehydrogenase, which have been so thoroughly studied, reflect molecular properties of the protein quite separate from details of the catalytic site structure. This paper thus presents a study of the cold inactivation of aspartic β-semialdehyde dehydrogenase showing striking similarity with the behavior of glyceraldehyde-3-phosphate dehydrogenase and adding evidence for close structural homology between the two proteins.

Materials and Methods

Reagents. Adenosine 5′-triphosphate (Sigma grade, disodium salt) was obtained from Sigma Chemical Co., St. Louis, Mo. All other reagents are described in Holland and Westhead, 1973a. The standard assay solution had the following composition: 0.1 M potassium phosphate buffer, pH 7.5, 0.01 M EDTA, 10^{-4} M Clelands reagent, 0.5 mM $NADP^+$, 2 mM aspartic β-semialdehyde, 0.03 M $KHCO_3$, and 0.003 M Tris. The last two components were stored as a concentrated solution of 1 M $KHCO_3$ plus 0.1 M Tris, pH 8.5, and were added to the assay solution in a ratio of 30 μl/ml.

The enzyme used in these studies was either partially puri-

† From the Department of Biochemistry, University of Massachusetts, Amherst, Massachusetts 01002. *Received July 11, 1972.* Supported by National Institutes of Health Grant AM11157. A preliminary account of this work has been given (Holland and Westhead, 1969). This work is part of the Ph.D. Thesis (1971) of Michael J. Holland, which is available from University Microfilms, Ann Arbor, Mich. 48106.

‡ Present address: Department of Biochemistry and Biophysics, University of California at San Francisco, San Francisco, Calif. 94122.