ticular twisted hydrophobic pocket in the proper relationship to a site favorable for binding the cationic iminium group.

Acknowledgment

We thank Professor Joseph Harris for suggesting the use of acetonitrile for recrystallizing auramine O.

Supplementary Material Available

Supplementary material describing the results of the molecular orbital calculations in more detail will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 20 X reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number BIO-73-3459.

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Growth, Isolation, and Characterization of a Yeast Manganese Alcohol Dehydrogenase†

Patrick L. Coleman‡ and Henry Weiner*

ABSTRACT: In order to elucidate the role of zinc in yeast alcohol dehydrogenase, zinc was replaced by manganese, and properties of the manganese enzyme were compared with the zinc enzyme. A wild type *Saccharomyces cerevisae* (YU 1001) was anaerobically grown in a zinc-free, manganese-rich medium as well as in a normal zinc-containing medium. The Mn enzyme was isolated and estimated to be greater than 50% homogeneous. It was less stable than the corresponding Zn enzyme, and a modified isolation and purification scheme was developed. It was necessary to eliminate all heat steps from the purification and EDTA from the buffers and to add sucrose and mercaptoethanol. Both the Mn and the Zn enzymes from YU 1001 interact with antibodies prepared against baker's yeast alcohol dehydrogenase suggesting that their structures were similar to each other as well as to the baker's enzyme. The K_m values for the two YU 1001 enzymes were similar and close to the values for the baker's yeast enzyme; however, their R_F values on gel electrophoresis as well as their heat stabilities and ammonium sulfate solubilities differed. The pH profile for the three enzymes also differs such that the YU 1001 enzymes have a narrow bell-shaped curve with pK_{app} values of 6 and 8.5 for the Zn enzyme and 7 and 8.5 for the Mn enzyme. It could not be ascertained whether the two YU 1001 enzymes were products of the same gene or whether the presence of manganese altered the structure of the protein accounting for the lower stability of the Mn enzyme.

Alcohol dehydrogenases characterized from many different sources are invariably zinc metalloenzymes (references in Sund and Theorell, 1963). For this reason the role of zinc in the enzyme, especially that isolated from horse liver, has been extensively studied. The zinc-free horse liver enzyme is catalytically inactive (Åkeson, 1964; Oppenheimer *et al.*, 1967); however, it binds coenzyme and coenzyme analogs (Weiner, 1969; Mildvan and Weiner, 1969; Hoagstrom *et al.*, 1969) and substrate and substrate analogs (Iweibo and Weiner, 1972; Coleman *et al.*, 1972a), and retains its native molecular weight (Green and McKay, 1969; Coleman *et al.*, 1972a). In addition, the lack of catalysis with an activated substrate, bromoacet-

† From the Department of Biochemistry, Purdue University, West Lafayette, Indiana 47906. *Received October 24, 1973.* Supported in part by National Science Foundation Grant GB 31963. Journal Paper No. 4920 from the Purdue University Agriculture Research Station.

‡ This work is taken from the dissertation submitted by P. L. C. to Purdue University in partial fulfillment of the requirements for the Ph.D. degree, Aug 1972. Present address: National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Md 20014.

aldehyde, by the zinc-free enzyme suggests that the general acid role for zinc may not exist (Iweibo and Weiner, 1972). Zinc is required to maintain the tertiary structure of the horse liver enzyme and is responsible for conformational changes required in order for catalysis to occur (Iweibo and Weiner, 1972; Coleman *et al.*, 1972a).

Relatively little is known about the role of metal in yeast alcohol dehydrogenase. The enzyme is composed of four subunits of mol wt 36,000 (Kägi and Vallee, 1960), binds 4 mol of coenzyme[1] (Hayes and Velick, 1954), and has four very tightly bound zinc atoms (Vallee and Hoch, 1955; Kägi and Vallee, 1960). The enzyme is inhibited by *o*-phenanthroline and many other metal chelators (Vallee, 1955; Vallee and Hoch, 1955). There is an irreversible loss of activity and zinc below pH 6.0 (Hoch and Vallee, 1959) and the loss of zinc is accompanied by a shift in mol wt from 150,000 to 36,000 (Snodgrass *et al.*, 1960). Therefore, of all the functions postulated for zinc in horse liver alcohol dehydrogenase (*vide supra*) the only one verified for the yeast enzyme is that the metal is necessary for quaternary structural integrity.

The study of horse liver alcohol dehydrogenase was facilitated by the isolation of a stable zinc-free protein. This is not possible with the yeast enzyme since it dissociates into monomers upon metal loss. Therefore, we attempted to study the effects on the enzyme of metals other than zinc. Curdel (1966) has grown yeast in the presence of excess cobalt or manganese and the absence of added zinc. With this method of *de novo* metal substitution he has isolated a cobalt and a manganese D-lactate dehydrogenase (Curdel, 1966; Curdel, 1968) and a cobalt alcohol dehydrogenase (Curdel and Iwatsubo, 1968) which contained 68% zinc and 32% cobalt. They obtained their enzyme after short growth (48 hr) with high levels of cobalt (10 mM) and with no precautions to remove zinc or determine whether it was a contaminant in any of the substituents of the medium.

This paper reports the growth, isolation, and partial characterization of a manganese yeast alcohol dehydrogenase isolated from a zinc-free culture.

Materials and Methods

The various strains of the yeast *Saccharomyces cerevisae* used were: baker's, purchased as air-dried cells from Fleishmann's; YU 1 and YU 1001, two wild-type strains from the Lindegren Carbondale Collection at the Southern Illinois University, the gifts of Drs. Edwin Umbarger and Harlan Brown, Purdue University; and a strain of cobalt-grown yeast, yeast Foam (Curdel and Iwatsubo, 1968), was the gift of Dr. J. M. Jallon, Centre de Genetique Moleculaire, Gif-sur-Yvette, France.

The growth medium was modified from that of Phaff *et al.* (1966) by the following changes and additions: tryptophan, methionine, and histidine, 1 mg/l. each; glucose, 4–5 g/l.; and lactic acid, 1 g/l., to stabilize pH. Vitamin mixture #40060 General Biochemical was added at 0.5 g/l. Each Co or Mn growth was in the absence of Zn. Solid cultures contained 1% Bacto-Agar. All constituents of the medium were autoclaved before yeast was added.

Polyethylene vessels were used where possible. When glass was necessary it was extensively freed of easily removed metals by extraction with warm sulfuric and nitric acid solution and hot EDTA and diethyldithiocarbamate solution. No zinc was ever detected in the medium after growth in glass or in plastic vessels.

Cultures were initially plated on agar slants and then transferred to 10 ml of liquid culture in screw cap culture tubes if growth occurred on the slants. The 10-ml cultures were transferred to 50-ml cultures in small flasks. They were then transferred to 1-l. flasks and then to 20-l. carboys. Finally, the largest growth chambers used were 200-l. plastic garbage cans (Sears, Roebuck & Co.) which were inoculated with 20-l. carboys of yeast culture. The 200-l. container was sanitized by using 95% ethanol freely on all internal surfaces. Also the warm autoclaved solutions were poured into the can as soon as practical after they were removed from the autoclave. For anaerobic growth, nitrogen gas was generously bubbled through the medium which also served to keep the solution agitated. Mineral oil was floated on top to prevent oxygen from dissolving through the surface.

Cultures were always collected by centrifugation during log phase growth as determined by light scattering of the medium at 620 nm. Collected yeast was suspended in a volume of pH 7.5 sodium phosphate buffer, 0.1 M, equal in mass to the mass of cells to be suspended, to which was added 100 mg of toluenesulfonyl fluoride in dioxane/l. of diluted cells. This was immediately frozen. Before the preparation of the enzyme another 2 vol of the buffer was added to the thawed yeast suspension.

The cells were lysed using a French Pressure cell (American Instrument Co.) at 1500 psi. The solution was passed through twice to ensure total cell lysis. Further passages gave no increase in enzymatic activity.

The solution was centrifuged at 4° at 28,000*g* for 40 min as were all subsequent preparative centrifugations. The pellet was resuspended in buffer at 20–25° and stirred slowly for 2 hr and centrifuged, and the supernatant added to the previous supernatant. The pellet was discarded.

The combined supernatants were brought to 65% saturation with ammonium sulfate in P_i buffer.[2] After precipitation and centrifugation the resulting supernatant was discarded and the pellet was redissolved in 30% saturated ammonium sulfate–buffer solution. This suspension was centrifuged and the pellet discarded. This supernatant was dialyzed overnight against 55% saturated ammonium sulfate. After centrifugation the pellet was resuspended in 50% saturated ammonium sulfate–buffer solution and centrifuged, and the resulting supernatant was assayed for enzymatic activity. After centrifugation each pellet was resuspended at 5% lower ammonium sulfate down to 30% saturation. Each new supernatant was assayed for enzymatic activity and discarded if none were found. The fraction or fractions with the highest specific activity were then dialyzed overnight against solutions of ammonium sulfate which were 2–3% greater in saturation. This produced a precipitate with two- to threefold greater specific activity after it was dissolved in buffer and assayed. These procedures were continued until specific activity no longer increased.

Acrylamide Electrophoresis. Disc gel electrophoresis was used to establish homogeneity and estimate purity. The electrophoretic procedures used were those of Davis (1964) using 7% acrylamide gels. Activity stains contained (per 10 ml of P_i, pH 8.5 buffer): 50 mg of NAD^+, 0.9 mg of phenazine methosulfate, 0.4 mg of Nitro Blue Tetrazolium, and 100 μl of 95% ethanol. Gels were stained for protein using a 1% Amido

[1] Dickinson (1970) reports that only 3 mol of coenzyme bind per mol of enzyme, a result confirmed in this laboratory (Sanderson and Weiner, 1973).

[2] Per cent saturation defined at 25° by Green and Hughes (1955).

Schwarz solution in 7% acetic acid–water. The stained gels were analyzed using a scanning accessory for a Gilford spectrophotometer. Gels stained for protein were scanned at 550 nm, and the activity gels were scanned at 600 nm.

Metal Analysis. Metal concentrations were determined with a Perkin-Elmer spectrophotometer Model 214. The metal content of all reagents used in the medium was determined at the saturation concentration of the compound where practical. This was necessary to estimate an upper limit for metal contamination when no contamination was observed. Using this we found no zinc, manganese, or cobalt in glucose, the vitamin mix, $(NH_4)_2SO_4$, KH_2PO_4, NaCl, or in the many other reagents of the medium. When contamination of any compound was found the bottle of reagent was discarded and a new one from a different lot was obtained. Zinc contamination seemed to be a random event and invariably the second bottle was zinc free. There was no detectable cross-contamination of trace levels of zinc in salts or pure elements of the metals used as zinc substitutes. Since the medium was concentrated at least 100-fold before analyses for metals were made, 0.3 μg/l. of Zn and 3 μg/l. of Mn would have been detectable.

Enzyme Assay. The enzyme was typically assayed using 1.8 mM NAD^+ and 160 mM ethanol at pH 7.5 in phosphate buffer, 0.1 M at 25°. In crude homogenates the reaction was started by addition of ethanol, in the purer fractions by the addition of enzyme. The assay was performed on either a thermostated Gilford spectrophotometer or a thermostated Aminco filter microfluorophotometer fitted with excitation filter No. 4-7113 and emission filter No. 4-7116 (Wratten #2). Using either instrument activities were obtained using a Sargent recorder and manually determining the slope of the initial straight line of NADH absorbance or fluorescence.

Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Purified baker's yeast alcohol dehydrogenase has an absorbance at 280 nm of 1.26/mg per ml (Hayes and Velick, 1954).

Kinetic Analysis. Initial velocities were determined with an Aminco filter microfluorophotometer at 25°, initiating the reaction by the addition of enzyme. The instrument was equipped with an American Instrument Co. zero-suppression photomultiplier No. 10-267 and the variable speed–variable ratio recorder was sufficiently flexible that all velocities measured were approximately of the same slope. This results in approximately equal per cent error in all measurements.

Acetaldehyde concentration was determined by oxidation to acetic acid with an excess of NAD^+ using an aldehyde dehydrogenase (Feldman and Weiner, 1972). The absorbance at 340 nm of the NADH produced when the reaction is complete corresponds to the acetaldehyde concentration.

pH Profile. Rates of ethanol oxidation were measured from pH 5.5 to 9.1 in 0.1 M sodium phosphate. Initial rates of reactions initiated by the addition of either ethanol or enzyme were identical above pH 6.5. The pH was measured before and after the reaction. To ensure that a V_{max} assay was being employed at each pH, selective points were assayed with other concentrations of NAD^+ and ethanol.

Antibody Preparation and Inhibition of Enzyme Activity. Antibodies were prepared by injecting 10 mg of baker's yeast alcohol dehydrogenase in 2 ml of complete Freund's adjuvant three times subcutaneously to white laboratory rabbits at 3-week intervals. The rabbits were bled by cardiac puncture and the antiserum prepared as described by Coleman *et al.* (1972b). The enzyme–antibody reaction was investigated by double diffusion gel analysis and by addition of the antiserum to a solution of enzyme, substrate, and coenzyme in the spectrofluorometric assay described above.

Data Treatment. All constants and other values were determined using linear regression programs with a Wang Laboratories Model 360E/370/371 computer. Correlation coefficients were also determined. Selective constants were verified using a Cleland weighted program (Cleland, 1963).

Reagents. Metal standards were prepared from Beckman Co. 1000-μg/ml standards for zinc, manganese, cobalt, and nickel. The salts used in the medium were chloride salts of the highest purity available. Glucose was purchased from Nutritional Biochemicals and was found to be zinc free. Baker's yeast alcohol dehydrogenase was purchased from Miles. Boehringer-Mannheim was the source for NAD^+, NADH, and horse liver alcohol dehydrogenase. Bacto-agar and complete Freund's adjuvant were purchased from Difco. Pyrazole was the product of Eastman Organic as was acetaldehyde which was redistilled before use. Toluenesulfonyl fluoride was purchased from Aldrich. Folin Ciocalteu reagent for Lowry protein determination was purchased from Gradwohl Laboratories. Chelex is the product of Bio-Rad.

Results

Growth and Isolation. Yeast cultures were first plated on a normal nondeficient solid medium and allowed to grow at 30° anaerobically. Samples were then transferred to various enriched slants. The stock medium was Zn free and 0.3 mM of the appropriate metal (Co, Mn, and Zn) was added before the agar gelled.

Those strains which grew (baker's yeast did not grow on cobalt) were transferred to 10 ml of liquid culture in similar screw cap culture tubes. Care was taken to keep the concentration of the varied metal at 30 μM.[3] Aliquots were removed every few days to ascertain growth rates. When the turbidity measured at 620 nm suggested that the culture was near the end of log phase growth it was either used to inoculate a larger culture or about 2–5% was used to inoculate a similar sized culture and the remainder was centrifuged, resuspended, and frozen.

Some of the cultures became poisoned, probably due to the continuous presence of high levels of the transition metal. Among these were YU 1 and baker's Mn strains. Cobalt strains failed to grow when transferred to 20-l. carboys and were also inviable when collected and resuspended in 200-ml or 1-l. volumes of medium. Since many cultures failed to continue growth, it is possible that their prolonged presence in 30 μM cobalt or manganese was toxic (Weinberg, 1970).

Modification of Enzyme Preparation Compared with the Baker's Yeast Preparation. The preparation of the two YU 1001 enzymes followed the general procedures outlined by Racker (1950) and Hayes and Velick (1954). A number of their steps proved detrimental for the Mn enzyme and had to be modified to either increase the yield or stabilize the enzyme.

Yeast proteolytic enzymes which can digest enzymes in crude homogenates (Lazarus *et al.*, 1966) were shown to be inhibited by either sulfonyl fluorides or phosphorofluoridates (Fosset *et al.*, 1971). Toluenesulfonyl fluoride, a specific and stoichimometric inhibitor of serine proteolytic enzymes (Weiner *et al.*, 1966), was added to the cell slurry before it was

[3] The values of 0.3 mM metal were chosen for the solid cultures and 30 μM for the liquid cultures because Nickerson and Zerahn (1949) reported that 0.3 mM cobalt is toxic to liquid cultures, but not to solid cultures.

first frozen. The resulting activity in the crude homogenates was two- to threefold greater than if the inhibitor were not present.

The typical cell disruption technique of autolysis had to be avoided because of loss of enzymatic activity, even in the presence of toluenesulfonyl fluoride. Temperature sensitivity experiments (to be discussed) suggest that the YU 1001 alcohol dehydrogenases probably denature as the cells were lysing for a few hours at 37°. Passage, twice, through a French pressure cell broke cells apart as efficiently and did not decrease the enzyme activity. The use of a French pressure device together with a hydraulic press succeeded in increasing our activity tenfold compared with the 37° autolysis step (Racker, 1950). The incubation at 54° for 5–10 min and the two extractions with organic solvent were also omitted from the preparation.

Racker (1950) indicated the necessity of having 1.0 mM EDTA in the buffer to protect yeast alcohol dehydrogenase from heavy metal inactivation of sulfhydryl groups. To test whether the Mn YU 1001 yeast alcohol dehydrogenase was similarly insensitive to EDTA, samples of a 40% saturated ammonium sulfate supernatant were first dialyzed to remove the salt and then were dialyzed at 4° against pH 7.5 sodium phosphate buffer, 0.1 M, anaerobically, in the presence of 0.1% mercaptoethanol and in the presence or absence of 1.0 mM EDTA. The Mn enzyme was inactivated by dialysis against 1.0 mM EDTA, whereas neither the baker's nor the Zn YU 1001 enzyme was inactivated.

Storing the Mn-containing enzyme under nitrogen in the presence of 20% sucrose and 0.1% mercaptoethanol stabilized the enzyme against denaturation. Neither glycerol (30%) and 0.1% mercaptoethanol nor 3 mM glutathione stabilized the enzyme. The partially purified Zn-grown enzyme was tested to determine whether it too required ~20% sucrose, 0.1% mercaptoethanol, and anaerobic conditions to remain reasonably stable. None of these conditions was found necessary for preservation of the activity of the Zn-grown alcohol dehydrogenase; however, in order to compare the results between the two enzymes, conditions were kept identical.

From a typical preparation of the Mn enzyme from 100 g of wet packed cells, 1–2 mg of protein was isolated which contained 0.5–1 mg of Mn yeast alcohol dehydrogenase. This represents a 28% yield and an 80-fold purification. In a typical preparation of the Zn enzyme from 100 g of wet packed cells 10 mg of protein was isolated which contained 9 mg of Zn yeast alcohol dehydrogenase. This represents a 40% yield and a 50-fold purification of the zinc-containing enzyme. In addition to the differences in yield and stability the Zn-grown alcohol dehydrogenase was invariably found in the supernatant from the 45% saturated ammonium sulfate precipitation, while its Mn-grown counterpart was solubilized in the 40% or even the 35% fraction.

Effect of Freezing Cells. The Mn-grown cells lost >95% of the alcohol dehydrogenase activity when frozen for 2 months. The cells, though, remained viable. The Zn-grown cells were more stable with respect to loss of enzymatic activity.

Properties of Manganese- and Zinc-Grown YU 1001 Alcohol Dehydrogenase. ELECTROPHORETIC ANALYSIS. The purest fractions of each enzyme were assayed for purity and homogeneity *via* 7% acrylamide gel electrophoresis. The gels were stained for both total protein and alcohol-active protein. A scan of the gel pattern for the Zn-grown enzyme (Figure 1) shows that the enzyme has one large active peak among five distinct though much smaller protein bands. The active peak comprises >70% of the total protein stain as determined with a Du Pont Model 310 curve resolver. Scans of other preparations showed 70–80% active protein but with two active bands. One contained >80% of all the activity present, but the protein ratios were closer to 60:40 than 80:20. The phenomenon of two active baker's enzymes has been observed frequently in this laboratory and others (Sund and Theorell, 1963; Vallee, 1960). The Mn-grown alcohol dehydrogenase was resolved into five protein bands of which two showed activity. The Mn alcohol dehydrogenase was estimated to be 50% pure. One striking difference between the Mn and Zn enzymes was the R_F. The Zn-grown enzyme has an R_F of 0.33 ± 0.03, while the Mn-grown enzyme had a major band at 0.45 ± 0.04 as well as a minor one at 0.37 ± 0.03. The value of 0.33 is identical with that obtained routinely in this laboratory for the commercial baker's yeast Zn alcohol dehydrogenase.

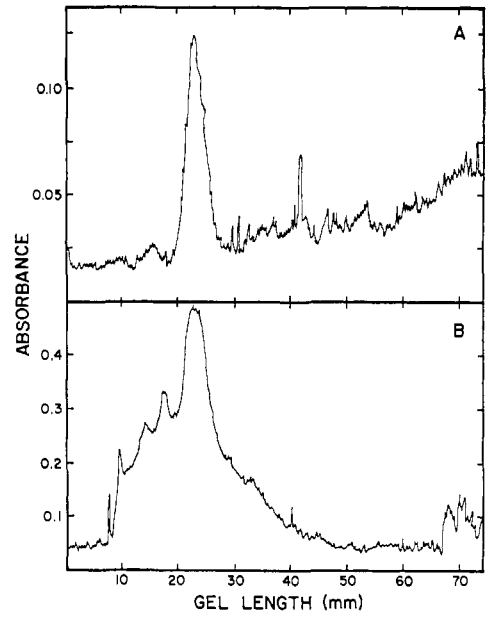

FIGURE 1: A comparison of active protein with total protein for Zn-grown YU 1001 alcohol dehydrogenase *via* acrylamide electrophoresis. Identical 200-μl samples of protein were electrophoresed and stained for activity (A) or protein (B). The gels were then scanned using a gel scanning accessory to a Gilford spectrophotometer and the absorbance changes were recorded. Many such gels were examined in order to obtain accurate values for R_F and the percentage composing the active band. This was obtained using a Du Pont Model 310 curve resolver to obtain the relative areas under the activity peak and entire scan.

METAL ANALYSIS. Both YU 1001 enzymes were analyzed for manganese and zinc after extensive dialysis against pH 7.5 phosphate, 0.1 M, containing 20% sucrose. Chelex was added outside the dialysis sac to bind all loosely bound metals. Results of metal analysis of different batches of enzyme revealed that there was no detectable Zn in the Mn enzyme. In a representative analysis a sample of Mn-grown protein, 0.035 mg/ml, contained 0.45 μM Mn and 0.00 μM Zn; analysis of a 0.104-mg/ml sample of Zn-grown protein resulted in 0.00 μM Mn and 2.46 μM Zn. Using the estimations of purity above, each enzyme has 3.7 ± 0.6 g-atoms of metal.

ANTIBODY BINDING. The inhibition of the activity of the two enzymes was similar (Figure 2). The addition of 62 μl of an antibody solution was needed to half-inhibit the Mn enzyme's activity while the Zn enzyme required 120 μl. The commercial baker's yeast enzyme required only 25 μl to half-inhibit (data not shown). All enzyme concentrations were 0.1 mg/ml. The

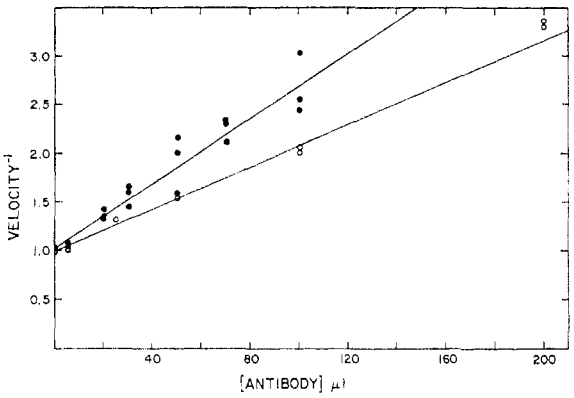

FIGURE 2: The inhibition of the Zn- and Mn-grown enzyme activities by antibody produced against baker's enzyme. The activity was measured in a V_{max} ethanol assay. Antibody was added after all reactants and 100 μg of enzyme were added. The slope of NADH absorbance *vs.* time measured immediately after the addition of antibody or 1 min later yielded identical. K_I values. For the YU 1001 enzymes K_I^{Mn} = 62 μl and K_I^{Zn} = 120 μl and for the inhibition of the commercial baker's yeast Zn enzyme (data not shown) K_I = 25 μl.

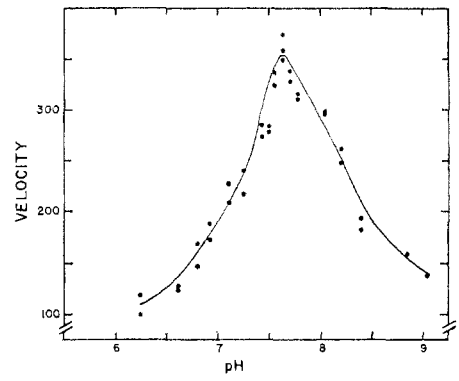

FIGURE 3: pH profile of Mn-grown YU 1001 alcohol dehydrogenase. NADH formation rate was measured fluorometrically in various phosphate solutions from pH 6.26 to 9.10. The reaction was initiated by the addition of enzyme to 160 mM ethanol and 1.8 mM NAD^+.

double diffusion experiments (not shown) also suggest that the antibodies react very similarly with all three enzymes. Only one line was found, and it contained no spurs.

TEMPERATURE SENSITIVITY. The baker's yeast enzyme is stable to moderate exposure to heat (Racker, 1950), a property utilized in the preparation of yeast alcohol dehydrogenase. Mn YU 1001 and Zn YU 1001 enzymes as well as a commercially prepared Zn baker's alcohol dehydrogenase were placed in a water bath at 31, 37, 46, or 54° and aliquots were withdrawn periodically and assayed at room temperature. The half-lives for the activity loss are given in Table I. The commercial baker's enzyme is far more stable at all temperatures than either of its counterparts. More significant is that the Zn YU 1001 enzyme is far more stable than the Mn enzyme.

FLOURESCENCE. The increase in NADH fluorescence upon addition of yeast alcohol dehydrogenase (Duysens and Kronenberg, 1957) has been used in the active-site titration of yeast alcohol dehydrogenase (Dickinson, 1970). Neither of the two YU 1001 enzymes gave fluorescence enhancement nor a blue shift of fluorescence.

KINETICS. Kinetic constants at pH 7.5 for the Zn and Mn YU 1001 enzymes are given in Table II. Even with the zero-suppression photomultiplier the light scattering and base fluorescence of NADH oxidation reaction solutions were too great to blank out and maintain a sensitivity sufficient to record the decrease in coenzyme fluorescence. Hence, we do not report values for the NADH–aldehyde side of the reaction catalyzed by Mn alcohol dehydrogenase. The K_m values for acetaldehyde and NADH for the baker's yeast enzyme in Table II are from Gierer (1955) while those for the NAD^+–ethanol reaction were performed in this laboratory.

pH–VELOCITY PROFILE. The pH profile of Mn YU 1001 alcohol dehydrogenase (Figure 3) shows a maximum velocity near pH 7.6 ± 0.1, whereas the maximum for the Zn YU 1001 (Figure 4) is 7.2 ± 0.2. Each of these values differs from the value of pH 8.6 given by Wallenfels and Sund (1957) and Wenger and Bernofsky (1971) for baker's enzyme. Assays at a few pH values between 7.8 and 9.3 confirm that the commercial enzyme had an optimum between pH 8.2 and 8.6. Also, the pH profiles for the two YU 1001 enzymes are narrow compared with the very broad peak of the baker's yeast alcohol dehydrogenase (Wallenfels and Sund, 1957; Wenger and Bernofsky, 1971).

EDTA AND PYRAZOLE INHIBITION OF YU 1001 ALCOHOL DEHYDROGENASE. Samples of all three enzymes were dialyzed 30 hr against pH 7.5 phosphate buffer at 4° in the presence and absence of 1.0 mM EDTA and then assayed for both enzymatic activity and metal content. The Mn content of Mn YU 1001 alcohol dehydrogenase was below the limits of detection for Mn and the enzymatic activity was <1% of the control. Both Zn enzymes dialyzed against EDTA had the same activity and metal content as their respective controls.

Pyrazole, a metal ligand and competitive inhibitor for ethanol binding in horse liver alcohol dehydrogenase (Theo-

TABLE I: Half-Lives for Loss of Enzymatic Activity at Elevated Temperatures.[a]

Alcohol Dehydrogenase	Half-Life (sec) at 54°	46°	37°	31°
Zn baker's	1850	2800		40,000
Zn YU 1001	68	540	2500	5,500
Mn YU 1001				60

[a] Enzyme was assayed at 25° immediately after removal from the incubation mixture. All protein concentrations were 0.25–0.60 μM.

TABLE II: K_m Values for the Four Active Ligands of the Yeast Alcohol Dehydrogenases.[a]

Enzyme	Ethanol (mM)	Acetaldehyde (μM)	NAD^+ (μM)	NADH (μM)
Zn baker's	20	300[b]	430	30[b]
Zn YU 1001	19.1	642	474	28
Mn YU 1001	57		137	

[a] The values for the K_m's were determined by a computer fit of the data. All experiments were at pH 7.5 phosphate, 0.1 M, at 25°. Errors were ±10% or less. [b] From Gierer (1955), values at pH 7.8 phosphate, 0.05 M (+ 1% glycine as heavy metal chelator).

rell and Yonetani, 1963), inhibits baker's yeast alcohol dehydrogenase (Singlevich and Barboriak, 1970). It is also a competitive inhibitor of the alcohol dehydrogenases (data not shown). Using nearly K_m ethanol and NAD^+ concentrations (20 mM and 300 μM, respectively), K_I values for pyrazole were obtained from inhibition of the initial velocity of the reaction with no preincubation of enzyme with pyrazole. The values were: Mn YU 1001, 55 μM; Zn YU 1001, 24 μM; Zn baker's yeast, 30 μM. The latter value differs greatly from the previous value of 1500 μM (Singlevich and Barboriak, 1970), possibly because theirs was a discontinuous assay which measured the total amount of product formed over a period of 1 hr.

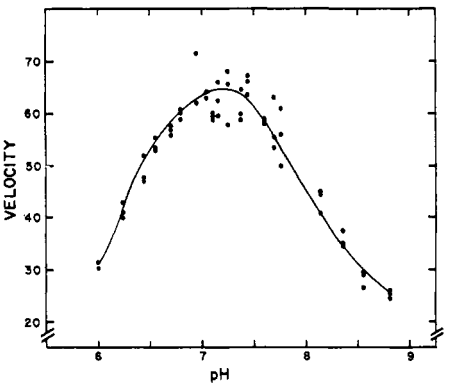

FIGURE 4: pH profile of Zn-grown YU 1001 alcohol dehydrogenase. All conditions were the same in as Figure 3.

Discussion

We have isolated a yeast alcohol dehydrogenase which contains manganese as its only metal. The Mn enzyme is less stable than either the zinc enzyme from the same strain (YU 1001) or from baker's yeast. Due to the lowered stability an altered preparation for the enzyme had to be used. The most significant changes in this isolation procedure compared with other procedures for isolating baker's yeast alcohol dehydrogenase (Racker, 1950; Hayes and Velick, 1954) are in the use of the French press to lyse the cells and omission of the heat step from the procedure and EDTA from the buffers. It was also necessary to have 20% sucrose and 0.1 M mercaptoethanol in all buffers in order to keep the Mn enzyme active. Parameters of structural integrity are drastically changed, suggesting a structural role for metal in the yeast enzyme. Thermal stability is decreased greatly in the Mn enzyme. The Mn enzyme is also much more sensitive to spontaneous oxidation as indicated by the requirement for mercaptoethanol and anaerobic storage conditions. We observed that the Mn enzyme required the presence of 20% sucrose which is generally used to keep easily dissociable proteins intact in order to retain activity, while Zn YU 1001 alcohol dehydrogenase did not.

It might be argued that these results suggest that two different enzymes from different structural genes are being studied, similar to the alleles of alcohol dehydrogenase found by Lutstorf and Megnet (1968) and Wenger and Bernofsky (1971). One important factor necessary to produce their observations was a difference in medium or growth conditions. The two YU 1001 enzymes were grown under conditions differing only in 2.5 μM ZnCl, a compound and concentration unlikely to cause the 100% repression–derepression. The substantial difference in the R_F values of the two YU 1001 enzymes, 0.33 compared with 0.45, may be more easily accounted for by differences in amino acid composition of the proteins, but structural differences caused by the different metals may also result in changes on the surface of the protein. This problem cannot be resolved until a homogeneous preparation of each YU 1001 enzyme is available so that at least the amino acid compositions can be compared.

The antibody results cannot be used to show that the two YU 1001 enzymes are products from the same gene since both enzymes interact with the baker's antibodies to the same extent as the commercial baker alcohol dehydrogenase. However, the YU 1001 enzymes are the only alcohol dehydrogenases which have been shown not to enhance the fluorescence of bound NADH. Secondly, both YU 1001 enzymes are more heat labile than the baker's yeast enzyme. However, the Mn enzyme being so much less stable compared with its zinc counterpart still leaves the two possibilities open: that is, the metal stabilizes the structure or the two enzymes have different primary structures and hence different stabilities.

In addition to a possible structural role, the metal could function in binding substrates or directly interact in the catalytic reaction at the active site.

The lack of any drastic changes in the K_m values for the ethanol–NAD^+ half of the reaction suggests that there is no metal influence on ethanol or NAD^+ binding.

From only the pH effects on V_{max} it is not possible to ascertain whether or not the metal performs a catalytic role. The bell-shaped curves presented in Figures 3 and 4 show that two different groups are involved in catalysis providing that one pK_{app} is not that of a group which influences the conformation of the enzyme. If the metal is directly involved in catalysis, it could be acting as a general acid, as postulated originally by Abeles *et al.* (1957). Recent model reactions investigated by Creighton and Sigman (1971) have reinforced this possibility.

Before it can be determined whether the Mn and Zn enzymes are products of the same gene or to determine unequivocally the role of metal in alcohol dehydrogenase it will be necessary to totally purify and stabilize both enzymes from this yeast strain. The magnetic resonance studies as reviewed by Mildvan and Cohn (1970) as well as a further comparison of the two YU 1001 enzymes then can be made.

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Stereochemistry of Reduction of D-Glyceraldehyde Catalyzed by a Nicotinamide Adenine Dinucleotide Phosphate Dependent Dehydrogenase from Skeletal Muscle†

D. J. Walton

ABSTRACT: Methods have been developed for determining the stereochemistry of the dehydrogenase-catalyzed reduction of D-glyceraldehyde by NADPH. To decide upon whether the A (*pro*-4*R*) or B (*pro*-4*S*) hydrogen of the dihydronicotinamide ring of NADPH is transferred to the substrate, D-[^{14}C]glyceraldehyde is reduced by A- and B-labeled [^{3}H]NADPH, and the ^{3}H:^{14}C ratios of the resulting samples of glycerol are measured. The direction of hydride attack at the carbonyl carbon is determined by examining the configuration of the tritiated carbon of the [^{3}H,^{14}C]glycerol resulting from the reduction involving tritium transfer. The [^{3}H,^{14}C]glycerol is successively phosphorylated (ATP, glycerokinase) and oxidized (NAD^+, glycerol-3-phosphate dehydrogenase) to form [1-^{3}H,^{14}C]dihydroxyacetone 1-phosphate. Hydrolysis of the latter (acid phosphatase) and phosphorylation (ATP, triokinase) of the resulting [^{3}H,^{14}C]dihydroxyacetone gave a mixture of [1-^{3}H,^{14}C]- and [3-^{3}H,^{14}C]dihydroxyacetone 1-phosphate. The configuration of the tritiated carbon of the latter, which is the same as that of the original [^{3}H,^{14}C]glycerol, is decided by determining which of the two enzymes, triosephosphate isomerase or muscle aldolase, labilizes the tritium atom. These procedures have been used to show that in the reduction catalyzed by the muscle glycerol dehydrogenase described by Kormann, A. W., Hurst, R. O., and Flynn, T. G. [(1972), *Biochim. Biophys. Acta 258*, 40] the A (*pro*-4*R*) hydrogen of NADPH attacks the *re* face of the carbonyl of D-glyceraldehyde.

A number of $NADP^+$-linked dehydrogenases which catalyze the reduction of aldehydes have been reported to occur in mammalian tissues (Kormann *et al.*, 1972; Bosron and Prairie, 1972). Since some of the enzymes are poorly characterized, and their substrate specificities overlap, the separate existence of each enzyme has been queried by the latter authors.

The preferred physiological substrate for the majority of these enzymes is D-glyceraldehyde, which is reduced to glycerol. It was assumed, *a priori*, that like most dehydrogenase-catalyzed reductions of carbonyl compounds (Levy *et al.*, 1962), the reduction of D-glyceraldehyde effected by each dehydrogenase is stereospecific. The overall objective of the present work is to explore the possibility of using the stereospecificity of reduction of D-glyceraldehyde by NADPH as an aid to the characterization of each dehydrogenase. This approach makes use of the ability of each enzyme to discrimi-

† From the Department of Biochemistry, Queen's University, Kingston, Ontario, Canada. *Received March 20, 1973.*