Subunit Structure and Photooxidation of Yeast Imidazoleglycerolphosphate Dehydratase†

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ABSTRACT: Imidazoleglycerolphosphate dehydratase has been purified from baker's yeast. Due to the instability of the enzyme, which was similar to the *Salmonella typhimurium* dehydratase, a low-fold purification was obtained; however, single protein bands were observed on both analytical polyacrylamide gel electrophoresis and gel electrophoresis in the presence of sodium dodecyl sulfate. The molecular weight of both purified and crude enzyme was 290,000 determined by Sepharose 6B gel filtration both in the presence and absence of 1 mM $MnCl_2$. The subunit molecular weight was 35,000 indicating the native enzyme is composed of eight polypeptide chains. The addition of the subunit molecular weights of the monofunctional yeast enzymes, imidazoleglycerolphosphate dehydratase and histidinol-phosphate phosphatase, equals the molecular weight of the subunit produced by *Salmonella typhimurium* which possesses both of these activities in a single polypeptide chain or aggregate of that chain. The yeast enzyme requires Mn^{2+} which adds first to the protein in a rapid equilibrium ordered mechanism. The $K_{i\,Mn^{2+}}$ is about 7 μM. The K_m for imidazoleglycerol phosphate was 0.54 mM at pH. 7.2 which is the optimal pH for activity. Photooxidation using Rose Bengal inactivates the enzyme by a first-order reaction. The rate of inactivation is dependent on the pH of photooxidation in a manner which tends to suggest the involvement of histidine in the active site. Mn^{2+} prevents inactivation by photooxidation and β-mercaptoethanol above 50 mM increases the rate of inactivation.

In *Saccharomyces cerevisiae* (Fink, 1964) and *Neurospora crassa* (Ahmed *et al.*, 1964), the genes specifying the synthesis of the enzymes involved in histidine biosynthesis are distributed over several different linkage groups. This can be compared to the gene-enzyme relationship in *Salmonella typhimurium* (Ames and Hartman, 1962) in which the histidine biosynthetic enzymes are specified by a cluster of genes organized into an operon (Ames and Martin, 1964). In yeast the only clustering of histidine biosynthetic genes seen is in the *his4* region, where three enzymes, a dehydrogenase, a pyrophosphatase, and a cyclase, are encoded (Shaffer and Fink, 1969). Shaffer *et al.* (1972) suggested that the latter three enzymes form a complex multifunctional unit and may even be synthesized as a single polypeptide chain, later to be cleaved into single enzymes. No such relationship has been demonstrated in *Salmonella typhimurium*.

In addition to the above differences between eucaryotic and procaryotic organisms, an important distinguishing characteristic exists in the gene-enzyme relationship of the activities specified by the *hisB* gene of *Salmonella typhimurium*. The available evidence suggests that *hisB* is a bifunctional gene coding for two dissimilar, nonconsecutive enzymatic reactions—imidazoleglycerolphosphate dehydratase and histidinol-phosphate phosphatase. The evidence includes both *in vitro* (Loper, 1961) and *in vivo* (Hartman *et al.*, 1971) complementation studies together with direct studies on the physical nature of the gene product including sedimentation behavior of the two activities (Whitfield *et al.*, 1964; Vasington and LeBeau, 1967), reaction to antisera specific for the *hisB* enzyme (Loper *et al.*, 1964) and copurification of the dehydratase and phosphatase (Brady and Houston, 1973). No evidence exists suggesting the presence of two cistrons within *hisB* each independently specifying one or the other of the phosphatase or dehydratase activities. Noncomplementing mutants are found distributed throughout the *hisB* region (Loper *et al.*, 1964).

Further studies in this laboratory using partially purified enzymes have shown that the active sites of the wildtype dehydratase-phosphatase of *Salmonella typhimurium* are separate and independent (Brady and Houston, 1973). Through the characterization of the enzyme produced by nonsense mutants with lesions at various points throughout the *hisB* region, the operator-proximal portion of *hisB* was demonstrated to specify the phosphatase activity, that is, mutants with polypeptide chain terminating lesions near the middle of *hisB* lacked imidazoleglycerolphosphate dehydratase activity, yet still contained histidinol-phosphate phosphatase with good specific activity (Houston, 1973a).

A mutant enzyme specified by the phosphatase subregion of *hisB* has been purified and characterized (Houston, 1973b). Although two forms were isolated, the molecular weight of the smallest active species was 38,000 consisting of two chains of equal size. Proteolytic degradation was thought to be responsible for the production of two species, the other species having a molecular weight of 49,000 consisting of a 38,000 molecular weight polypeptide and a 11,500 molecular weight polypeptide. A single chain of approximately 49,000 molecular weight was presumably the original gene product. More recent evidence using another nonsense mutant strain of *Salmonella typhimurium* is consistent with this interpretation (Houston, 1973c). The agreement of this figure (38,000 molecular weight) for the size of the "phosphatase core polypeptide" is in remarkable agreement with the size (38,000 molecular weight) of the histidinol-phosphate phosphatase polypeptide isolated from baker's yeast by Millay and Houston (1973). If the hypoth-

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esis were considered that the *hisB* gene simply "split" during the evolutionary process yielding now two genes independently specifying dehydratase and phosphatase enzymes in yeast and other eucaryotes, it might be anticipated that the subunit polypeptide of imidazoleglycerolphosphate dehydratase in yeast would have a molecular weight of about 37,000—the difference between the suggested 75,000 molecular weight of wildtype dehydratase-phosphatase subunit enzyme in *Salmonella typhimurium* (Vasington and LeBeau, 1967; Brady and Houston, 1973) and both the "phosphatase core polypeptide" and yeast histidinol-phosphate phosphatase. Therefore, the purification and characterization of imidazoleglycerolphosphate dehydratase were undertaken in order to further compare the bifunctional enzyme with its two monofunctional counterparts.

Materials and Methods

Ammonium sulfate and Rose Bengal were purchased from Fisher. Triethanolamine, triethylaminoethyl-cellulose, histidine, and histidinol dihydrochloride were purchased from Sigma. D-*erythro*-Imidazoleglycerol phosphate and β-mercaptoethanol were purchased from Calbiochem. Manganous chloride and magnesium chloride were purchased from Baker. Zinc chloride was purchased from Mallinckrodt. Acrylamide, obtained from Canalco, was recrystallized from hot 20% chloroform in methanol (v/v). Methylenebisacrylamide was obtained from Eastman and used without further treatment. Hydroxylapatite was prepared by the method of Bernardi (1971) and discarded after one use. Red Star compressed baker's yeast was purchased from a local bakery and stored at −20° until needed.

Enzyme Assay. Imidazoleglycerolphosphate dehydratase was assayed by a slight modification of the method described by Ames (1957). The assay was performed at 37° in a final volume of 85–100 μl containing final concentrations of 0.1 M triethanolamine hydrochloride (pH 7.2), 0.2 mM $MnCl_2$, and 85 mM β-mercaptoethanol. For routine assays, 3 mM imidazoleglycerol phosphate was used and 5–25 μl of enzyme was added. A stock solution of 0.1 M imidazoleglycerol phosphate was made in 0.1 M triethanolamine hydrochloride, adjusted to pH 7.2, and stored at 4°. If high concentrations of substrate were used, imidazoleglycerol phosphate dissolved in water was sufficiently acidic to partially overcome the buffering capacity of the triethanolamine buffer, resulting in changes in enzyme activity due to pH change. The reaction was initiated by the addition of enzyme and terminated by addition of 175 μl of 1.43 N NaOH. After further incubation at 37° for 30 min, the absorbance at 290 nm was determined using a blank containing all reagents including enzyme which was added after the addition of sodium hydroxide. The molar extinction coefficient at 290 nm of the product of dehydratase action, imidazoleacetol phosphate, in base was calculated to be 5100 from the data of Ames (1957).

Purification of Imidazoleglycerolphosphate Dehydratase. Baker's yeast was thawed at room temperature and suspended in 1.3 l. of cold 0.02 M triethanolamine hydrochloride (pH 7.2) for each pound of cells used. The suspension was added to 225 g of cold 0.2 mm diameter glass beads (Thomas Scientific) and the slurry subjected to the action of an Eppenbach mill for 15 min at 5–15°. The cell debris was removed by centrifugation at 40,000*g* for 1 hr at 4°. The crude extract at 0° was brought to 40% saturation with ammonium sulfate and centrifuged at 4° after 1 hr. The ammonium sulfate concentration of the supernatent was increased to 50% saturation and centrifuged after standing 1 hr at 0°. The precipitate contained most of the dehydratase activity and was taken up in a minimal volume of 0.02 M triethanolamine hydrochloride at pH 7.2. The enzyme was dialyzed at 4° overnight against two changes of this buffer (2 l. each) to remove residual ammonium sulfate.

Triethylaminoethyl-Cellulose Chromatography: TEAE-cellulose[1] was washed as described by Peterson and Sober (1962), equilibrated with 0.02 M triethanolamine hydrochloride at pH 7.2, deaerated *in vacuo,* and packed into a 2.5 × 40 cm column using 10 psi of pressure. This and subsequent operations were carried out at 4° unless otherwise specified. After washing the column with about 250 ml of 0.02 M triethanolamine hydrochloride buffer at pH 7.2, a linear gradient was applied using 500 ml of 0.02 M pH 7.2 buffer and 500 ml of 0.02 M buffer made to 0.6 M in ammonium sulfate. A flow rate of 75 ml/hr was used and fractions of about 6 ml were collected. The dehydratase enzyme was found to elute in the midst of a large protein peak near the beginning of the gradient. These fractions were pooled and dialyzed overnight against two changes of pH 7.2 buffer (2 l. each).

Hydroxylapatite Chromatography. A column (4.5 × 19 cm) of hydroxylapatite equilibrated with 0.02 M triethanolamine hydrochloride at pH 7.2 was used to absorb the dialyzed enzyme from the TEAE-cellulose step. A gradient made with 300 ml of 0.175 M ammonium sulfate in pH 7.2 buffer and 300 ml of 0.725 M ammonium sulfate in buffer caused the elution of dehydratase activity between 0.25 and 0.32 M ammonium sulfate. The flow rate was 50 ml/hr. The fractions containing dehydratase activity were pooled, extensively dialyzed against pH 7.2 buffer, and concentrated by ultrafiltration to 10–20 ml.

Sephadex G-200 Gel Filtration. A column (2.5 × 85 cm) of Sephadex G-200 equilibrated with pH 7.2 buffer containing 10 mM mercaptoethanol was used as the final step before preparative polyacrylamide gel electrophoresis. The enzyme was found to elute very near the void volume of the column and the fractions with dehydratase activity were pooled and concentrated by ultrafiltration in the cold using a Amicon Diaflo apparatus with a PM30 membrane.

Preparative Polyacrylamide Gel Electrophoresis. The final step of purification by preparative gel electrophoresis on polyacrylamide was accomplished at 2° using a 2-cm separating gel of the composition T = 3.1%, C = 2.0% using the nomenclature of Hjerten (1962). A 2-cm stacking gel T = 3.5%, C = 19.9% overlayed the separating gel. A Canalco preparative gel electrophoresis apparatus was used with a PD/320 column. The high molecular weight of the dehydratase necessitated the use of an open gel matrix to allow penetration of the enzyme and the completion of the separation in a reasonable time. The necessary shortness of the gel and its large pore size made the electrophoretic separation particularly difficult since the gel tended to easily break away from the glass side walls. The electrode reservoir buffer was buffer F of Ornstein and Davis (Davis, 1964). The slit elution buffer was 0.02 M triethanolamine hydrochloride–0.01 M mercaptoethanol at pH 7.5. A potential of 600–800 V was usually employed, drawing about 15 mA. Fractions were collected at a flow rate of 1 ml/min. Bromophenol Blue was included with the enzyme to mark

[1] Abbreviations used are: TEAE-cellulose, triethylaminoethyl-cellulose; TEA-HCl, triethanolamine hydrochloride.

TABLE I: Purification of Imidazoleglycerolphosphate Dehydratase from Baker's Yeast.

Fraction	Total Protein (mg)	Total Activity (μmol/hr)	Specific Activity (μmol hr mg^{-1})	Activity Yield (%)	−Fold Purification
Crude	58,140	5608	0.098	100	
Ammonium sulfate	23,140	2946	0.13	51.7	1.32
TEAE-cellulose	4,464	2768	0.62	48.6	6.32
Hydroxylapatite	793	283	0.36	5.0	3.67
Sephadex G-200	308	252	0.82	4.4	8.37
Preparative gel electrophoresis	4	73.5	18.4	1.3	187.8

the front. Fractions containing dehydratase activity were combined and concentrated by ultrafiltration.

Analytical Polyacrylamide Gel Electrophoresis. Purity of the enzyme was assessed by the analytical method of Ornstein and Davis (Davis, 1964) using gels of different pore sizes. Staining was accomplished with Amido Schwarz in 7% acetic acid and destained by lateral electrophoresis in 5% acetic acid.

Molecular Weight Estimation. The enzyme was filtered through a Sephadex G-200 column with triethanolamine hydrochloride–mercaptoethanol buffer and its molecular weight estimated by the procedure of Whitaker (1963). Since the enzyme elution volume was so near to the void volume of the column, making estimation of the molecular weight even more subject to error, a 1.5 × 100 cm column of Sepharose 6B was calibrated using standard proteins and the molecular weight was reestimated.

Electrophoresis Containing Sodium Dodecyl Sulfate. The method of Weber and Osborn (1969) was used to estimate the molecular weight of subunits of the dehydratase enzyme. Denaturation in sodium dodecyl sulfate and staining were done as described by Houston (1973b). Bovine serum albumin, ovalbumin, rabbit γ-globulin H and L chains, pepsin, aldolase, trypsinogen, cytochrome *c*, and ribonuclease were used to obtain the standard plot.

Photooxidation. Rose Bengal was used to photooxidize the dehydratase enzyme (approximately 0.3 mg/ml) in 0.02 M triethanolamine hydrochloride–0.01 M mercaptoethanol adjusted to pH 7.2. The enzyme was brought to room temperature in a small (10 × 75 mm) test tube placed 30 in. in front of a bank of three fluorescent lamps (General Electric Mazola 15-W white lamps). At intervals samples were withdrawn and added to an assay solution contained in a test tube tightly wrapped with aluminum foil to prevent further exposure to light. The tube was stoppered and incubated at 37°, and the reaction with substrate was quenched with the addition of sodium hydroxide. Since only small volumes on the order of 500 μl were used with a relatively large surface area, stirring during illumination was found not to be necessary and did not change the course of photoinactivation compared to an unstirred enzyme solution.

Protein Determination. The protein concentration of crude extract and preparations at early purification steps was determined by the method of Lowry *et al.* (1951). In later stages of purification, either the Lowry procedure or the procedure of Layne (1957) using the ratio of the absorbance at 280 and 260 nm was employed.

Results

Breakage of the yeast cells using the Eppenbach mill was found to be the most successful procedure for releasing the enzyme; other methods such as sonication, toluenization, lyophilization, autolysis, and treatment with snail gut enzymes before sonication either inactivated the enzyme or failed to give efficient cell breakage in our hands. Mercaptoethanol is not required at this stage of purification. The specific activity of imidazoleglycerolphosphate dehydratase in crude extracts was found to be approximately 0.1 μmol of imidazoleacetol phosphate formed per mg per hr at 37°.

Klopotowski and Wiater (1965) have previously partially purified this enzyme from yeast. Their procedure included ammonium sulfate precipitation, heat treatment, and DEAE-cellulose chromatography. They did not use mercaptans in their buffers, but did find that the enzyme easily inactivated. It was necessary to preincubate the enzyme in mercaptoethanol and manganese chloride before activity could be measured. No evidence was given as to the purity of the enzyme other than a 118-fold increase in specific activity. We found it necessary to include mercaptoethanol in the buffer after hydroxylapatite chromatography, a point at which some 90% of the applied activity was frequently lost even in the presence of mercaptoethanol. Other preparations were stable for up to 2 months when stored at 4° in buffer. Inactivated enzyme could not be restored to activity by either short or prolonged treatment with mercaptoethanol at either 37 or 0° with or without manganese chloride (1 mM).

Table I outlines the results of a typical purification. If activity is used as a basis of comparison, a 188-fold purification was obtained for this particular preparation. The final specific activity of other preparations was variable and was sometimes much lower even though the enzyme was electrophoretically homogeneous as judged by both analytical polyacrylamide gel electrophoresis and electrophoresis in sodium dodecyl sulfate. Using three different concentrations of acrylamide monomer on disc gel electrophoresis, only a single band could be observed even with high protein loads with or without sodium dodecyl sulfate (Figure 1).

The instability of the dehydratase activity of *S. typhimurium* has been encountered by several laboratories (Loper, 1961; Vasington and LeBeau, 1967; Martin *et al.*, 1971; Brady and Houston, 1973) and has presented a large problem in purification of the enzyme. Vasington and LeBeau (1967) obtained only a fivefold purification after ammonium sulfate fractionation and DEAE-cellulose chromatography and noted the loss of total dehydratase activity throughout the procedure. No method has been found in this laboratory to completely stabilize dehydratase activity throughout the purification process. We found that inclusion of Mg^{2+}, Mn^{2+}, or Mg^{2+} and Mn^{2+} in combination did not appreciably stabilize the dehydratase activity, nor did the inclusion of glycerol or dithiothreitol in the buffers.

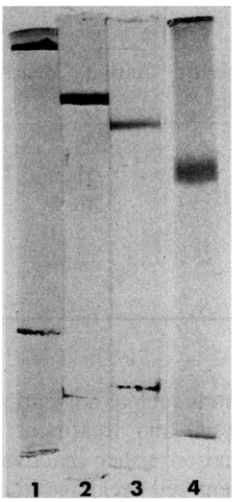

FIGURE 1: Polyacrylamide gel electrophoresis of imidazoleglycerol-phosphate dehydratase. 1, 7.5%; 2, 5.6%; and 3, 4.7% polyacrylamide gels using Ornstein–Davis conditions (Davis, 1964); 4, sodium dodecyl sulfate–polyacrylamide gel using the procedure of Weber and Osborn (1969).

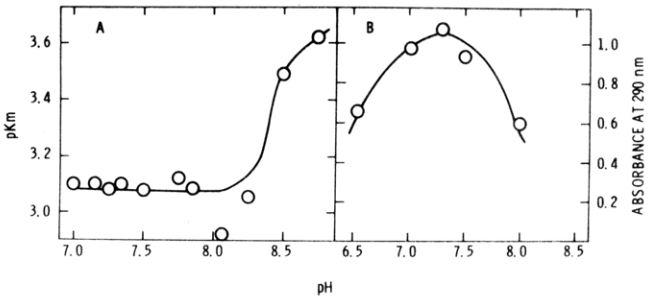

FIGURE 2: Effect of pH on velocity and K_m. (A) The dehydratase activity at 37° was determined at various pH values in 0.1 M TEA or Tris base titrated to pH with hydrochloric acid. No difference was noted between these buffers. The assay (100 μl) was made 88 mM in mercaptoethanol and 0.2 mM in $MnCl_2$. Reaction was initiated by addition of enzyme and terminated by 175 μl of 1.43 N NaOH. Double reciprocal plots were linear. (B) Enzyme activity was determined at various pH values in 0.1 M TEA or 0.1 M Tris (titrated with HCl) containing 88 mM mercaptoethanol, 0.2 mM $MnCl_2$, and 10 mM imidazoleglycerol phosphate.

Hydroxylapatite chromatography was a necessary step even though considerable activity was lost. In the preparative gel electrophoresis step, the specific activity was constant throughout the peak. In view of these problems associated with the enzyme and in view of the electrophoretic purity, the enzyme is probably pure and not contaminated with other proteins of unknown function. The enzyme as purified, however, probably contains an unknown and variable proportion of crippled or inactive enzyme molecules.

Molecular Weight and Subunit Composition. The molecular weight of the dehydratase was estimated at 290,000, a value obtained by Sephadex G-200 chromatography and confirmed by calibrated gel filtration experiments using a Sepharose 6B column. As described below, the dehydratase is dependent on the presence of manganese ion for the expression of activity. In addition to this, Brady and Houston (1973) have shown Mn^{2+} promotes polymerization of the *S. typhimurium* bifunctional *hisB* enzyme. Furthermore, the polymerization reaction is reversible and the multimer can be dissociated by the removal of Mn^{2+} unpon dialysis. These gel filtration experiments with the yeast dehydratase were performed without Mn^{2+} in the buffer; however, the same molecular size was observed if 1 mM $MnCl_2$ was included. Crude extract enzyme gave the same results. We have tried to dissociate the enzyme as can be done with the *S. typhimurium* bifunctional enzyme. Dialysis against EDTA or EDTA and mercaptoethanol to remove metal ions resulted in no change in molecular weight when either crude or purified enzyme was used.

The behavior of the enzyme on disc gel electrophoresis was also consistent with a large molecular weight. Preliminary experiments using the method of Hedrick and Smith (1968) varying the acrylamide monomer concentration also indicated a molecular weight of about 300,000.

Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate at pH 7.1 gave evidence for the presence of only one size of subunit with a molecular weight of 35,000 ± 1000. No other bands could be observed on the gel (Figure 1). Therefore, assuming no proteolytic degradation of the enzyme (Houston, 1973c), it would appear from these data that the molecular weight of the native imidazoleglycerol-phosphate dehydratase can be entirely accounted for by eight 35,000 molecular weight polypeptide chains. In this case, proteolytic digestion is unlikely since single bands were observed on both types of gels in contrast to yeast histidinol-phosphate phosphatase (Millay and Houston, 1973).

Effect of Heat on Dehydratase Activity. The dehydratase activity remains at a constant level when heated at 54° over a period of 1 hr. The dehydratase activity, but not the phosphatase activity, of the bifunctional *hisB* enzyme of *Salmonella typhimurium* was also shown to be stable to heating (Brady and Houston, 1973). Klopotowski and Wiater (1965) did note that heat treatment of the enzyme resulted in a less stable enzyme which could not be stored for long periods of time.

The energy of activation was determined for dehydratase activity by an Arrhenius plot. A value of 15,900 cal/mol was determined between the temperature range of 23 and 45°. This compares well with the value for the *S. typhimurium* enzyme, 14,700 cal/mol.

Effect of pH on Velocity and K_m for Imidazoleglycerol Phosphate. Previous estimates have been made of the K_m toward imidazoleglycerol phosphate for the dehydratase enzyme from a variety of sources. These values range from 1.7 mM for the enzyme in crude extracts of oats and 0.6 mM for barley (Wiater *et al.*, 1971c) to 0.24 mM for *N. crassa* enzyme (Ames, 1957). *Salmonella typhimurium* enzyme has a K_m of 0.70 mM (Brady and Houston, 1973) at pH 7.5 using 50 μM $MnCl_2$ at 37°. The enzyme purified in this work exhibits optimal activity at pH 7.2 (Figure 2B). The dehydratase of the bifunctional *S. typhimurium* enzyme exhibits a higher pH optimum around pH 8 (Brady and Houston, 1973). The K_m at pH 7.2 was determined by a Lineweaver–Burk plot, which was linear, to be 0.54 mM using 0.02–11.75 mM imidazoleglycerol phosphate and 0.2 mM $MnCl_2$ in the assay at 37°. Even though the enzyme is fairly labile as described above, the K_m value does not change during purification. The effect of varying pH on K_m is shown in Figure 2A.

Initial Velocity Studies with Mn^{2+}. The K_m of the enzyme for manganous chloride was evaluated by a Lineweaver–Burk plot. The plot was linear and a value of 7.6 μM was determined by using 125 mM triethanolamine hydrochloride–0.01 M mercaptoethanol. This value is similar to the value of 7.3 μM found for *S. typhimurium* dehydratase (Brady and Houston, 1973) and is in good agreement

with values previously determined with *Neurospora* enzyme (Ames, 1957). Double reciprocal plots showed that Zn^{2+} was a competitive inhibitor of Mn^{2+} and had an inhibition constant of 38 μM.

Double reciprocal plots were made at various constant manganous chloride concentrations and the concentration of imidazoleglycerol phosphate was varied. The data in Figure 3A show the stimulation of activity by Mn^{2+} and illustrate the fact that the maximal velocity is unaltered since all lines intersect on the ordinate. According to Cleland (1970), this pattern is consistent with a sequential mechanism in which manganese must bind to the enzyme before imidazoleglycerol phosphate. Therefore, while the dehydratase cannot be regarded as a metalloenzyme in the strict sense of the word, since Mn^{2+} can easily be dialyzed away especially in the presence of chelating agents, it would appear that an activated metal–enzyme complex is an obligatory intermediate which is established in a rapid equilibrium ordered mechanism before binding of substrate and subsequent catalysis can occur. We have not tested this supposition of a metal–enzyme obligatory intermediate by determining whether imidazoleglycerol phosphate can bind in equilibrium dialysis experiments independently of Mn^{2+}. A replot of the slopes of the intersecting lines of Figure 3A is shown in Figure 3B. The intersection on the abscissa gives a value of 6.0 μM for $K_{i\,Mn^{2+}}$ in good agreement with the value determined by a double reciprocal plot varying Mn^{2+}.

Effect of Inhibitors. While it is well known that 3-amino-1,2,4-triazole (the herbicide amitrole) is a competitive inhibitor of dehydratase from *S. typhimurium* and cereal grasses (Wiater *et al.*, 1971b), the inhibition constant was determined for the purified enzyme instead of with crude enzyme used in other studies. Lineweaver–Burk plots using 0.59 mM aminotriazole or Dixon plots, employing 0.059–0.59 mM aminotriazole concentrations at imidazoleglycerol phosphate concentration of either 0.29 or 0.59 mM, gave comparable competitive inhibition constants for aminotriazole of 23 μM at pH 7.2. The value is in good agreement with that of Wiater *et al.* (1971a) in yeast, but differs by about tenfold from the K_i of 3.2 μM for the bifunctional dehydratase of *Salmonella typhimurium* (Brady and Houston, 1973).

The yeast dehydratase in crude and partially purified form has been reported to be sensitive to phosphate inhibition (Klopotowski and Wiater, 1965) as is the *S. typhimurium* enzyme (Brady and Houston, 1973). The latter enzyme shows the synergistic inhibitory effect[2] between phosphate and aminotriazole which is also found in crude yeast dehydratase (Wiater *et al.*, 1971a). In contrast to this, we have not been able to demonstrate any inhibition of dehydratase activity by phosphate even at a concentration up to 25 mM and, furthermore, phosphate does not act synergistically with the competitive inhibitor, aminotriazole. Histidinol phosphate, histidinol, and histidine all at 25 mM concentration separately had no effect on enzymatic activity at pH 7.2.

Effect of Photooxidation on Dehydratase Activity. Photooxidation has been proposed as a general method to indicate the nature of the residues within an active site of an enzyme that are necessary for catalytic activity (Ray, 1967). To show the possible effect of Rose Bengal dye alone, assays were first performed in the dark by including 10 μM to 1 mM Rose Bengal dye with the substrate. It was shown that these concentrations of Rose Bengal had no effect on the level of dehydratase activity if light were rigorously excluded during the assay before the addition of sodium hydroxide. The possibility existed that photooxidation could oxidize the protecting agent, mercaptoethanol, to such an extent that activity loss could result from a reduction in the mercaptan level. Photooxidation of 0.10 mM mercaptoethanol in 0.10 M Tris-HCl by 10 μM and 1 mM Rose Bengal was followed at pH 7.25–9.0 by reaction of mercaptoethanol with 5,5′-dithiobis(2-nitrobenzoic acid). During 60 min of photooxidation, less than 1% of the mercaptoethanol was oxidized under these conditions as judged by the absorbance at 412 nm after 400-μl samples were reacted with 0.4 mM Ellman reagent at pH 7.5 in sodium phosphate buffer. The inclusion of 0.22 mM manganese chloride in the mixture increased the rate of thiol oxidation to a small extent.

A concentration of 1 μM Rose Bengal led to the loss of 50% of the activity in 60 min (Figure 4A) under the conditions employed. The loss of activity followed first-order ki-

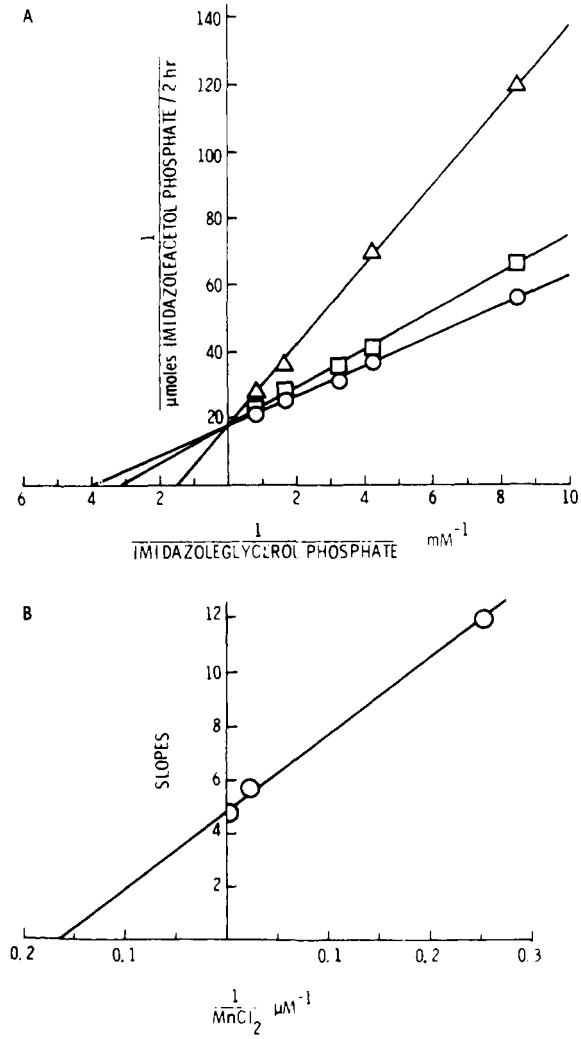

FIGURE 3: Effect of Mn^{2+} on initial velocity. (A) The dehydratase was assayed for 2 hr at 37° in 0.1 M TEA-HCl–88 mM mercaptoethanol using 4 μM (△), 40 μM (□), and 400 μM (○) $MnCl_2$ and varying the concentration of substrate between 0.59 and 4.7 mM. (B) The slopes of the lines from Figure 3A plotted against the reciprocal of the $MnCl_2$ concentration.

[2] L. L. Houston, unpublished observations. The bifunctional *hisB* enzyme purified from *Salmonella typhimurium* as previously described (Brady and Houston, 1973) is synergistically inhibited by the combination of phosphate and aminotriazole with an α value of 0.17, similar to that found by Klopotowski and Wiater (1965).

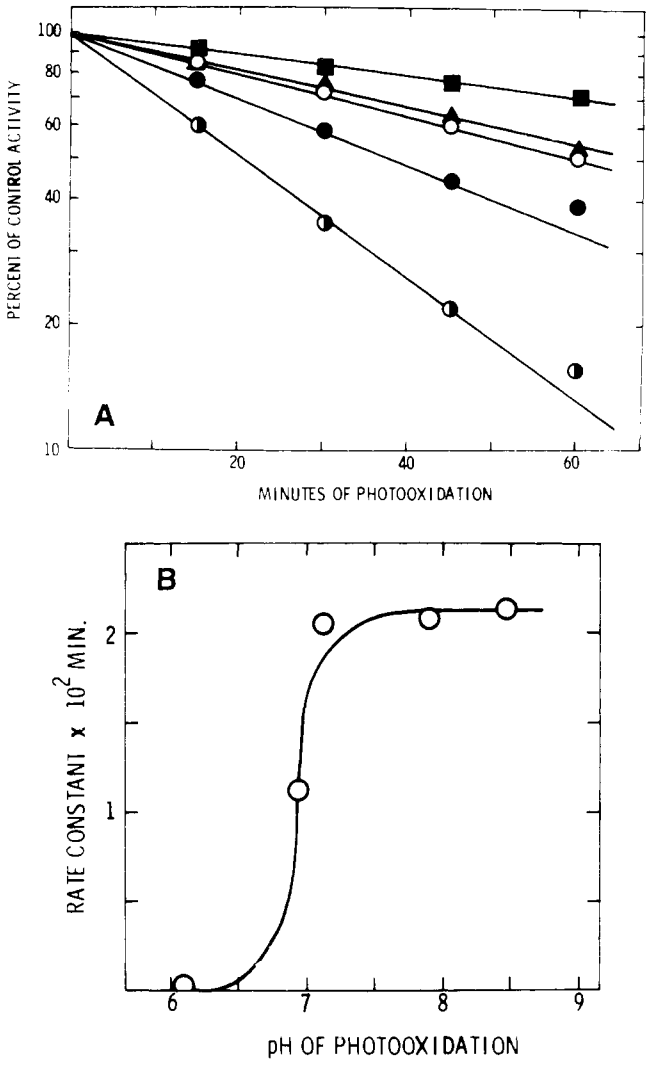

FIGURE 4: Photooxidation of dehydratase in mercaptoethanol and pH dependency. (A) The dehydratase (125 μl), stored in 0.02 M TEA-HCl and 88 mM mercaptoethanol, was diluted into 500 μl of 0.1 M TEA-HCl buffer containing various concentrations of mercaptoethanol and 1 μM Rose Bengal. Photooxidation was carried out at 22° as described in the Materials and Methods section and the activities were compared to an untreated control. $MnCl_2$ was not present except in the assay. Final concentrations of mercaptoethanol are 17.8 mM (○), 36.2 mM (▲), 54.6 mM (■), 73.0 mM (●) and 110 mM (◑). (B) The dehydratase was photooxidized as in Figure 4A without the addition of mercaptoethanol. The rate constants were determined from first-order plots. All plots were linear.

netics. Increasing the concentration of mercaptoethanol above about 60 mM served not to protect the sulfhydryl dependent enzyme, but to potentiate the effect of photooxidation on the rate of destruction of activity (Figure 4A). Protection against loss of activity was observed at lower mercaptoethanol concentration. This is further evidence that destruction and oxidation of mercaptoethanol is not the limiting factor resulting in loss of dehydratase activity. Furthermore, the buildup of some kind of dehydratase inhibitor does not occur during the time of photooxidation (as opposed to a direct modification of the enzyme), since addition of solutions of buffer containing mercaptoethanol which had been subjected to photooxidation for 60 min under identical conditions showed no ability to inhibit the dehydratase activity. The pH of the photooxidized solutions did not change during the experiment.

Effect of Mn^{2+} on Photooxidation. In contrast to the increased sensitivity of the dehydratase to the effects of photooxidation with increased mercaptoethanol, the dehydratase was stabilized by the addition of increasing concentration of manganese ion. Addition of either 0.1 or 0.2 mM $MnCl_2$ completely stabilized the enzyme to the effects of photooxidation for 60 min during which the unprotected enzyme was 50% inhibited. This stabilization may reflect a conformational change in the enzyme which changes the accessibility of the Rose Bengal dye to the site(s) responsible for inactivation or it may be due to direct prevention of access of the dye to the site. A specific effect is indicated since no protection was afforded by inclusion of divalent magnesium ion.

Sensitivity of Photooxidation to pH. Various side chains of residues typically found to participate in the catalytic mechanism of enzymes are known to show differential sensitivity to photooxidation at various pH values (Ray and Koshland, 1962). Since it was difficult to isolate enough enzyme to do direct amino acid analysis in order to determine what residues were photooxidized, we resorted to determining the pH sensitivity of photoinactivation to try to tentatively identify functional catalytic residues. Dehydratase shows a marked sensitivity to photooxidation at high pH and is more stable when the oxidation is performed at pH values below 7 (Figure 4B). These kinetics are consistent with the interpretation that an un-ionized imidazole group is the species sensitive to photooxidation. Photooxidation of histidine would be expected to exhibit a similar sensitivity with a pK in the region of 7. Appropriate controls were carried out to show that the enzyme was stable at each pH value under the conditions of photooxidation in the absence of illumination, but in the presence of Rose Bengal at 37°. Therefore, there is no instability of the enzyme at higher pH values and inactivation can be attributed directly to the effect of photooxidation.

Discussion

Imidazoleglycerolphosphate dehydratase from baker's yeast was purified to apparent homogeneity as judged by polyacrylamide gel electrophoresis. Instability was a continual problem and activity was variable especially at steps after TEAE-cellulose chromatography. The enzyme was especially sensitive in the absence of mercaptoethanol, a behavior noted previously (Klopotowski and Wiater, 1965). Klopotowski and Wiater (1965) using a partially purified preparation of the dehydratase from yeast found that activity could be restored if the enzyme was preincubated with mercaptoethanol and manganese chloride before imidazoleglycerol phosphate was added. Their buffer system did not contain mercaptoethanol. While preincubation did serve to increase activity slightly at the initial purification steps in this work, it had little effect in the latter stages. The enzyme was extremely labile in the absence of mercaptoethanol and could not be stabilized with glycerol or Mn^{2+}. This may mean that a sulfhydryl group on the enzyme is necessary for the catalytic function of the dehydratase.

Photooxidation using sensitizing dyes is a general method known to modify a variety of amino acid side chains including tryptophan, tyrosine, histidine, methionine, and cysteine (Weil, 1965). As such, it has been used by Ray and Koshland (1962) to correlate the rate of loss of enzyme activity with the rate of side chain modification determined by amino acid analysis. The pH and temperature dependency of photooxidation of these free amino acids has been investigated (Weil, 1965; Westhead, 1965). Studies of histidine

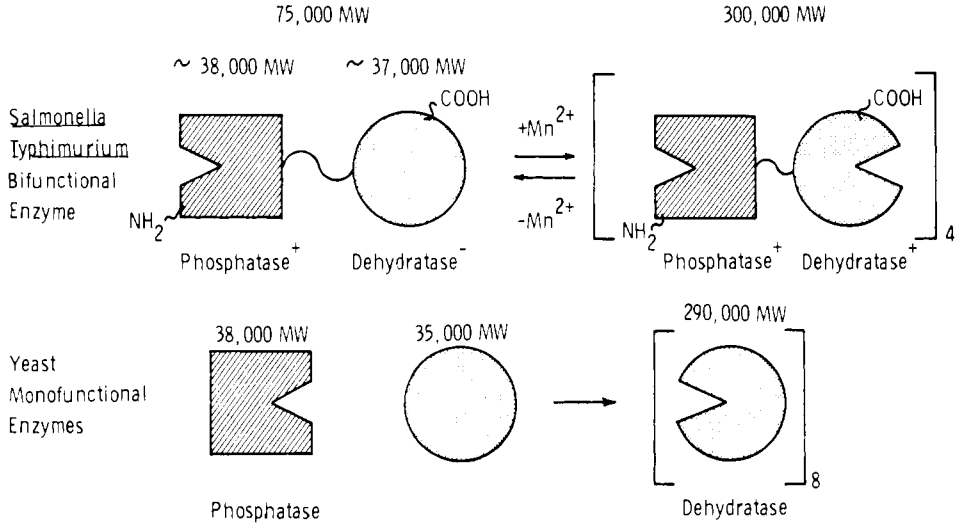

FIGURE 5: Hypothetical scheme of relationships between the monofunctional yeast and the bifunctional *Salmonella typhimurium* dehydratase-phosphatase enzymes.

and histidine containing peptides (Weil, 1965; Westhead, 1965; Martinez-Carrion *et al.*, 1967) have shown that the free base form of the imidazolium group is the species that is sensitive to photooxidation by either Methylene Blue or Rose Bengal. While functional groups cannot always be identified with precision by pH-sensitivity relationships, the pH profile toward imidazoleglycerol phosphate and the pH-photoinactivation relationship was consistent with the involvement of histidine in the dehydratase activity. The pK of enzyme activity loss is similar to that of other studies where it was shown that histidine was involved in the catalytic process (Westhead, 1965; Martinez-Carrion *et al.*, 1967).

The ionizable groups that are responsible for the pH dependency of activity could reside on the substrate which contains an imidazolium group and a phosphate ester. The identification of histidine by photooxidation must also remain tentative until it can be confirmed by amino acid analysis. Other groups as well are likely to be photooxidized, but now show the same pH dependency. Inactivation may be due not to a direct effect on a catalytic residue, but to changes which result in dissociation of the polymeric structure or some other change in enzyme structure. It has been suggested that the *S. typhimurium* bifunctional enzyme expressed dehydratase activity only in a polymeric state. Mn^{2+} is necessary for activity for both dehydratase enzymes and has the additional effect of promoting the aggregation of the *S. typhimurium* polypeptide chain. Mn^{2+} has no effect on either wild-type phosphatase, but does kinetically interact with the mutant enzymes of *S. typhimurium* (Houston and Graham, 1974).

Since manganese is required for activity, apparently first forming a Mn^{2+}-enzyme complex in a rapid equilibrium ordered mechanism to which imidazoleglycerol phosphate adds, and protects from photoinactivation, the idea is strengthed that photooxidation acts directly on a catalytic residue. The possibility exists that paramagnetic metals such as Mn^{2+} can protect against photooxidation by reacting with sensitized dye. This cannot be ruled out at this time; however, it should be noted that Mn^{2+} slightly stimulated photooxidation of mercaptoethanol. Where mercaptoethanol protects activity during storage, enhanced inactivation by photooxidation was promoted by the thiol, although some protection was shown at a lower mercaptoethanol concentration.

It is curious that the purified enzyme is not inhibited by phosphate ion. Using a partially purified yeast enzyme, Wiater *et al.* (1971a) has shown that the dibasic phosphate ion is the inhibiting species. Lack of phosphate inhibition and, consequently, the ability of phosphate and aminotriazole to act synergistically was not a property that was lost during purification of the enzyme used here, since synergism could not be demonstrated in crude extract or after TEAE-cellulose chromatography. Inhibition by aminotriazole, however, agrees quite well with other work. It is possible that differences in the strains of yeast used are responsible although Wiater *et al.* (1971b) have shown that dehydratase from oats is not synergistically inhibited by phosphate and aminotriazole.

The data on molecular weight of the native enzyme, 290,000 and the subunit molecular weight, 35,000, determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicate that the enzyme is an octomer. These data are interesting in the light of other data on the bifunctional *hisB* enzyme from *Salmonella typhimurium* which has both histidinol-phosphate phosphatase activity and imidazoleglycerolphosphate dehydratase activity. The polypeptide specified by *hisB* has been estimated to be 75,000 molecular weight. The operator-proximal part of the gene corresponding to the amino-terminal portion of the polypeptide chain has been identified by Houston (1973a) as a subregion which is of sufficient size to specify a structure with only the phosphatase activity. Using mutants with nonsense lesions, further studies identified the smallest enzyme isolated as having a molecular weight of 38,000 (Houston, 1971b,c). Subtracting the 38,000 polypeptide with phosphatase activity from the native chain would leave remaining a residual portion of the chain toward the carboxyl end equal to 37,000 molecular weight. It is, therefore, possible that if the bifunctional *hisB* enzyme resembles two independently acting and separately folded regions, such as is thought to exist in other bifunctional enzymes (Shaffer *et al.*, 1972), then the yeast dehydratase corresponds quite closely and is evolutionarily related to the structure found in the "dehydratase globule" of the *hisB* enzyme. Figure 5 summarizes these hypothetical relationships. Histidinol-

phosphate phosphatase also purified from baker's yeast by Millay and Houston (1973) exactly corresponds in molecular weight, 38,000, to the molecular weight of the phosphatase from the UGA nonsense mutant which defines the region comprising the "phosphatase globule" of the bifunctional *hisB* enzyme described by Houston (1973b).

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Molecular Kinetics of Beef Heart Lactate Dehydrogenase†

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ABSTRACT: The dehydrogenation of lactate to pyruvate, catalyzed by beef heart lactate dehydrogenase, proceeds by an ordered ternary complex mechanism. A study has been made of the steady-state and pre-steady-state kinetics of the reaction in both directions, at various substrate and coenzyme concentrations, and over a range of temperatures (5–50°). Analysis of the results allowed eight rate constants to be determined, together with their enthalpies and entropies of activation. The concentration of active enzyme was also calculated from the kinetic results, and is consistent with the value obtained from the protein concentration and the molecular weight. The entropy changes during the course of the individual reactions suggest that the enzyme is most folded in the ternary complex. Biological implications of the results are considered, and it is noted that beef heart lactate dehydrogenase is particularly well suited for interconverting pyruvate and lactate at normal body temperature and at substrate concentrations commonly found in living systems.

Reactions catalyzed by lactate dehydrogenase and alcohol dehydrogenase have been studied extensively in the steady-state and the pre-steady-state (Holbrook and Gutfreund, 1973; for a review see Laidler and Bunting, 1973). The reactions are found to occur largely by an ordered ternary complex mechanism. The present paper describes an investigation of the pre-steady-state and steady-state kinetics of the beef heart lactate dehydrogenase system, over a range of temperature, and has allowed kinetic parameters to be obtained for eight elementary reactions.

By lowering the temperature to 15° and below, it is possi-

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