Purification and Properties of a Mutant Bifunctional Enzyme from the HisB Gene of Salmonella typhimurium*

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SUMMARY

A distinct change occurs in the molecular size of the enzyme, histidinol phosphate phosphatase, specified by nonsense mutants with lesions near the middle of hisB. The hisB gene of Salmonella typhimurium controls two different activities, imidazolglycerolphosphate dehydratase and histidinol phosphate phosphatase, both of which reside in a single protein. The phosphatase activity from mutants with lesions distal to this transition point appears in or near the void volume of Sephadex G-200, whereas from mutants with lesions proximal to the transition point produce a polypeptide with little or no activity. One mutant with its lesion near this transition point was chosen for further study. The phosphatase enzyme was purified from strain TR691, a non-complementing UGA hisB278 mutant. Two forms of the enzyme were found by preparative polyacrylamide gel electrophoresis. One form had a molecular weight of 38,000 and was a dimer of 18,000 molecular weight polypeptide chains as shown by electrophoresis in sodium dodecyl sulfate. The other form of the enzyme had a molecular weight of 47,000 and was a dimer of dissimilar chains with molecular weights of 38,000 and 11,500. The pH optima for catalytic activity of both forms was 7.0 in the absence of MnCl2, and 6.5 in the presence of 1.0 mM MnCl2. The Kₐ for histidinol phosphate was 0.83 mM and was unchanged by the addition of Mn²⁺

There is convincing evidence that the hisB enzyme is composed of subunits and it has been suggested that 75,000 is the molecular weight of the monomeric units (3-7). Genetic studies indicate that a single functional unit of the gene controls both activities. Only two of the three possible classes of hisB mutants have been detected, those lacking only the imidazolglycerolphosphate phosphatase activity and those lacking both activities. In vitro and in vitro complementation patterns are nonlinear and show a complex relationship (1, 2). Recent reviews by Hartman et al. (6) and Brenner and Ames (7) have discussed these findings.

The perplexing behavior of the wild type enzyme has led us to approach the investigation of the structure of the enzyme through the characterization of mutant enzymes. Recent evidence obtained in this laboratory has demonstrated a transition point near the middle of hisB that defines a subregion within the gene (8). Nonsense mutants with their lesions in the operator proximal region specify a polypeptide with little or no histidinol phosphate phosphatase activity; however, strains with nonsense lesions in the distal region specify a protein which has a high molecular weight similar to the native enzyme. In contrast, nonsense mutants with a lesion in approximately the middle of hisB specify a protein which has a molecular weight near 40,000 and shows good specific activity in crude extracts. In order to explore further the nature of the hisB enzyme, we have purified and partially characterized the enzyme specified by a nonsense mutant, strain TR691, which has a lesion near the transition point.

MATERIALS AND METHODS

Histidinol phosphate was purchased from Cyelo Chemical Co. and imidazolglycerolphosphate from Calbiochem. Acrylamide was obtained from Canalo and recrystallized from hot 20% chloroform in methanol (v/v). Methylene bisacrylamide was from Eastman and was used without further purification. Triethylaminoethyl cellulose was purchased from Sigma and hydroxyapatite was prepared according to the procedure described by Bernadi (9). For preparative gel electrophoresis a Canalo Prep Gel apparatus was used with cooling at 2°C.

Dr. John Roth provided the Salmonella typhimurium strain TR691 (hisB278 hisT1829 aroD5) used in this study. Relatively high levels of the histidine biosynthetic enzymes were obtained from the strain since physiological derepression occurs...
due to the mutation in hisT. Cultures were grown in formyl-histidine as previously described (1, 8) and stored at -20° as a paste.

The assay for histidinol phosphate phosphatase has been described (4, 8) and is based on the method of Ames et al. (10).

**TEAE-cellulose Chromatography**—A crude extract of strain TR691 was made by suspending the thawed cells (33.5%, w/v) in 0.05 M Tris chloride-0.01 M mepacrinethanol-1.0 mM MnCl₂, pH 8.5, at 0°. The suspension was sonified in 200-ml portions in a Rosett cell with a Bronson sonifier using three 1-min bursts with 1-min rests. After centrifugation at 49,000 × g, the cell debris was washed twice with half-volume portions of the buffer. Dialysis against 10 volumes of cold buffer (two changes) was carried out overnight and the extract was placed on a column (4.5 × 55 cm) of TEAE-cellulose. The TEAE-cellulose was first washed as described by Peterson and Sober (11), deaerated in vacuo, and packed using 10 p.s.i. of pressure. All operations were performed at 4° unless otherwise specified. The column was washed with about 250 ml of buffer and a linear gradient was applied to the column using 1500 ml of 0.05 M Tris chloride 0.01 M mepacrinethanol-1.0 mM MnCl₂, pH 8.5, versus 1500 ml of 0.3 M ammonium sulfate in the same buffer at pH 8.5. A flow rate of 80 ml per hour was used.

**Hydroxyapatite Chromatography**—The fractions between 1250 and 2100 ml of the gradient from the TEAE-cellulose column with high activity were pooled and dialyzed against buffer to remove ammonium sulfate. The dialyzed pool was concentrated using an Amicon Diaflo ultrafiltration apparatus with a PM-10 membrane. This concentrate was placed on a column (2.5 × 70 cm) of hydroxyapatite which had been equilibrated with buffer. Gradient elution was employed to chromatograph the enzyme using 1 liter of 0.05 M ammonium sulfate in buffer versus 1 liter of 0.22 M ammonium sulfate in buffer at pH 8.5. A Radiometer CDE-2e conductivity meter was used to monitor the gradient.

**Sephadex G-75 Chromatography**—The active fractions between 900 and 1500 ml of the gradient from the hydroxyapatite column with high activity were pooled and dialyzed against buffer to remove ammonium sulfate. The dialyzed pool was concentrated using an Amicon Diaflo ultrafiltration apparatus with a PM-10 membrane. This concentrate was placed on a column (50 × 47.5 cm) of Sephadex G-75-40 μ was used to fractionate the enzyme further.

**Preparative Gel Electrophoresis**—The active fractions in the low molecular weight range from Sephadex G-75 chromatography were pooled and concentrated to about 10 ml by ultrafiltration using a UM-2 membrane; PM-10 membranes were not used because they allow substantial quantities of the dilute enzyme to escape. A Canalco preparative gel electrophoresis apparatus (PD2/320 column) was used as the final step in purification of the enzyme. A 7.5% separating gel was made using the Ornstein Davie formulation (12) without potassium ferrocyanide. Separating gels approximately 10 cm long were used underneath a 3-cm stacking gel. The system was run at 2° although some runs have been performed at 10° with equally good separation. The eluting buffer was the Tris chloride-mepacrinethanol-MnCl₂ buffer at pH 8.5. A constant current of about 15 ma was used which required 400 to 600 volts. Bromphenol blue was included in the enzyme solution as a marker. Fractions were collected at a flow rate of about 2 ml per min and assayed for phosphatase activity. Purity was checked by analytical polyacrylamide electrophoresis using the procedure of Ornstein and Davis (12). Amido Schwarz in 7% acetic acid was used to stain for protein and the gels were destained by lateral electrophoresis in 5% acetic acid. These electrophoretic results were used as a guide to obtain a pool of the protein.

When sufficient purity was not reached on the first electrophoretic separation, a second separation with selected fractions was carried out using a PD2/150 column.

**Sodium Dodecyl Sulfate Gel Electrophoresis**—Electrophoresis in sodium dodecyl sulfate on polyacrylamide was employed to detect the presence of and to determine the molecular weight of subunits. Weber and Osborn’s method (13) was used with one modification. Staining was accomplished by first soaking the gels for a minimum of 4 hours to remove sodium dodecyl sulfate in at least four changes of a mixture made with an equal volume of 20% acetic acid and methanol. The gels were then incubated in Amido Schwarz in 7% acetic acid for at least 4 hours (usually 12 hours) and laterally destained in 5% acetic acid. In our hands, Coomassie blue was more easily removed from the protein band by electrophoretic destaining; therefore, when this stain was used, destaining was accomplished by soaking in 5% acetic acid. The bromphenol blue band was marked with a needle dipped in India ink. Bovine serum albumin, rabbit γ-globulin H and L chains, ovalbumin, pepsin, and cytochrome c were used as standard proteins for calibration.

**Urea Gel Electrophoresis**—Electrophoresis in 8 M urea was accomplished by making up some of the reagents with recrystallized urea. Reagents C and D of Ornstein and Davis (12) were made to a final concentration of 8 M urea and E and the ammonium persulfate reagent to 9.5 M urea. Where the addition of water is specified, 9.5 M urea was substituted. The final concentration of urea using this procedure is 7.94 M in both separating and stacking gels. The reagents containing urea were made fresh in small amounts and electrophoresis was carried out within 1 to 2 hours. Urea was not added to the reservoir buffer. The run required approximately 2 hours. Gels were stained overnight with Amido Schwarz.

**RESULTS**

**Purification of Two Forms of Histidinol Phosphate Phosphatase**—The specific activity of histidinol phosphate phosphatase of TR691 crude extract varied between 0.6 and 5.9 amoles of histidinol phosphate hydrolyzed per hour per mg of protein after dialysis. The level of derepression was good as judged by the specific activity of histidinol dehydrogenase (14), which was between 0.27 and 0.83 unit per mg. A single peak of activity which showed considerable tailing was observed to elute from TEAE-cellulose between 50 and 75 mM ammonium sulfate. Assay blanks without histidinol phosphate show the presence of a non-enzyme component between 1000 and 1150 ml of the gradient that gives an intense reaction with the ascorbate-nitroblue reagent. No activity towards 0.01 M p-nitrophenylphosphate was found within the peak of histidinol phosphate phosphatase activity. The histidinol phosphate phosphatase activity eluted from hydroxyapatite in a single peak at an ammonium sulfate concentration between 0.090 and 0.115 M, just in front of a large, inactive protein peak. Phosphate was not eluted from the resin at sufficient concentrations to interfere with the assay.

Upward flow gel filtration on a column of Sephadex G-75 was used to purify the enzyme further. The active fractions were concentrated and the final purification was accomplished by preparative gel electrophoresis. Fig. 1 represents the profile of a typical run on a 7.5% polyacrylamide gel. Two distinct

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1 The abbreviation used is: TEAE, triethylaminoethyl.
peaks of activity were observed. On some runs the peaks were less well separated and the second appeared as a trailing shoulder which necessitated a second separation. Acrylamide concentrations of up to 15% have been successfully used, resulting in a considerable reduction of the mobility but yielding a product with the same purity as that resolved on a 7.5% gel. Fractions across the peak were analyzed for purity by analytical disc gel electrophoresis. The peak tubes from Fraction 1 exhibit a single protein band upon analytical gel electrophoresis, while the fractions on either side show the presence of impurities. A component is present in tube 95 that runs faster than Fraction 1 which is found in pure form in tube 98. A single slower moving component is present in tube 107 and two bands are observed in tube 113 that are close together. When portions of tubes 98 and 113 were combined and run; three protein bands were clearly distinguished. The fastest moving band represents one form of the enzyme and the second form resides in one of the slower moving components. This second form (Fraction 2) has been obtained in a pure state after an additional pass through preparative gel electrophoresis. If the purification was performed in the presence of 0.1 mM phenylmethylsulfonyl fluoride, an inhibitor of serine proteases, the same pattern of two active fractions was observed on electrophoresis.

Only two peaks of histidinol phosphate phosphatase activity were found when slices of an analytical disc gel were assayed for activity. Slices of about 1 mm were obtained using a Canalco lateral gel slicer and eluted for 18 hours at 4°C with pH 7.5 buffer. The assay procedure was not entirely satisfactory since blank gels gave a considerable absorbance at 820 nm as shown in Fig. 2 in the slices from the top of the gel which contained no protein. The enzyme used for the experiment was from a preparative gel run that was incompletely resolved. The activity peaks corresponded to the two protein bands stained with Amido Schwarz on a duplicate gel.

It would appear that, whatever the nature of the two forms of the enzyme, they are reasonably stable at this point and no conversion of one to the other can be easily detected. Electrophoretic analyses of Fractions 92 to 96, 97 to 102, 103 to 105, and 106 to 123 after they have been kept at 4°C for 2 weeks are identical with those initially obtained. No additional bands have been generated in this time and the relative intensity of the two bands in Fraction 2 appears to be the same as that in the original pattern.

The purification data of a typical run are presented in Table I. The total recovery of activity, including two preparative gel electrophoresis runs, is 24% of that in the initial crude extract. The specific activity (umoles of phosphate released per hour per mg) of Fraction 1 varied between 125 and 240, most preparations giving a value of about 175. The specific activity of Fraction 2 was lower and varied much less; however, the disparity of specific activities of Fraction 1 and 2 may be less than it appears to be since it is based on A280 and not on a per mole basis. Fraction 1 typically accounted for 15 to 25% of the total activity recovered. The 280:260 absorbance ratio is 1.52 and was determined with a Cary 14 spectrophotometer after dialysis. An amount of enzyme that would result in the hy-
drolysis of 92 μmoles of histidinol phosphate in 1 hour shows no detectable nonspecific phosphatase activity in 3 hours at 37°.

Both Fractions 1 and 2 were examined for carbohydrate content by the method of Sieben et al. (15). No detectable reaction was found; if carbohydrate is present it must be no greater than 0.1%.

Effect of pH and Mn⁺⁺ Concentration—The effect of pH on the hydrolytic activity of Fraction 1 was investigated by incubating the enzyme in 0.1 M triethanolamine that was adjusted to the desired pH with succinic acid. The effect of Mn⁺⁺ was also examined by including 1.05 mM MnCl₂ in the assay. The assays indicated by the open circles in Fig. 3 do have some Mn⁺⁺ (0.87 μM) in them since the enzyme was added in 1.0 mM MnCl₂. In the presence of this small amount of Mn⁺⁺ the pH optimum was 7.6; inclusion of MnCl₂ at a concentration of 1.05 mM shifted the optimum for phosphatase activity to 6.8 and also resulted in some stimulation of activity. The activity at the optimal pH increased about 30% upon the addition of Mn⁺⁺. Fraction 2 enzyme was treated in a similar manner with similar results. The two fractions could not be differentiated by this experiment.

Kinetic Properties—The rate of hydrolysis with varying concentrations of histidinol phosphate is shown in the double reciprocal plot for Fraction 1 enzyme (Fig. 4A). A value of 0.83 mM was calculated for the K₉ of histidinol phosphate at pH 7.5 which did not change when the assay was done at pH 6.5 in the presence of 0.28 mM MnCl₂. The expected change in velocity can be observed from the changed intercept. When Fraction 2 was tested, an identical K₉ value was determined, 0.83 mM. No deviation from linearity of the double reciprocal plot was observed and the K₉ values were similar to that of wild type enzyme, 0.30 mM (4).

As a further check on the similarity of Fractions 1 and 2, the inhibition constant for the binding of histidinol to the enzymes was determined using a Dixon plot (16). Three concentrations of histidinol phosphate, 11.76 mM, 2.01 mM, and 0.588 mM, were used at pH 7.5. The final concentration of MnCl₂ added with the enzyme was different for Fractions 1 and 2 due to different dilution factors; for Fraction 1 it was 7.95 μM and for Fraction 2 it was 13.0 μM. Fig. 4B shows the results obtained with Fraction 2. The three lines intersect at a single point and yield a Kᵢ for histidinol of 45 μM in good agreement with the constant found for the native enzyme, 52 μM (4). A Dixon plot of the results with Fraction 1 using the same histidinol phosphate and histidinol concentrations yielded a value of 32 μM, the same as Fraction 2 within the experimental error of the determination. Therefore, no significant kinetic differences have been observed between Fractions 1 and 2. Neither of the phosphatases has any detectable imidazoleglycerolphosphate dehydratase activity.

Molecular Weight—The molecular weight of the two enzymes was estimated by Sephadex G 100 gel filtration using a standardized column. The elution volumes of duplicate runs of each standard and blue dextran were within 0.5 to 1.0 ml. A plot of the log of the molecular weight of four standard proteins resulted in a straight line (Fig. 5, top). The elution volume of Fraction 1 (Fig. 5, bottom) corresponded to a molecular weight of 38,000 and of Fraction 2, to a molecular weight of 47,000.

Sedimentation Rate—The sedimentation velocity of the two fractions before storage was determined under identical conditions at pH 7.5 at a concentration of 1.5 mg per ml. The symmetrical peaks moved with sedimentation constants of 3.26 S and 3.67 S for Fractions 1 and 2, respectively, and, assuming no change in the shape of the molecules, added further substantiation that the molecular weights of Fractions 1 and 2 are different. It is clear that there is no tendency for either fraction to polymerize in the presence of Mn⁺⁺ as there is with wild type enzyme (4).

Subunit Structure from Sodium Dodecyl Sulfate Gel Electrophoresis—In order to learn whether each of the enzymes is composed of a single polypeptide or whether additional polypeptide chains are present, polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out. In addition to denaturing the enzyme by simply incubating in sodium dodecyl sulfate-mersaptoethanol at 37° for 2 to 24 hours, some samples were heated for either 6 or 12 min in a boiling water bath or heated and then incubated at 37° for 2 to 24 hours. Fig. 6 shows the sodium dodecyl sulfate gels of Fractions 1 and 2 along with their analytical gels. Fraction 1, the lower molecular weight form, shows single bands on each type of gel. A linear
relationship of molecular weight to mobility was obtained with five standard proteins. The molecular weight of Fraction 1 enzyme was estimated to be 18,000 in sodium dodecyl sulfate; therefore, the native enzyme of 38,000 molecular weight must be a dimer. All methods of denaturation gave identical results.

On the other hand, Fraction 2 yielded two polypeptide chains on sodium dodecyl sulfate gels, one having a molecular weight of 38,000 and one of about 11,500. These two chains then account for the molecular weight, 47,000, of the Fraction 2 phosphatase. When gels are loaded with much larger amounts of protein, additional minor bands can be observed with various molecular weights between 11,500 and 38,000; however, the analytical gels indicate a high degree of purity.

**Urea Gel Electrophoresis**—A single diffuse major band for Fraction 1 was observed when gel electrophoresis was performed in 8 M urea. Three minor bands were present which are substantially less intense. If the 38,000 molecular weight dimer consisted of two nonidentical chains that were able to be separated upon electrophoresis, it would be expected that they would stain with roughly equal intensity, but this was not observed.

**Discussion**

The hisB gene can be divided into four complementation groups by abortive transduction or by in vitro enzyme complementation experiments (1, 2, 6, 7). By characterizing the enzyme specified by nonsense mutants, the bifunctional gene can be divided into subregions based on molecular weight and
activity considerations (8). The genetic data (2, 6, 7) indicate that only 1 functional unit is available for the production of a gene product having both phosphatase and dehydratase activity. Nevertheless, due to the complex behavior of the enzyme, it is not entirely clear that a single polypeptide chain is synthesized from the gene. However, it is highly probable that this is the case and the enzymes purified here are derived from that polypeptide which has been modified after synthesis. If the synthesis of two chains does in fact occur, an initiation and termination signal in the middle of hisB would be required. The subunit structure of Fraction 2 then could be accounted for by synthesis of one complete chain and a fragment of a second. One should be able to observe two distinct cistrons if the synthesis of one chain is not linked to the second in some unusual manner. The structure of Fraction 1 could not be explained, however, even if the small component of Fraction 2 were lost from some molecules.

Since two cistrons have not been observed in hisB (1, 2, 6), a single polypeptide chain of 75,000 molecular weight (3, 7) is envisioned in this model as the basic subunit. The primary gene product from the nonsense mutant would be envisioned as a chain of 47,000 molecular weight which is subject to limited proteolysis cleaving it into smaller segments in a manner similar to polio virus-specific proteins (17–19), DNA polymerase (20), or the fused histidinol dehydrogenase-imidazoleacetol transaminase enzyme (21). Fig. 7 shows a hypothetical scheme which incorporates this general idea. The restricted cleavage of a peptide bond(s) would result in the form isolated as Fraction 2. Although it is conceivable that the small component may represent contamination with a small protein which just happens to bind to this enzyme, cleavage of one or a few bonds in the 38,000 fragment may proceed after dissociation of the small molecular weight component or cleavage of the large component may be a requirement for dissociation of the 11,500 molecular weight chain. Limited attempts at both direct conversion of Fraction 2 to 1 and protection from proteolysis during purification were unsuccessful and it should be emphasized that this scheme is hypothetical and the evidence presented here is circumstantial. However, in view of the genetics of hisB, it is difficult to conclude that Fractions 1 and 2 are not related in some direct fashion although the relation may possibly be more in the nature of an artifact of the isolation procedures used; i.e. the spurious action of cellular proteases. Proteolysis may not be limited strictly to a single bond and several bonds may be cleaved, leading to some heterogeneity in both Fractions 1 and 2. This would explain the multiplicity of bands on sodium dodecyl sulfate gels that are heavily loaded with protein as compared to the apparent homogeneity of the “native” enzyme as shown by analytical gel electrophoresis.

If limited proteolysis in strain TR691 occurs, it does not result in loss of phosphatase activity, nor does it result in a change of the Keq for histidinol phosphate or the inhibition constant for the competitive inhibitor, histidinol, between Fraction 1 and 2, and it does not result in substantial changes with regard to the respective value for wild type enzyme (4). Whether such post-translational events as postulated here occur with the bifunctional wild type enzyme is not known.

The missing polypeptide chain for the distal region of hisB must be important both for the development of imidazoleglycerol phosphate dehydratase activity and for the ability to polymerize to large molecular weight forms of the enzyme (8). While Mn++ effects the polymerization of the wild type enzyme into a large molecular weight form and is required for dehydration activity, it does not affect the phosphatase activity of wild type enzyme. The pH profile of both Fractions 1 and 2 is modified by the inclusion of Mn++ in the assay buffer. Perhaps this is reflecting some essential change in the enzyme structure that is necessary for polymerization and one that may occur in wild type enzyme.

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Fig. 7. Hypothetical scheme proposed for the relationship between the phosphatase enzymes of strain TR691 of Salmonella typhimurium.
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