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PURIFICATION OF THE BIFUNCTIONAL ENZYME, IMIDAZOLEGLYCEROLPHOSPHATE DEHYDRATASE-HISTIDINOL PHOSPHATASE, OF *SALMONELLA TYPHIMURIUM*

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Summary

Mn^{2+} precipitation, antibody affinity chromatography, and selective proteolysis have been used to purify a bifunctional core of the *hisB* enzyme from *Salmonella typhimurium*. The core is homogeneous in subunit molecular weight; however, it is heterogeneous in its charge properties, probably as a result of multiple cleavage points produced by limited proteolytic digestion. The resistance of the enzyme to irreversible denaturation by urea allowed the use of urea to elute the *hisB* enzyme from a column of anti-*hisB* IgG immobilized on Sepharose. Heterogeneous oligomeric forms of the enzyme were demonstrated by electrophoretic analysis and exist as multiples of the 46 000 molecular weight monomer.

Introduction

The *hisB* gene codes for the seventh (imidazoleglycerolphosphate dehydratase (D-*erythro*-imidazoleglycerolphosphate hydro-lyase), EC 4.2.1.19) and ninth (histidinol phosphatase, (histidinol-phosphate phosphohydrolase), EC 3.1.3.15) steps of the histidine biosynthetic pathway in *Salmonella typhimurium*. Recent work [1] in our laboratory on the proteolytic degradation of the *hisB* gene product has confirmed the interpretation of genetic results [2—4] that the *hisB* gene product is bifunctional. We demonstrated the presence of multiple bifunctional *hisB* polypeptides, all of which crossreacted with antibody produced in response to a 46 000 molecular weight polypeptide and had

similar phosphatase to dehydratase activity ratios. These bifunctional polypeptides had molecular weights ranging from 46 000 to 95 000. The 46 000 molecular weight polypeptide was selectively resistant to proteolysis by trypsin and α-chymotrypsin. These results are incorporated into a preparative procedure for the *hisB* enzyme described here, which uses Mn^{2+} precipitation, an antibody affinity column, and proteolytic digestion to obtain a significant quantity of bifunctional core enzyme which is homogeneous with respect to subunit molecular weight.

Materials and Methods

$(NH_4)_2SO_4$ and urea were analytical grade reagents from Mallinckrodt. 'A' grade D-*erythro*-imadazoleglycerolphosphate hydrate was from Calbiochem and histidinol phosphate was purchased from Cyclo Chemical Co. Worthington α-chymotrypsin and trypsin were used. Triethanolamine and 2-mercaptoethanol were from Sigma.

Cell growth procedure. The derepressed *S. typhimurium* mutant *his01242* (parent strain LT-2) was grown on 0.4% Difco nutrient broth, 0.2% glucose and Vogel-Bonner salts in a 200-l New Brunswick Fermacell Fermentor, harvested by centrifugation during mid-log phase, and stored as a frozen paste at −80°C.

HisB enzyme purification. Cells were suspended in buffer A (0.5 M triethanolamine-HCl (pH 7.5), 0.01 M 2-mercaptoethanol, 0.1 mM $MnCl_2$) and sonicated as before [1]. The enzyme was isolated by Mn^{2+} precipitation (6 h) and subsequent extraction into 0.4 M $(NH_4)_2SO_4$ [1]. The $(NH_4)_2SO_4$ suspension was stirred for 20 min, centrifuged at 125 000 × *g* for 30 min and the precipitate reextracted. The combined supernatants were dialyzed twice against 10 vols. buffer.

The dialyzed protein was washed onto a Sepharose CL-4B anti-*hisB* IgG affinity column [1] with 500 ml buffer, followed by 750 ml buffer containing 7 M urea. The eluted fractions were assayed for protein content and both enzyme activities. Active fractions were pooled, concentrated to 50 ml using an Amicon Diaflow apparatus and dialyzed. None of the small amount of precipitate which formed during dialysis was discarded.

The amount of protein in the concentrate was determined and the solution was digested with 1% (w/w) α-chymotrypsin at 4°C in buffer A. The reaction mixture was stirred for 5 h and adjusted to 1.6 mM phenylmethyl sulfonyl fluoride. Stock solutions of 1 mg/ml protease in buffer and 10 mg/ml phenylmethyl sulfonyl fluoride in ethanol were freshly made.

The digested enzyme preparation was immediately applied to a 2.5 × 87 cm Sephacryl S-200 column equilibrated with buffer A. Active fractions were pooled and concentrated to 1—10 mg/ml. This procedure produced a high yield of homogeneous subunits as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis. SDS-gel electrophoresis was performed by Laemmli's procedure [5] using 2.0 mm thick slab gels and 11% acrylamide. Proteins in sample buffer were heated at 100°C for 10 min. Polyacrylamide gel electrophoresis (7.5% acrylamide) was carried out using either System I or III

described by Gabriel [6] with our modifications [1]. Gels containing 8 M urea were made using the same procedure. All gels were stained with Coomassie blue G-250 using the procedure of Fairbanks et al. [7].

Isoelectric focusing. The isoelectric focusing gels [8] contained 8 M urea, 5% acrylamide, 0.134% *N,N'*-methylenebisacrylamide, 2.5% pH 3.5—10 Ampholine and 1% each of pH 9—11, 4—6, and 5—7 Ampholine. The initial voltage was 200—400 V and the final voltage was 1000 V. The pH gradient was determined by cutting a gel strip into 10-mm slices and measuring the pH after overnight agitation in 1 ml of distilled water.

Protein determination. Three protein assays were utilized: Warburg's procedure, as summarized by Layne [9], Waddell's method [10,11], and the procedure of Lowry et al. [12]. Protein samples were dialyzed against phosphate buffer to remove all interfering substances. Averages using all three methods were obtained when very accurate determinations were desired.

Enzyme assays. The substrates histidinol phosphate and imidazoleglycerolphosphate were stored as 10 mM solutions in 0.4 M triethanolamine-HCl (pH 7.5), at 4°C. Imidazoleglycerolphosphate dehydratase and histidinol phosphatase were assayed as described previously [1].

Reaction conditions were varied to see where, in each case, the product formation for phosphatase or dehydratase began to increase nonlinearly. *HisB* enzyme levels above 1 μg/100 μl assay led to nonlinearity for both enzyme activities. The phosphatase reaction became nonlinear with incubation times greater than 30 min, while the dehydratase reaction remained linear for incubation periods less than 50 min. For both activities, nonlinearity occurred at substrate levels above 2 mM. Preincubation of the enzyme at low pH (below 7.0) or 37°C destroys either activity irreversibly, but inactivation at high pH (9.5) can be reversed by adjusting the assay mix to pH 7.5.

Results

Mn^{2+} precipitation

About 90% of the total phosphatase and dehydratase activities were recovered with one extraction of the cell debris. The inclusion of either (0.1 mM) Mn^{2+} or Mg^{2+} in the sonication mixture had no effect on enzyme recovery. Preliminary experiments showed that Mn^{2+} served as a more selective precipitating agent than Mg^{2+}. The optimal Mn^{2+} concentration for precipitation was determined to be 60 mM. Although ammonium sulfate tended to inactivate the *hisB* enzyme when used for precipitation, it had little effect on recovery at the concentration used to resolubilize the enzyme.

Affinity chromatography using a specific immunoglobulin

The elution behavior of partially purified *hisB* enzyme over an anti-*hisB* affinity column revealed that an equilibrium of enzyme forms may exist which involves a minor (>10%) population of enzyme. Even if small enzyme samples were applied in an attempt to eliminate all fall-through enzyme activity, some enzyme activity was always found not to bind to the column. In view of the small size (<10 mg) of some of these samples, *hisB* enzyme was probably not being carried through the column by nonspecific protein interactions.

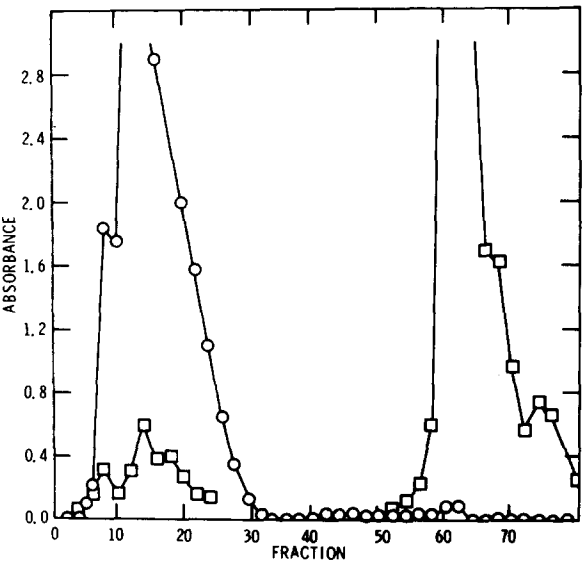


Fig. 1. Sepharose CL-4B anti-*hisB* IgG affinity column chromatography. Resolubilized enzyme obtained by Mn^{2+} precipitation was washed on a 5 × 8.3 cm Sepharose CL-4B anti-*hisB* IgG affinity column with 400 ml buffer followed by 500 ml buffer containing 7 M urea. 10-ml fractions were collected and monitored at 280 nm for protein (○). 10-μl samples were assayed for 15 min for phosphatase activity (□).

Since the instability of the *hisB* enzyme at low pH values was already known to us, urea was used to elute the enzyme. Buffer containing 7 M urea was both necessary and sufficient to remove all enzyme from the column. The ability of the antibody column to selectivity bind *hisB* enzyme is indicated by Fig. 1, which shows that the major portion of the protein immediately eluted from the column and that only a small percentage of the total protein was eluted by urea. In addition, only a small amount of activity was not retained by the column and the major proportion of phosphatase activity was eluted by urea.

We have put crude extract directly on the anti-*hisB* column and eluted the enzyme with urea. A considerable amount of enzyme (approx. 90%) was obtained which precipitated in urea containing buffer and was not resolubilized after dialysis. The soluble form after centrifugation contained mainly 46 000 M_r polypeptides on SDS gels while the precipitate contained many more higher M_r bands. The specific activities for both dehydratase and phosphatase of the soluble enzyme was about 10 times lower than those of the insoluble enzyme, which was in turn more than 50 times lower than the preparation reported here.

Preparative use of limited proteolysis

The initial *hisB* gene product appears to be digested by intracellular proteases [1], and it is these products which were observed on SDS-polyacrylamide gels (Fig. 2, lane C) after isolation on the anti-*hisB* column. Many products have higher molecular weights than the 46 000 M_r species against which the antibody was raised. We have taken advantage of the selective resis-

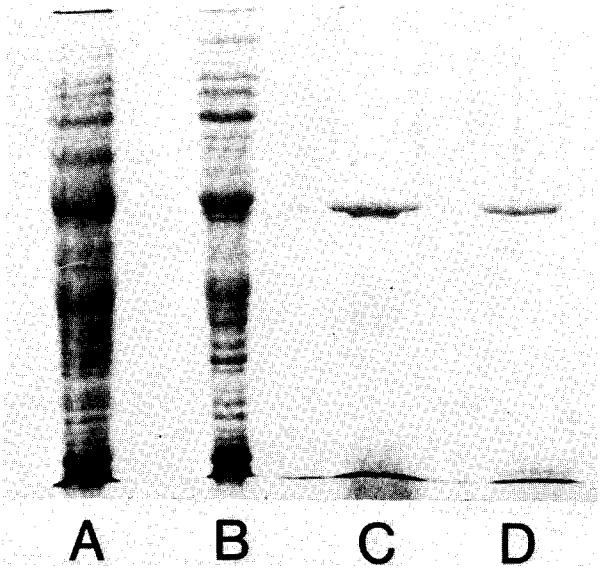


Fig. 2 Purification of the *hisB* enzyme. Samples containing 25 μg protein were incubated for 10 min at 100°C in 1% SDS and 0.1 M 2-mercaptoethanol and applied to an 11% SDS-polyacrylamide slab gel. A, crude sonicate; B, resolubilized Mn^{2+} precipitate; C, pooled, concentrated, and dialyzed fractions from the antibody affinity column (see Fig. 1); D, the enzyme preparation following digestion by α-chymotrypsin and removal of small polypeptides.

tance to digestion of the 46 000 M_r species to produce a relatively homogeneous preparation. Conditions were optimized with respect to the amount of protease, incubation time, temperature, and the amount of phenylmethylsulfonyl fluoride used to stop the reaction by examining the samples by SDS gel electrophoresis. The digestion seemed to plateau between 3 and 7 h and produced the maximum yield of 46 000 M_r species. The 46 000 M_r form was not impervious to proteolysis and began to digest after about 7 h of incubation at 4°C. The digestion products obtained with trypsin, α-chymotrypsin, or a combination of both proteases yielded the same patterns of degradation. Sephacryl S-200 column efficiently separated a single active enzyme peak from excess phenylmethylsulfonyl fluoride, α-chymotrypsin, and most of the small polypeptides. The slight heterogeneity which was sometimes seen in the active, pure enzyme pool was probably due to small polypeptides which retained the ability to associate with the aggregated enzyme.

The decrease in the number of bands and the increase in intensity of the 46 000 M_r band with each successive stage in the purification illustrates the significant purification associated with each step in this procedure. Table I shows that the final preparation obtained by the purification procedure results in a 93-fold purification of dehydratase and a 16-fold purification of phosphatase. The variation in the ratio of specific activities can probably be attributed to the difficulty in measuring activity accurately in crude sonicate and the effects of proteolysis. Other *his* operon enzymes purified from the same

TABLE I

PURIFICATION OF THE *hisB* BIFUNCTIONAL CORE

IGP, imidazoleglycerolphosphate; HP, histidinol phosphate.

Stage of purification	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Substrate	K_m (mM)	Specific activity (mmol/min per mg)	Total activity (mol/min)	Yield (%)	Specific activity phosphatase/-dehydratase
Crude sonicate	161	9.62	1550	HP	0.198	12.6	19.0	100	2.81
				IGP	0.606	4.49	6.96	100	
Resolubilized enzyme	114	4.97	567	HP	0.253	49.9	28.3	145	1.34
				IGP	0.932	45.7	25.9	372	
Concentrate from antibody column	39.3	1.33	52.3	HP	0.30	120	6.28	32	0.74
				IGP	0.60	163	8.52	122	
Final preparation	11.3	1.27	14.4	HP	0.15	202	2.90	15	0.48
				IGP	0.71	417	6.00	86	

derepressed *S. typhymurium* mutant have shown similar levels of purification [13].

Enzyme response to preparative conditions. The level of phosphatase and dehydratase activities was measured following a 24 h incubation period in 0—8 M urea solutions because, during the enzyme preparation, the enzyme remained 6—12 h in buffer-containing urea. We found that 60% of both activities persisted in 4 M urea, although no attempt was made to renature the enzyme from the urea and the assays themselves contained one-fourth the level of urea in which the enzyme samples were incubated. Both activities continued to increase in the preparative procedure following exposure to urea and renaturation (Table I). Furthermore, both activities are resistant to SDS treatment [1].

Assay mixtures containing up to 10% ethanol or phenylmethylsulfonyl fluoride at levels as high as 5 mM (the limit of solubility in aqueous solution) have virtually no effect on either enzyme activity.

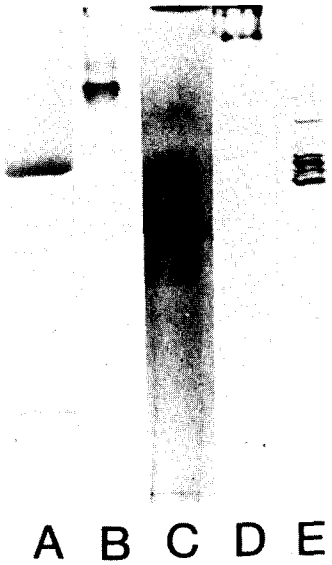
Gel filtration and polyacrylamide gel electrophoresis of the hisB enzyme

An accurate estimation of the molecular weight of the purified *hisB* core enzyme was difficult to obtain due to aggregation properties of the enzyme and the presence of proteolytic degradation products. *HisB* enzyme which had been applied to a standardized Sepharose CL-6B column eluted at a position corresponding to a 260 000 ± 40 000 M_r aggregate. This value is only approximate and is not very different from a 300 000 M_r value cited earlier [3] in which chymotryptic digestion was not employed. Because of the presence of an unknown amount of smaller degradation products, it is impossible to accurately say how many 46 000 M_r chains are contained in the aggregate, but it cannot be more than six.

SDS gels of *hisB* enzyme purified according to the procedure reported here provided evidence that the subunit population was reasonably homogeneous with respect to molecular weight (Fig. 3, lane A). This enzyme was subjected to a number of additional electrophoretic techniques in order to further characterize the protein (Fig. 4). There was one major band in all three analytical gels and several minor bands. Interestingly, the isoelectric focusing gel containing urea (Fig. 3, Lane E) contained several well-resolved bands. The gels pictured in Fig. 3 show that the *hisB* enzyme monomer obtained by this purification procedure was only homogeneous as measured by SDS electrophoresis, that a major and several minor forms constituted a large percentage of the aggregated enzyme population, and that a considerable number of enzyme forms differed in pK_i values. The many enzyme forms observed after isoelectric focusing would be consistent with the proteolytic nicking process producing a 'frayed end'.

Oligomeric hisB enzyme species

An estimate of the number and molecular weight of oligomeric species of the enzyme was made by analytical gel electrophoresis. The procedure of Hedrick and Smith [14] was modified by using a pH 6.7 stacking gel and a pH 8.9 separating gel to detect size or charge isomers of the oligomeric *hisB* enzyme. This enzyme was prepared using modifications of our earlier procedure [15]. A



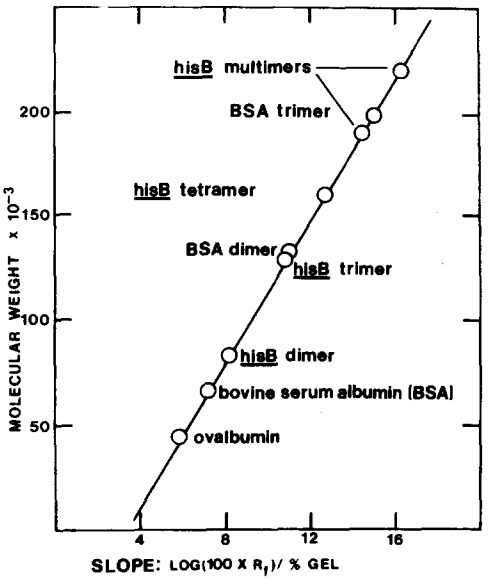


Fig. 3. Polyacrylamide gel electrophoresis of *hisB* enzyme. 100-μg enzyme samples were applied to each slab gel and electrophoresed according to the procedures in Methods. A, the sample was heated for 10 min at 100°C in 1% SDS and 0.1 M 2-mercaptoethanol before application to the gel; B, the sample was incubated for 2 h at 37°C in 8 M urea and 0.1 M 2-mercaptoethanol preceding application to an 8 M urea, pH 8.3 analytical gel; C, untreated enzyme was applied to a pH 8.3 analytical gel; D, untreated enzyme was applied to a pH 3.8 analytical gel; E, the enzyme sample was used to soak a small square of filter paper which was then applied to the middle of an isoelectric focusing slab gel. The isoelectric points of most of the bands in Lane E were between pH 4.5 and 5.0.

Fig. 4. An analysis of *hisB* enzyme oligomers by analytical gel electrophoresis. About 60 μg of *hisB* enzyme was run on analytical gels of varying polyacrylamide concentration. The mobility of different size isomers was measured as a function of the percent gel. The procedure was carried out according to the method of Hedrick and Smith [14].

plot of the logarithm of the enzyme mobilities on a series of gels versus the percent of polyacrylamide used converged at a single point, indicating that the protein species were size isomers. A plot of the slope [log ($R_f \times 100$)] from the above plot versus the molecular weight of protein standards, indicated the presence of isomers differing by multiples of approx. 46 000 M_r (Fig. 4).

Enzymatic activity of hisB aggregates

Fig. 5 shows the existence of phosphatase and dehydrase activities in all of the enzyme forms (same enzyme used in Fig. 4). Although the experimental procedure was not quantitative, the relationship between the peaks produced by densitometer scanning of the stained gel and enzyme activity peaks obtained from gel slices was approximately proportional. The different aggregate species primarily resulted from the aggregation of different numbers of identical subunits, since the subunit molecular weight in this sample was homogeneous.

The *hisB* enzyme can undergo reversible changes in size [3] which are dependent on Mn^{2+}, pH and ionic strength. In the presence of 0.2 mM Mn^{2+} in 0.05 M triethanolamine-HCl at pH 7.5, the bifunctional core enzyme purified

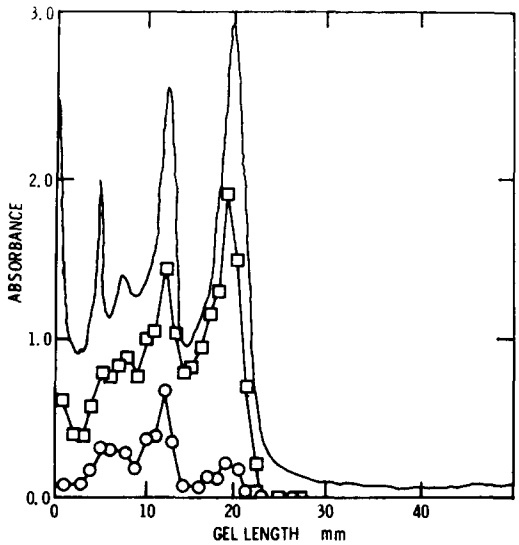


Fig. 5. Enzyme activity of gel slices from analytical polyacrylamide gels. Samples containing 100 μg of *hisB* enzyme were run on two 7.5% polyacrylamide gels, pH 8.3, at 4°C. One gel was sliced into 1-mm sections and the duplicate gel was stained for protein and subjected to densitometric scanning at 620 nm. Gel slices were incubated for 24 h in 200 μl buffer to elute the enzyme. Sample volumes and incubation times were 25 μl for 20 min in the dehydratase reaction (○) and 10 μl for 15 min for the phosphatase assay (□).

after chymotryptic digestion as described here predominantly existed in a highly aggregated form which eluted at a single active peak corresponding to a molecular weight of about 260 000 on a Sepharose 6B column. Disaggregation was induced by dialyzing the enzyme against 0.05 M glycine at pH 9.0 containing 0.5 M NaCl and lacking any Mn^{2+}. Sephadex G-200 chromatography revealed that most of the enzyme existed as the dimer form ($M_r \cong 90\,000$) under these conditions. After restoring the enzyme to the original buffer conditions, the aggregate once again eluted at a position corresponding to a 260 000 M_r on a Sepharose 6B column.

Discussion

Precipitation of the *hisB* enzyme by Mn^{2+} is an efficient first step in the isolation procedure. Because antibody generated against a 46 000 M_r species crossreacted with both larger and smaller enzyme species, all of which possessed bifunctional activity [1], it was possible to use immobilized antibody to isolate relatively large amounts of the enzyme. The use of mild proteolytic digestion to reduce the molecular weight heterogeneity proved successful in that essentially all the material migrated with a M_r = 46 000 in SDS gel electrophoresis. However, the *hisB* bifunctional core enzyme was clearly not homogeneous with respect to its charge properties. This probably resulted from slightly different chain lengths produced by proteolytic digestion.

Electrophoresis of the purified core enzyme demonstrated that multiple aggregates of renatured 46 000 M_r monomers persist, although they may be

produced by the electrophoretic conditions. The fact that gel filtration produced a symmetrical peak, which was considerably improved over previous work [3], suggests that different sized aggregates do not exist in solution. All of these forms appear to possess similar specific activities for both *hisB* enzyme activities. Furthermore, the proteolytic digestion and urea treatment did not change the metal ion dependency of aggregation which was observed previously [3,15]. The relative constancy of kinetic parameters shows the maintenance of native catalytic properties, and the ratio of phosphatase and dehydratase specific activities does not vary greatly throughout the procedure and emphasizes the bifunctional character of the enzyme.

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