Some Properties of the Catalytic Sites of Imidazoleglycerol Phosphate Dehydratase-Histidinol Phosphate Phosphatase, a Bifunctional Enzyme from Salmonella typhimurium*

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DONALD R. BRADY† AND L. L. HOUSTON

From the Department of Biochemistry, University of Kansas, Lawrence, Kansas 66044

SUMMARY

Kinetic and aggregatory properties of a partially purified preparation of D-erythro-imidazoleglycerol phosphate dehydratase-histidinol phosphate phosphatase from a derepressed mutant of Salmonella typhimurium (his01212) have been investigated. The molecular weight in crude extracts is 300,000. Purification of the enzyme causes disaggregation, the main component being 75,000 in molecular weight. Addition of MnCl₂ causes reaggregation resulting in a molecular weight greater than 300,000. Aminotriazole (Kᵢ = 3.2 μM) and phosphate ion (Kᵢ = 1.3 mM) competitively inhibited only the dehydratase activity. Zinc chloride, at micromolar concentrations, inhibits only the dehydratase activity. Both histidinol (Kᵢ = 52 μM) and histidine (Kᵢ = 10 mM) are competitive inhibitors of only the phosphatase activity. Heating at 54° results in a very rapid loss of only the phosphatase activity. T½ is 2 min; the dehydratase activity is stable under these conditions. Imidazoleglycerol phosphate is not hydrolyzed and does not inhibit the phosphatase activity. Histidinol phosphate does not affect the dehydratase activity. We conclude that the dehydratase and phosphatase active sites are separate and distinct, although they appear to reside in a single protein.

The energies of activation for both substrates were determined to be 15,100 cal per mole for the hydrolysis of histidinol phosphate and 14,700 cal per mole for the dehydration of D-erythro-imidazoleglycerol phosphate to produce imidazoleacetol phosphate and H₂O and the hydrolysis of histidinol phosphate to histidinol and phosphate. This conclusion is supported by both genetic and biochemical studies. The hisB gene product appears to be a bifunctional enzyme; a single protein responsible for two enzymatic activities, the dehydration of D-erythro-imidazoleglycerol phosphate to produce imidazoleacetol phosphate and H₂O and the hydrolysis of histidinol phosphate to histidinol and phosphate. This conclusion is supported by both genetic and biochemical studies. The hisB gene is characterized by four complementation groups (6, 7) each of which is distributed throughout the hisB gene. Mutants exist which lack only dehydratase activity; however, all mutants lacking phosphatase activity also lack dehydratase activity (4), some of those being single site mutations involving one DNA base pair (5, 7, 8). Phosphatase activity cannot be restored by in vitro complementation (8). Noncomplementing mutants are distributed throughout the gene (8). Antisera towards wild type enzyme reacts with enzyme isolated from phosphatase⁺-dehydratase⁻ mutants (7). No procedure has yet been found to physically separate the two activities during attempted purification of the enzyme (4, 8-11).

The enzyme has been reported to be difficult to purify (4) and as a result, little information concerning the structure and mechanism of action of this enzyme is available. It is assumed that two catalytic sites are responsible for the activities since aminotriazole was shown to inhibit only the dehydratase activity (12), and the nature of the reactions that are catalyzed are quite different. Because of the complex patterns of aggregation observed (10, 11), a multimeric subunit structure has been proposed (8, 11). Both 150,000 and 75,000 molecular weight components have been reported (8, 10, 11). It has been suggested that the enzyme consists of subunits with phosphatase activity which when aggregated yield the dehydratase activity (11).

The work reported here was undertaken in an attempt to more clearly define the identity of the two sites as well as to characterize some kinetic properties of the enzyme from Salmonella typhimurium his01242.

MATERIALS AND METHODS

D-Erythro imidazoleglycerol phosphate was purchased from Calbiochem and histidinol phosphate from Cyclo Chemical Co.

The histidine biosynthetic enzymes are encoded by an operon in Salmonella typhimurium and have been the subject of many elegant genetic studies in several laboratories (1-5).
Aminotriazole was purchased from Aldrich and histidinol from Sigma. *Salmonella typhimurium hisB1242* was a gift from Dr. Philip Hartman, Johns Hopkins University, and Dr. John Roth, University of California, Berkeley.

Preparation of Cell Extract of *Salmonella typhimurium*-The derepressed mutant *hisB1242* was grown on Vogel-Bonner (13) minimal medium in 0.2% glucose in 15-liter carboys by the method of Ames et al. (14). The cells were vigorously aerated for 20 hours and the harvested cells were stored as a frozen paste at -20°C until needed. Cell breakage was accomplished by sonification of a 33% (w/v) weight to volume suspension of frozen cells in 0.05 M Tris chloride-0.01 M mercaptoethanol at pH 8.5. A Branson sonifier was used to deliver three 1-min bursts. After sonification in a Rosett cell at 0°C, the extract was centrifuged at 40,000 × g for 30 min at 4°C.

**TEAE-cellulose Chromatography**—TEAE-cellulose was prepared for chromatography by the procedure of Peterson and Sober (15) and equilibrated with buffer. The extract was applied to a column (2.5 × 80 cm for the extract from 25 g of cells), washed with 1 to 2 volumes of buffer, and developed with a linear gradient using 500 ml of 0.05 M ammonium sulfate in buffer versus 500 ml of 0.5 M ammonium sulfate in buffer.

**Sephadex G-200 Gel Filtration**—A pool of the active fractions from TEAE-cellulose chromatography was concentrated on an Amicon Diaflo using a PM-10 membrane and applied to a column (6.0 × 42 cm) of Sephadex G-200 at 4°C. The pH of the elution buffer was adjusted to 7.5 for this and subsequent steps.

The fractions containing activity were pooled and concentrated as described above. The concentrate was made 1 mM in MnCl₂ and heated at 37°C for 10 min. Following cooling to 4°C, the concentrate was chromatographed on the same G-200 column after equilibration with fresh buffer containing 1 mM MnCl₂. Columns were usually developed at 20 to 30 ml per hour. The fractions containing dehydratase and phosphatase activity were pooled and concentrated. Unless otherwise stated, the enzyme preparation used for all assays was the one obtained from this second passage through Sephadex G-200.

**Enzymatic Assays**—Imidazoleglycerol phosphate dehydratase was assayed by a modification of the method used by Ames (16). The assay was performed at 37°C in a final volume of 100 μl which contained 25 mM triethanolamine hydrochloride at pH 7.5, 50 μM MnCl₂, 18 mM mercaptoethanol, 1 mM imidazoleglycerol phosphate and 5 to 10 μl of enzyme. The reaction was initiated by the addition of enzyme and was terminated with 200 μl of 1.43 N NaOH. After further incubation at 37°C for 30 min, the absorbance at 290 nm was determined against a blank containing 1 mM MnCl₂, 25 mM imidazole, 0.05 M phosphate and 5 to 10 μl of enzyme. The reaction was initiated by the addition of enzyme and was terminated with 200 μl of molybdate-ascorbate reagent. After 20 min incubation at 45°C, the absorbance was read at 820 nm against a blank using all reaction components but with 10 μl of water in place of histidinol phosphate. For these conditions, a molar extinction coefficient of 820 nm of imidazolacetyl phosphate is 5100 as calculated from the data of Ames (16).

Histidinol phosphate phosphatase was assayed by a modification of the procedure of Ames et al. (14). The reaction was carried out at 37°C in a final volume of 100 μl containing 25 mM triethanolamine hydrochloride at pH 7.5, 1 mM histidinol phosphate, and 5 to 10 μl of enzyme. The reaction was terminated by the addition of 200 μl of molybdate-ascorbate reagent. After 20 min incubation at 45°C, the absorbance was read at 820 nm against a blank using all reaction components but with 10 μl of water in place of histidinol phosphate. For these conditions, a molar extinction coefficient of 820 nm of histidinol phosphate was calculated from the data of Ames et al. (14) and was verified in this laboratory. Protein concentrations were measured by the procedure of Lowry et al. (17). All inhibitors used were buffered to pH 7.5.

**RESULTS**

**Enzyme Isolation**—The results of the chromatography of a crude extract of *Salmonella typhimurium hisB1242* on TEAE-cellulose can be seen in Fig. 2. The phosphatase and dehydratase activity chromatograph together as a single, rather broad peak toward the end of the ammonium sulfate gradient. The activities appear to be inseparable on this column. Changing the steepness of the salt gradient, adjusting the pH from 8.5 to 7.5, or using a NaCl gradient had no effect upon the coincident appearance of both activities within the peak. The concentrated eluant from TEAE-cellulose chromatography was passed through a Sephadex G-200 column resulting in the appearance of a complex activity profile with at least two peaks of activity (Fig. 3A). Both activities were found in each of the peaks; however, the ratio of phosphatase activity to dehydratase activity was different between Fraction I and Fraction II. The peaks

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1 The abbreviation used is: TEAE-cellulose, triethylaminoethylcellulose.
weight species were observed.

and Fractions I and II were estimated on standardized Sephadex G-200 columns. A column (1.5 x 92 cm) of Sephadex G-200 was calibrated using standard proteins and the profile of a l-ml elution volume identical with Fraction III; no smaller molecular weight species were observed. Further attempts to purify the enzyme have not been successful.

Effect of pH—The effect of pH on both dehydratase and phosphatase activities is shown in Fig. 4. For the phosphatase assay, buffers were made to a final concentration of 0.1 M in triethanolamine and titrated with succinic acid to the desired pH. Hydrochloric acid was used to adjust the pH for the buffers used in the dehydratase assay. Maximal dehydratase activity occurs near pH 7.5, dropping off sharply below that pH and more slowly at a more alkaline pH. Phosphatase activity was maximal in the region of pH 6.5 to 7. The inclusion of 1 mM MnCl₂ in the phosphatase assay had no effect on the pH profile. A compromise pH was chosen, pH 7.5, so that both assays could be done under conditions that were as similar as possible.

Effect of Manganese—Manganese is known to be required for imidazolglycerol phosphate dehydratase activity in Neurospora (16) and it is used in the assay for the Salmonella enzyme (14). Using the TEAE-cellulose preparation, the requirement for Mn⁺⁺ was shown for the Salmonella enzyme and a K₅ for MnCl₂ of 7.3 μM was determined.

Aminotriazole Inhibition—Aminotriazole has been demonstrated to be an inhibitor of imidazolglycerol phosphate de-
concentrations of histidinol phosphate, 0.42 mM (○) and 0.83 mM (●). Phosphatase inhibition using this assay presented obvious difficulties. Therefore, an assay was used in which the phosphatase was coupled to histidinol dehydrogenase, and the reduction of NAD$^+$ was followed (22). Using this assay, phosphatase was found not to inhibit the phosphatase at concentrations up to 20 mM. Histidinol dehydrogenase activity was unaffected by phosphate over the same concentration range.

Arsenate has been reported to be an inhibitor of phosphatase activity (7, 8), but in our hands proved to be a poor inhibitor of phosphatase activity (D). The concentration of histidinol was varied from 9.5 to 90 mM (○). Histidine inhibition of phosphatase activity (C). Histidine was varied at concentrations, up to 200 mM (○), and 2 mm (●). Phosphatase inhibition of dehydratase activity (B). The concentration of sodium phosphate (pH 7.5) was varied up to 26.1 mM and the effect on dehydratase activity was measured in the presence of 0.19 mM d-erythro-imidazoleglycerol phosphate (●) and 2.63 mM d-erythro-imidazoleglycerol phosphate (○). Histidinol inhibition of phosphatase activity (A). The concentration of histidinol was varied from 9.5 μM to 0.19 mM (○). Three concentrations of histidinol phosphate were used, 0.48 mM (○), 2.38 mM (●), and 9.5 mM (○). Histidine inhibition of phosphatase activity (D). Histidine was varied at concentrations up to 30 mM and the phosphatase activity measured at two concentrations of histidinol phosphate, 0.42 mM (○) and 0.83 mM (●). A concentration of 15 mM sodium arsenate was required to cause 50% inhibition of phosphatase activity.

Histidinol Inhibition—Histidinol is one of the products of dephosphorylation of histidinol phosphate and it is, therefore, not surprising that inhibition of the phosphatase by histidinol was observed. A Dixon plot of that data revealed the inhibition to be competitive with an inhibition constant of 52 μM (Fig. 5C). On the other hand, dehydratase activity, using 1 mM imidazoleglycerol phosphate, was not affected by the presence of histidinol at concentrations some 500 times (25 mM) as high as those giving 50% inhibition of the phosphatase.

Histidine Inhibition—A Dixon plot of the histidine inhibition of phosphatase activity (Fig. 5D) shows that the conversion of NAD$^+$ to NADH is rapid and that the reaction is first order in NAD$^+$ (23). The $K_I$ for histidine was found to be 10 mM or about 200 times larger than the $K_I$ for histidinol. Dehydratase activity is not affected by histidine up to a concentration of 20 mM. 

Zn$^{2+}$ Inhibition—During the course of examining the effect of other metal ions, it was found that zinc inhibits imidazoleglycerol phosphate dehydrogenase. At a ZnCl$_2$ concentration of 1.1 mM, dephosphorylation of imidazoleglycerol phosphate is inhibited by about 500%, even though Mn$^{2+}$ is present at a concentration seven times its $K_M$. No inhibition of phosphatase activity by zinc ion could be detected over the concentration range that resulted in inhibiting 50% of the dehydratase activity.

Inhibition by Heat—Heat treatment of the dehydratase-phosphatase enzyme allowed an additional means of showing a differential effect on the two activities. Heating to 54°C at pH 7.5 results in a very rapid first order loss of phosphatase activity with a half-life of about 2 min. In contrast to the marked sensitivity of the phosphatase, no loss of dehydratase activity was detected upon heating for up to 16 min. Loss of dehydratase activity showed some variability, but the rate of loss of dehydratase activity was always greatly different from that for phosphatase activity.

Nonreciprocal Inhibition by Substrates—If mutually exclusive and independent sites existed for the two activities, then no reciprocal inhibition would be observed between imidazoleglycerol phosphate and histidinol phosphate. Accordingly, it was first demonstrated that no phosphate was released from imidazoleglycerol phosphate when varied from 5 to 50 mM. To determine whether imidazoleglycerol phosphate could inhibit phosphatase activity, concentrations from 5 to 50 mM were added to the enzyme in 6.2 mM histidinol phosphate. The activity of the phosphatase was not affected under these conditions.

In order to investigate the converse effect, histidinol phosphate inhibition of dehydratase activity, it was first necessary to inhibit the phosphatase activity since continual release of phosphate from histidinol phosphate would in itself result in an observed inhibition of the dehydratase. Therefore, the experiment was carried out using a concentration of 50 mM histidinol which inhibits the phosphatase activity, but not the dehydratase activity. Even at this high histidinol concentration, it was not possible to completely inhibit the phosphatase activity and some phosphate was released at high histidinol phosphate concentrations. However, the presence of histidinol phosphate up to a concentration of 20 mM had no significant effect on the dehydratase activity. A slight decrease in velocity (15%) of 2 Elimination of the terminal alcoholic function yields an even weaker competitive inhibitor for the phosphatase activity. The $K_I$ for histamine is $1.9 \times 10^{-3}$ M.
imidazoglycerol phosphate dehydration was observed at 20 mM histidinol phosphate concentration and could be attributed to the small quantities of phosphate released by histidinol phosphate hydrolysis.

**Energy of Activation**—The energy of activation for the phosphatase and the dehydratase was determined from an Arrhenius plot. A value of 15,100 cal per mole was calculated for the hydrolysis of histidinol phosphate. The energy of activation for the dehydration of imidazoglycerol phosphate was calculated to be 14,700 cal per mole.

**K_m Values for Histidinol Phosphate and Imitazoglycerol Phosphate**—Histidinol phosphate was varied from 0.03 mM to 33 mM and imidazoglycerol phosphate from 0.06 mM to 33 mM. It was essential to adjust the pH of the substrates to 7.5 in order not to have the pH change at high concentrations of substrate, thus affecting the enzyme activity. A complete blank was used for each point. Lineeweaver-Burk plots were linear and yielded a $K_m$ value of 0.30 mM for histidinol phosphate and 0.70 mM for imidazoglycerol phosphate.

**DISCUSSION**

The data presented here on the inhibition patterns of the bifunctional hisB enzyme using a variety of different inhibitors indicate that the dehydratase and phosphatase sites are separate and distinct. The dehydratase activity is inhibited by aminotriazole and phosphate (Fig. 5) as well as by Zn^{2+}. The phosphatase activity is inhibited by none of these, but rather is inhibited by heating at 54°C, histidinol and histidine (Fig. 5). Dehydratase activity is not inhibited by the latter two and is considerably more stable to heat. Aminotriazole and phosphate act competitively with imidazoglycerol phosphate and, therefore, presumably bind at the active site. Since all of these inhibitors differ widely in either charge, size, or structure, and each one inhibits competitively only one of the two activities, and in view of the dissimilarity of the two reactions, it seems reasonable to conclude that two separate sites on the protein must be responsible for the two activities.

However, as Dixon and Webb (23) point out, competitive inhibition can occur by two possible modes of binding: at the active site itself (fully competitive inhibition), or at some other site which affects catalytic activity in a partially competitive manner. If the inhibitors used in this study are binding at the active site, then the lack of inhibition of one of the two activities speaks strongly for there being two separate sites. If, however, binding of the inhibitors occurs at a site other than the active site, the possibility then exists that parts of the two active sites may overlap. This possibility was ruled out, however, by observing the effects of incubating both substrates together. Because no reciprocal inhibitory effects were observed, it can be concluded that there is no overlap between the two sites; that the sites are separate and do not share common catalytic residues.

Our data on the molecular weight forms are somewhat at variance with those found by others. We have not observed any distinct species with a molecular weight of 150,000 which has been previously reported (10, 11). Rather, we observe 300,000 and 75,000 molecular weight forms as the major species under the conditions used as well as larger aggregates much higher than 300,000. Vasington and LeBeau (11) report a 75,000 molecular weight species upon further purification of the enzyme (without Mn^{2+}) or freezing and thawing of a crude preparation. Our data indicate that the 75,000 molecular weight unit is capable of aggregation to produce species of molecular weight greater than 150,000. The numerical values for these molecular weight forms must be considered tentative in light of the limitations of the method used to determine molecular weights. However, aggregation does indeed appear to occur. Although Mn^{2+} influences this aggregation, other factors may be operative as well, since further attempts to purify the enzyme by ion exchange chromatography or preparative disc gel electrophoresis even with Mn^{2+} present resulted in broad peaks or multiple bands of activity.

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Donald R. Brady and L. L. Houston

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