SEDIMENTATION PROPERTIES OF THE ENZYMES OF THE HISTIDINE B GENE

by

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In Salmonella typhimurium, the enzymes histidinol phosphate phosphatase and imidazoleglycerol phosphate dehydrase are specified by a single gene designated as the histidine B gene by Hartman et al. (1-4). These two enzymes catalyze non-sequential reactions involved in histidine biosynthesis. The histidine B gene of Salmonella typhimurium is a complex genetic region composed of four basic complementation units, designated Ba, Bb, Bc and Bd (1, 3). While only imidazoleglycerol phosphate dehydrase activity is lost when mutations occur in the Ba, the Bb or the Bd complementation units, both enzyme activities are lost if mutation occurs in the Bc complementation unit (4). The genetic and biochemical evidence reported in the literature suggest that imidazoleglycerol phosphate dehydrase is a polymer of histidinol phosphate phosphatase subunits and that the polymer is bifunctional, exhibiting both phosphatase and dehydrase activities (3-7).

Whitfield et al. (7) have determined the molecular weights of many of the enzymes specified by the histidine operon by the sucrose gradient centrifugation method described by Martin and Ames (6). The molecular weight reported by them for partially purified imidazoleglycerol phosphate dehydrase and histidinol phosphate phosphatase was 145,000 (7). We have confirmed their observations in crude extracts of these two enzymes but on further study we have observed that the molecular weight of the dehydrase and phosphatase decreases to about one half the original molecular weight following either

partial purification or aging of crude extracts. These findings suggest that the dehydrase-phosphatase enzyme complex may exist in an aggregated form in vivo. Some of our observations on this phenomenon are presented in this paper.

Methods and Experimental Procedure

Preparation of Histidinol Phosphate and Imidazoleglycerol Phosphate. L-histidinol phosphate was synthesized by the method of Ames and Horecker (8) as modified by Levin and Hartman (9). Imidazoleglycerol phosphate was synthesized by a slight modification of the method of Ames (10).

Growth and Harvest of Bacteria. A Salmonella typhimurium histidine mutant, hisIF135 was the source of histidinol phosphate phosphatase and imidazole-glycerol phosphate dehydrase. The deletion mutation in this organism does not involve the genes specifying imidazoleglycerol phosphate dehydrase and histidinol phosphate phosphatase; hence this mutant is wild type with respect to these two enzymes. The following routine procedure was used for obtaining hisIF135 cells derepressed approximately 20-fold for imidazoleglycerol phosphate dehydrase and histidinol phosphate phosphatase. Overnight cultures were obtained by inoculating the mutant bacteria into a bubbler tube containing 5-10 ml of the E medium of Vogel and Bonner (12) containing 20 μg L-histidine-HCl per ml and either 0.2% glucose or 0.02 M glycerol and aerating at 37° for about 15 hours. The overnight culture was subinoculated at a 1/100 dilution into the E medium of Vogel and Bonner containing 0.02 M glycerol and 20 μg of L-histidine-HCl per ml and shaken vigorously on a rotatory shaker for about 8 to 10 hours. These cultures were then subinoculated at 1/50 dilutions into similar E-glycerol medium containing 0.05 mM histidinol in place of histidine. Final cultures (14-15 liters) were grown by vigorous aeration at 37°. The generation time in the histidinol-containing cultures was approximately 6 hours. The cells were harvested by centrifugation when the cultures reached about 10^9 cells per ml as determined by absorbance at 650 mμ. The harvested cells were washed once in cold 0.01 M Tris-HCl, pH 7.5, and stored in pellet

form at -30° until used.

Preparation of Cell-Free Extracts. The washed cells were suspended in 0.01 M Tris-HCl containing 0.01 M mercaptoethanol, pH 7.5, and subjected to sonic vibration for 30 to 60 seconds in a Branson Sonicator. The sonic extracts were centrifuged at 104,000 x g for 1 hour at 2°C. The supernatant was decanted and used for enzyme assays. Extracts were stored at -30°C.

Purification of Enzymes. The enzymes were partially purified from crude extracts by precipitation with protamine sulfate (1 mg protamine sulfate per 4 mg protein). Following centrifugation at 35,000 g for 20 minutes, the enzymes were extracted from the pellet with 0.5 M Tris-HCl, pH 7.5. The enzymes were further purified by precipitation with ammonium sulfate (35-55 per cent saturation) followed by dialysis and DEAE-chromatography. This procedure usually yields about a 5-fold purification of the enzymes.

Enzyme Assays. L-histidinol phosphate phosphatase was assayed by the procedure of Ames et al. (11) as modified by Levin and Hartman (9). Imidazoleglycerol phosphate dehydrase was assayed by the procedure of Ames (10).

Molecular Weight Determination. The molecular weights of histidinol phosphate phosphatase and imidazoleglycerol phosphate dehydrase were determined by the method described by Martin and Ames (6) and by Whitfield et al. (7). Histidinol dehydrogenase, present in the enzyme preparation, was used as a standard. Histidinol dehydrogenase has a molecular weight of approximately 75,000 (13) and a sedimentation coefficient of 5.1S (7, 13). The sedimentation behavior of histidinol dehydrogenase was the same in the crude and partially purified preparations used in this study.

Results and Discussion

When a freshly prepared crude sonicate of hisIF135 was subjected to sucrose density gradient centrifugation, imidazoleglycerol phosphate dehydrase and histidinol phosphate phosphatase were found to sediment together (Fig. 1), confirming the observations of Martin and Ames (6) and Whitfield et al. (7).

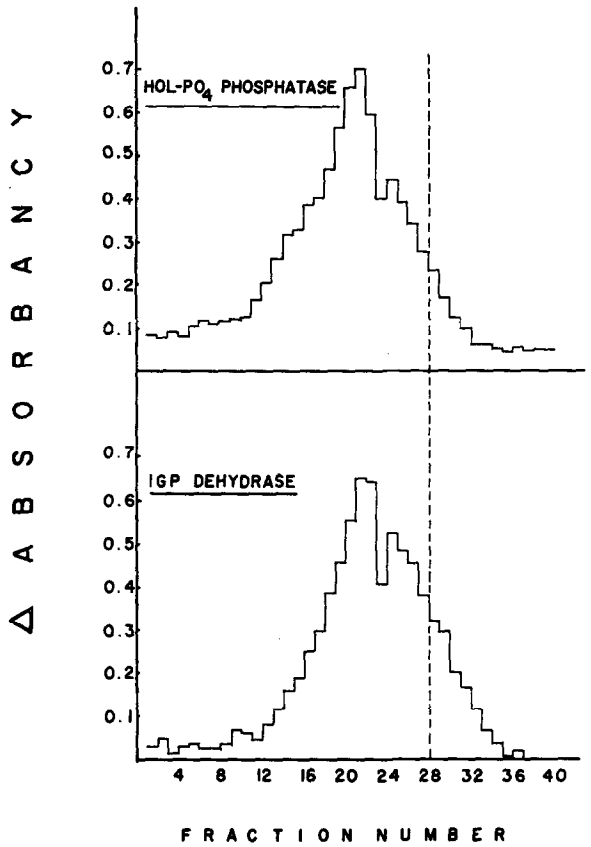

Figure 1. The centrifugation pattern of imidazoleglycerol phosphate dehydrase and histidinol phosphate phosphatase in a freshly prepared crude sonicate of hisIF135 cells. A 5-20 per cent sucrose gradient in 0.01 M Tris-HCl, pH 7.5, containing 0.01 M mercaptoethanol was used. The volume of each gradient was 4.6 ml. On top of each gradient was placed 0.1 ml of the appropriate enzyme solution containing 0.01 M Tris-HCl, pH 7.5. Centrifugation at 33,000 rpm for approximately 16 hours was carried out in a Spinco Model L2 preparative centrifuge at 2°. The SW 39L rotor was used. The incubation time for the phosphatase and dehydrase assay was 30 and 60 minutes, respectively, at 37°. Histidinol dehydrogenase was used as a standard and the dashed line indicates the fraction containing maximal dehydrogenase activity.

The molecular weight of these enzymes was calculated by the method of Martin and Ames (6) to be about 150,000 which is essentially the same molecular weight determined by Whitfield et al. (7) for these two enzymes. When a partially purified enzyme preparation was centrifuged in a sucrose density gradient, both enzyme activities still sedimented together (Fig. 2 and 3)

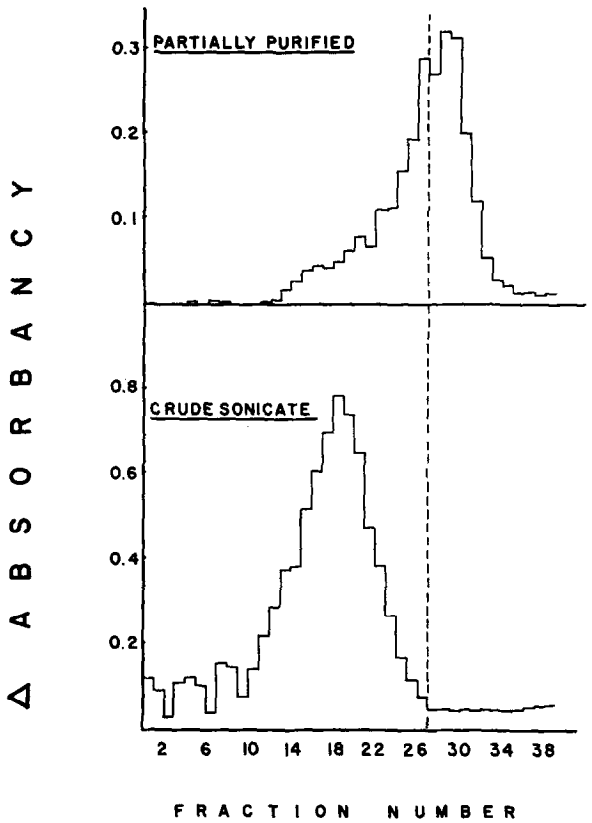

Figure 2. Comparison of the sedimentation behavior of histidinol phosphate phosphatase from a partially purified preparation (3-5 fold) and the crude sonicate from which the partially purified enzyme was obtained. The partially purified enzyme used in this experiment was obtained by precipitation with protamine sulfate (1 mg protamine sulfate/4 mg protein) followed by extraction with 0.5 M Tris-HCl, pH 7.5. The incubation time for the phosphatase assay was 30 minutes at 37°. The dashed line indicates maximal histidinol dehydrogenase activity.

but in a fraction corresponding to a molecular weight of approximately 75,000 which is one half the molecular weight of these enzymes in the freshly prepared crude sonicate. Extensive purification of the enzymes is not necessary to observe this phenomenon. Precipitation of the enzymes from the crude sonicate with protamine sulfate followed by extraction of the enzymes from the precipitate with 0.5 M Tris-HCl, pH 7.5, is sufficient to cause this change in molecular weight (Fig. 2 and 3).

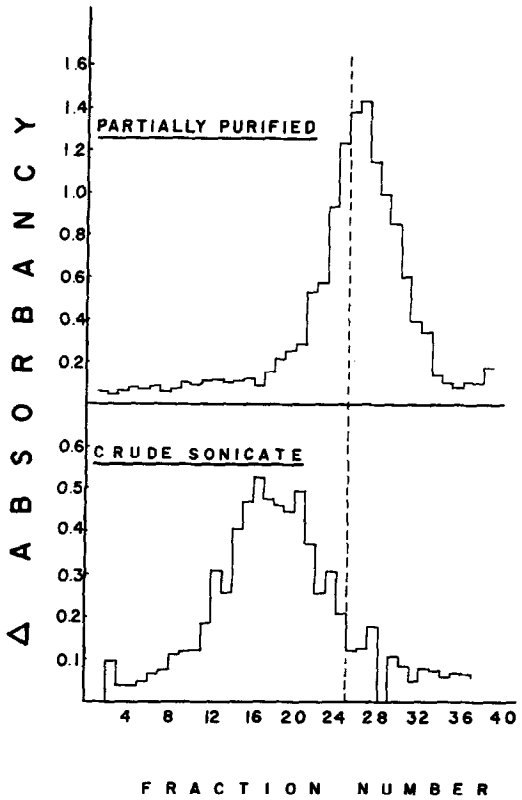

Figure 3. Comparison of the sedimentation behavior of imidazoleglycerol phosphate dehydrase from a partially purified preparation and the crude sonicate from which the partially purified enzyme was obtained. The enzyme preparations and the centrifugation procedure were the same as described in Fig. 2. The incubation time for the dehydrase assay was 60 minutes at 37°. The dashed line indicates maximal histidinol dehydrogenase activity.

Two distinct peaks containing both enzyme activities were obtained when a mixture of a partially purified enzyme preparation and a crude sonicate was centrifuged in a sucrose density gradient. The two peaks of activity correspond exactly to the activity peaks obtained when the crude and partially purified enzymes were centrifuged separately (Fig. 4). Only histidinol phosphate phosphatase activity is shown in Fig. 4 but imidazoleglycerol phosphate dehydrase activity shows the same distribution pattern as observed for the phosphatase.

It is not necessary to purify the enzymes to show phosphatase and dehydrase activity in a peak corresponding to a molecular weight of 75,000. By aging

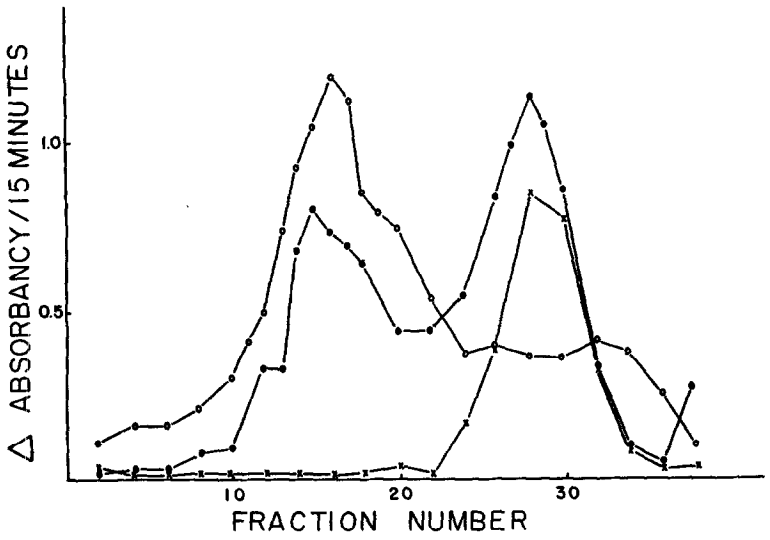

Figure 4. The centrifugation pattern of histidinol phosphate phosphatase activity in a mixture of a crude sonicate and a partially purified preparation obtained from the crude sonicate. The enzyme was partially purified by protamine sulfate precipitation followed by extraction with 0.5 M Tris-HCl, pH 7.5, ammonium sulfate precipitation and chromatography on DEAE cellulose. The procedure for centrifugation was the same as indicated in Fig. 1. —o—o—, crude sonicate; —x—x—, partially purified enzyme; —•—•—, mixture of crude sonicate and partially purified enzyme.

crude sonicates at -30° with daily freezing and thawing, the enzyme activities can be shown to exist in two peaks following sucrose density centrifugation, one corresponding to a molecular weight of about 150,000 and the other about 75,000. With progressive aging of a crude sonicate there is a decrease in the dehydrase and phosphatase activities found in the heavier peak and an increase in the activities found in the lighter peak. However, imidazoleglycerol phosphate dehydrase tends to be rather sensitive to freezing and thawing and thus total dehydrase activity decreases during this procedure.

These results indicate either that the molecular weight of the dehydrase and the phosphatase is half that previously reported (7) or that the enzymes exist as a complex which can be dissociated into smaller units retaining both enzyme activities. If imidazoleglycerol phosphate dehydrase is a dimer composed of two histidinol phosphate phosphatase subunits and if the molecular

weight of this dimer is about 75,000, the phosphatase molecule would have a molecular weight of about 40,000 which is approximately the molecular weights reported for the enzymes corresponding to the E, I, F and H histidine genes (7).

Several possible explanations can be offered to explain the above observations. It is possible that although the dehydrase-phosphatase complex can be dissociated into smaller active subunits in vitro, the larger complex with a molecular weight of about 150,000 is the active form in vivo. A second possibility is that the dehydrase-phosphatase complex exists as an aggregate in vivo which is broken down during purification or aging. Another possibility is that the dehydrase-phosphatase complex is non-specifically bound to some other protein(s) in the crude sonicate causing the dehydrase-phosphatase complex to sediment more rapidly in a sucrose gradient. Partial purification or aging of crude sonicates either removes these proteins or dissociates the enzymes from them. However, as shown in Fig. 4, when a partially purified enzyme preparation is mixed with a crude sonicate, little or no binding of the partially purified enzymes to the dehydrase-phosphatase complex in the crude sonicate occurs. This suggests that one of the first two possibilities is more likely.

We are not able to explain why our results with the partially purified dehydrase and phosphatase enzymes differ from those obtained by Whitfield et al. (7). Their studies which yielded a molecular weight of 145,000 for these enzymes were carried out with a 5-fold purified enzyme preparation. We generally obtained results similar to theirs with crude sonicates but when 3- to 5-fold purified enzyme preparations are used, a molecular weight of about 75,000 is obtained for the dehydrase and phosphatase.

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