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INOSINE-5'-PHOSPHATE DEHYDROGENASE OF *AEROBACTER AEROGENES*

STUDIES OF TERTIARY STRUCTURE

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SUMMARY

1. An improved procedure for the isolation of inosine-5'-phosphate (IMP) dehydrogenase (IMP:NAD^+ oxidoreductase, EC 1.2.1.14) is described which gives micromolar quantities of enzyme of greater than 95% purity with loss of only 30% of the total activity.

2. Ultracentrifugation and sucrose density sedimentation show that the enzyme undergoes dissociation as the ionic strength is increased. Under the following conditions two species of the indicated molecular weight were present in approximately equal amount: 0.02 M phosphate (pH 7.4) mol. wt. 560 000 and 950 000; 0.1 M Tris–citrate (pH 8.1) alone or 0.02 M buffer (pH 7.4 or 8.1) containing 0.1 M KCl, 0.1 M NaCl or 0.25 M KCl, molecular weight 185 000 and 300 000; 0.38 M Tris–HCl, molecular weight 90 000 and 180 000.

3. In the presence of sufficient (3 M) urea reversible loss of catalytic activity occurs and the enzyme shows with the ultracentrifuge a single peak of molecular weight of approx. 100 000 and with polyacrylamide electrophoresis a single protein with no enzyme activity.

4. The basic catalytic molecular species are believed to have a molecular weight of 90 000–100 000 and to possess, on the average, one IMP site per molecule. It is tentatively concluded that the species of this molecular weight are of more than one type. They appear to be converted to non-catalytic forms by 3 M urea.

INTRODUCTION

Under conditions similar to those used for its assay, inosine-5'-phosphate (IMP) dehydrogenase (IMP:NAD^+ oxidoreductase, EC 1.2.1.14) of *Aerobacter aerogenes*

Abbreviation: Cl-IMP, 6-chloro-9-β-D-ribofuranosylpurine 5'-phosphate.

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reacts rapidly with a stoichiometric amount of the 6-chloro analog (Cl-IMP) of IMP[1]. The reaction is accompanied by inactivation of the enzyme and ultraviolet spectral changes characteristic of reaction of the Cl-IMP with a sulfhydryl-containing amino acid (presumably cysteine). Under these conditions Cl-IMP does not appear to react with other amino acid residues of the enzyme. Ultracentrifugation showed that the enzyme was present, partly, as particles of molecular weight of approx. 200 000 and the above spectrophotometric titration indicated that approximately two molecules of Cl-IMP react with one such particle to cause inactivation[1]. This communication describes disc electrophoretic, ultracentrifugal and sucrose gradient sedimentation studies by means of which a survey has been made of the molecular species of the enzyme which are present at various ionic strengths and urea concentrations. Urea induces the formation of particles which on the average possess one IMP reaction site.

A variety of evidence indicates that the site of reaction of Cl-IMP is the IMP binding site(s) of IMP dehydrogenase[1-3]. Accordingly, it has become of interest to investigate the usefulness of Cl-IMP as a tool for the elucidation of amino acid sequences adjacent to the cysteine at the IMP site of this enzyme. Such studies would be facilitated by the ready availability of relatively large (micromolar) amounts of the enzyme. Improvements of our previous purification procedure[3] are now described which furnish the desired quantities of highly purified IMP dehydrogenase reproducibly and with high recovery of the available enzyme activity.

MATERIALS AND METHODS

Enzyme purification

The enzyme preparation was obtained as an $(NH_4)_2SO_4$ precipitate ("Step 3") from *A. aerogenes*, Strain P-14 (ref. 4), by the method of MAGASANIK *et al.*[5]. The bacteria were obtained from the Miles Chemical Co., Indiana. Our previous procedure[3] employed dialysis to remove excess of $(NH_4)_2SO_4$ from the Step-3 fraction prior to phosphocellulose chromatography. This dialysis led to 60–70% of the total loss of activity during purification. In the present studies, desalting was carried out at 4° with columns of Sephadex (G-25, fine) previously equilibrated with 0.02 M potassium phosphate buffer (pH 7.4). This procedure caused no loss of enzyme activity.

For column chromatography, Whatman phosphocellulose (7.4 mequiv/g) was subjected to the alkali and acid cycling procedure of PEDERSON AND SOBER[6]. The phosphocellulose was not re-used. Columns of 2.5 cm × 50 cm or 5.0 cm × 50 cm were packed at 10 and 5 lb/inch^2, respectively, on teflon supporting disks to a height of 40 cm with the phosphocellulose. The columns were equilibrated at 4° with approx. 10 vol. of 0.02 M potassium phosphate buffer (pH 7.4). To the 2.5 cm × 40 cm column was applied dialyzed Step-3 fraction from 60 g of wet packed bacteria. The phosphocellulose-to-protein weight ratio was almost 50% higher than that used previously[3]. The flow rate was maintained at 45 ml/h with a Milton Roy piston pump.

For hydroxyapatite chromatography, hydroxyapatite powder (Calbiochem) was suspended in 1 mM potassium phosphate buffer at pH 7.4, and washed by decantation until the supernate was not cloudy. The hydroxyapatite was poured as a thick slurry into a 2.5-cm Sephadex column and packed under atmospheric pressure to a height of 7 cm and equilibrated overnight with 20 vol. of buffer. All operations were carried out at 4°. Phosphocellulose (Step 4) protein (20–25 mg) was precipitated

by addition of solid $(NH_4)_2SO_4$ to 65% saturation, and was applied to the hydroxyapatite column as a solution in 1 mM potassium phosphate buffer.

Details of the enzyme assay procedure were given previously[1]. A unit of enzyme activity was defined as that which effected an increase in absorbance at 290 mμ of 1.0 in 1 min. Protein concentration was determined by the method of LOWRY *et al.*[7] using bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis

This was carried out with a Canalco Model 66 electrophoresis apparatus using the gel formation procedures described by Canalco. Protein samples of 25–200 μg were incorporated in a 3.5% gel at pH 6.8. An equal amount of a 3.5% stacking gel was subsequently polymerized on top of the sample gel. The separating gel at pH 9.0 contained 4, 6, 8 or 10% acrylamide by weight. The sample and stacking gels were light-polymerized utilizing riboflavin as the free radical initiator while the separating gel was polymerized with ammonium persulfate. A Tris–glycine buffer at pH 8.3 was used for the electrophoretic runs which were carried out in a cold room at 4° for 1–1.5 h at a constant current of 3 mA per gel. Bromphenol Blue was used to indicate local overheating or gel inconsistency.

The gels were stained for protein with Aniline Blue Black (0.5%) in 7% acetic acid or for IMP dehydrogenase activity with the tetrazolium reagent mixture, as previously described[1]. Aniline Blue Black destaining was carried out electrophoretically at 12.5 mA per gel after a staining period of at least 1 h.

Analytical ultracentrifugation

Phosphocellulose-purified IMP dehydrogenase preparations of the highest specific activity were examined with the schlieren optical system in the Spinco Model E ultracentrifuge at 60 000 rev./min. When feasible, protein fractions direct from the chromatographic columns were used, otherwise several such fractions were pooled and the protein precipitated with solid $(NH_4)_2SO_4$. The precipitate was redissolved in 25 mM Tris–citrate (pH 8.1) containing 0.1 M KCl. Sedimentation was carried out at 8 or 20° in either a 4° Kel-F cell or a 2.5° double-sector cell. An arbitrary value of 0.725 cm^3/g was assumed for the partial specific volume of IMP dehydrogenase.

Sucrose gradient centrifugation

Sucrose gradients (4.5 ml) of 5–20% in 0.02 M potassium phosphate buffer of pH 7.4 were kept at 4° for at least 2 h, after which 0.1 ml of a phosphocellulose-purified enzyme preparation containing 200–250 μg of protein was layered on the top. Centrifugation was carried out at 4° in a Beckman Model L centrifuge with a SW-39 rotor and speeds of 30 000 or 35 000 rev./min. After centrifugation, a 50% sucrose solution was forced into the bottom of the gradient tube by means of a hypodermic needle and syringe pump. Samples of 0.125 ml were collected from the top and assayed by the standard assay for IMP dehydrogenase and also for protein by the method of LOWRY *et al.*[7]. Horse blood catalase ($s_{20,w}$ of 11.2 S) was used throughout as a reference protein.

RESULTS

Enzyme purification

Two protein–activity elution profiles from phosphocellulose columns are shown in Fig. 1. The majority of the IMP dehydrogenase activity was eluted in either the latter portion (Fig. 1B) or the trailing edge (Fig. 1A) of the 0.25 M KCl protein peak. This differs from the earlier purification[3] where activity was eluted in the 0.50 M KCl peak. In addition, partial resolution of this protein peak was observed in the present studies. The increased resolution is associated with use of a higher ratio of phosphocellulose to protein. Approximately eighteen columns, packed from about twelve washed batches of phosphocellulose, gave results similar to those shown in Fig. 1. Two different batches of Whatman phosphocellulose gave the same results.

The specific activity of the IMP dehydrogenase fractionated on the column of Fig. 1A was 31.4 units/mg of protein, corresponding to a 195-fold purification from the initial crude extract. The recovery of activity from the phosphocellulose columns ranged from 70 to 85%. In the dialysis–phosphocellulose procedure[3] the specific activity was 4.5–5.0 units/mg of protein.

The larger 5.0 cm × 40 cm columns of phosphocellulose were also employed using the same relative sample size, *i.e.*, the desalted Step-3 fraction from approx. 240 g of wet packed bacteria. Essentially the same protein and activity profiles as shown in Fig. 1 were obtained.

The forementioned purification together with increased IMP dehydrogenase production by the derepressed P-14 mutant gives rise to a 5500-fold purification over the level of IMP dehydrogenase found in the wild type *A. aerogenes*.

Additional column chromatography of IMP dehydrogenase

As seen in Fig. 1, the IMP dehydrogenase activity was sometimes eluted well out in the trailing edge of the 0.25 M KCl protein peak while at other times it appeared slightly earlier. It was necessary to further purify preparations, such as shown in Fig. 1B, in which dehydrogenase activity eluted earlier in the 0.25 M KCl peak. Hydroxyapatite chromatography is suitable for this purpose. Fig. 2 shows a stepwise

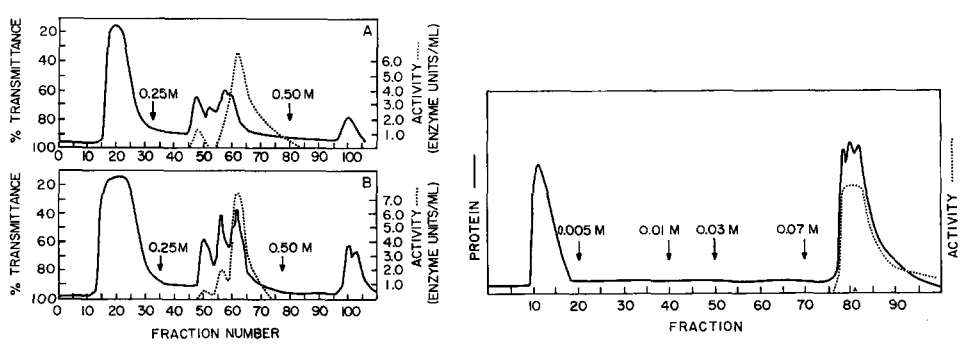

Fig. 1. Typical elution profiles of protein and IMP dehydrogenase activity from phosphocellulose columns. The arrows indicate stepwise changes in KCl concentration.

Fig. 2. Elution from a hydroxyapatite column of Fractions 58–70 of the phosphocellulose column of Fig. 1B. The concentration of phosphate buffer was increased stepwise as shown. Six 4-ml fractions were collected per h.

elution profile using increasing concentrations of phosphate buffer. A breakthrough protein peak was followed by an IMP dehydrogenase activity peak. This breakthrough peak was not present when the protein corresponding to the IMP dehydrogenase activity which had eluted well out in the trailing edge of the 0.25 M KCl phosphocellulose peak was chromatographed on hydroxyapatite. Specific activities of the hydroxyapatite preparations varied from 24 to 28 activity units/mg of protein and the recovery of activity was usually in excess of 80%. The electrophoretic and sedimentation behavior of these preparations was similar to those obtained with the most active phosphocellulose fractions.

Column chromatography of Step-4 IMP dehydrogenase on Whatman DEAE-cellulose with a linear KCl gradient in 0.02 M potassium phosphate buffer of pH 7.4 was not as successful as hydroxyapatite since activity became much more diluted. Concentration of these fractions by freeze-drying resulted in a 50% loss in enzymatic activity and concentration with Sephadex G-25-C caused a 10% loss of activity.

Preliminary experiments were carried out with molecular sieve chromatography. With Sephadex G-100 the IMP dehydrogenase activity was eluted at the void volume of the column thus indicating the molecular weight to be in excess of 100 000. With Sephadex G-150 or G-200 broad protein and activity profiles were obtained, suggesting the IMP dehydrogenase activity was present in forms of different molecular size. Similar results have been reported by POWELL *et al.*[10] who, during purification of an *Escherichia coli* IMP dehydrogenase, found three activity peaks on Sephadex G-200 columns.

Polyacrylamide gel electrophoresis

The phosphocellulose-purified preparations of highest specific activity showed four distinct protein bands migrating towards the anode (Fig. 3). Two bands of similar intensity comprised about 80% of the total stained protein. The two minor bands moved slower than the two major bands. All four bands stained for IMP dehydrogenase

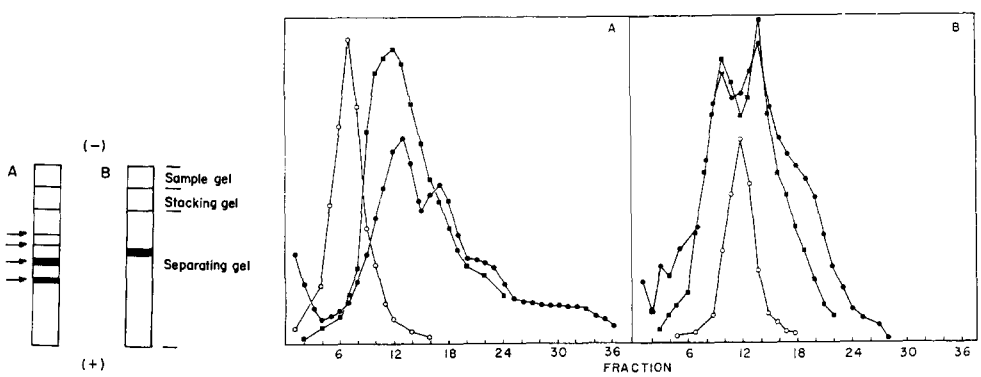

Fig. 3. Diagram of polyacrylamide electrophoresis of 60–70 μg of purified IMP dehydrogenase in a 10% gel. A shows protein bands (shown by arrows) which stain for IMP dehydrogenase activity. B shows one protein band (no activity) when the enzyme is run in gels containing 3 M urea.

Fig. 4. Sucrose gradient centrifugation of IMP dehydrogenase. A. 5 h at 35 000 rev./min. B. 8 h at 35 000 rev./min in the presence of 0.1 M KCl. ●—●, protein; ■—■, IMP dehydrogenase activity; ○—○, catalase activity.

activity. With heavier applications of protein (200 μg), four or five trace bands of protein were discernible moving faster than any of the dehydrogenase-containing bands. Reversing the electrodes in the Canalco apparatus revealed no protein which moved toward the cathode. When the protein sample was applied in a 5% sucrose solution directly on the top of the separating gel, the same band pattern was obtained, indicating that the polymerization process was not causing artifacts due to protein degradation. As the porosity of the gel was increased, all four enzyme bands migrated farther in a given time, but the relative positions of the bands were unaltered. With any given gel, the mobilities of the protein bands were very nearly a linear function of the time of the run.

Sucrose gradient centrifugation

The protein and enzyme activity curves are shown in Fig. 4A. The activity profile of IMP dehydrogenase is skewed on the bottom side of the gradient and two main protein bands are apparent. Since the IMP dehydrogenase reaction is specifically activated by K^+ it was of interest to include 0.1 M KCl in the sucrose gradients. When this was done (Fig. 4B), two activity peaks which corresponded to two protein peaks were observed. The presence of KCl had no effect on the mobility of catalase but greatly reduced the rate at which the IMP dehydrogenase peaks sedimented. The profiles were identical if catalase and the IMP dehydrogenase preparation were placed on the same or separate gradients. All of the protein applied to the gradients could be accounted for, together with 60–70% of the IMP dehydrogenase activity applied.

By the method of MARTIN AND AMES[8], and with the horse blood catalase as the reference protein, values of 19.2 and 27.2 S were calculated for the sedimentation coefficients of the protein peaks which were observed in the absence of KCl and 9.3 and 13.1 S for the peaks observed in the presence of 0.1 M KCl. From the known sedimentation coefficient and molecular weight (250 000) of catalase, molecular weights of 560 000 and 945 000 (without KCl) or 190 000 and 315 000 (with KCl) were calculated[9] for the protein species present in the IMP dehydrogenase preparations.

Analytical ultracentrifugation

The enzyme preparations consistently showed two major peaks of approximately equal areas. In some runs traces of two faster sedimenting components were also observed. The pattern was similar in either 0.02 M potassium phosphate buffer (pH 7.4) with 0.25 M KCl or in 25 mM Tris–citrate buffer (pH 8.1) with 0.10 M KCl. Likewise, 2 mM GSH had no effect on the observed schlieren pattern. Fig. 5 shows the

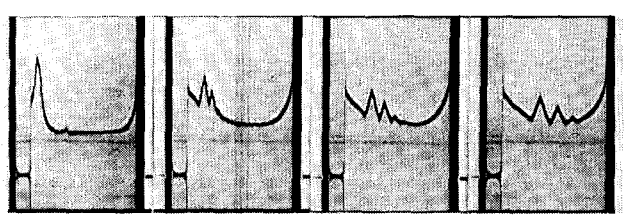

Fig. 5. Ultracentrifugation of IMP dehydrogenase at 60 000 rev./min and 8°. The protein (0.2%) was in a phosphate buffer (0.02 M) at pH 7.4 containing 0.25 M KCl. The left frame was taken about 10 min after the rotor reached full speed and the remaining frames were taken at 16-min intervals. The bar angle was 50°.

schlieren pattern for the same enzyme preparation used in the sucrose gradient experiments of Fig. 4. The $s_{20,w}$ values for the two major and one minor peaks were calculated[9] to be 9.1, 12.1 and 15.1 S.

Urea treatment

The addition of urea to the standard assay mixture for IMP dehydrogenase resulted in the enzyme inhibition seen in Fig. 6. Full inhibition occurred within the 30 sec required to start the assay and remained constant during the 15-min assay period.

When 0.1 ml of a complete 1-ml assay mixture containing urea (2.6 M) was used as the source of enzyme in another assay mixture which contained no urea, the inhibition was 16% that of the control (10-fold dilution of the enzyme gave 10% the initial rate). Inhibition by the 0.26 M urea present in the mixture would be expected to be 17–18% (Fig. 6), thus showing that the urea inhibition is rapidly and completely reversed by reduction of the concentration of urea.

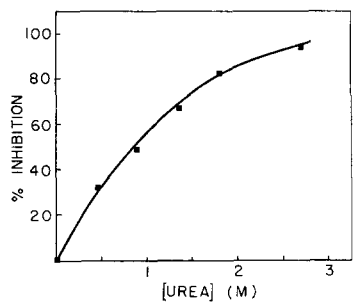

Fig. 6. Inhibition of IMP dehydrogenase by urea. Various concentrations of urea were incorporated in the standard assay. Phosphocellulose-purified enzyme was the last component added.

In the presence of 3 M urea, ultracentrifugation of IMP dehydrogenase under the conditions previously described (0.2% protein) resulted in the appearance of a single peak. After corrections for temperature, viscosity, and density were made, a $s_{20,w}$ of 6.4 S was calculated for this peak. Using the sedimentation constant and molecular weight of catalase, molecular weights of 105 000, 185 000, 290 000 and 390 000, respectively, for the urea peak and the three protein peaks of Fig. 5 were calculated[9].

Polyacrylamide gels for disc electrophoresis were made 3 M in urea prior to polymerization. When the IMP dehydrogenase was run in this system, a single band of protein (Fig. 3B) was produced. This band did not correspond to any of the four IMP dehydrogenase bands seen in the absence of urea. The band produced by 3 M urea did not stain for IMP dehydrogenase activity with the tetrazolium reagent mixture.

DISCUSSION

The four-step purification procedure resulted in a loss of only 30% of the enzyme activity present in the bacterial extract. This estimate is based on the total activity

of the Step-2 fraction which is in excess of that apparently present in the initial extract[3,5]. On the average, 75 mg of enzyme was obtained from 240 g of wet packed *A. aerogenes* cells and our chromatographic studies indicate that processing of larger amounts of bacteria should be successful provided that the correct ratio of phosphocellulose to protein is employed in the final stage of the purification. Polyacrylamide gel electrophoresis indicated that more than 95% of the total protein of the purified enzyme preparation possessed IMP dehydrogenase activity.

IMP dehydrogenase was found to dissociate progressively as ionic strength increased. Thus, in 0.02 M buffer, sucrose gradient centrifugation indicates that the two predominant species have molecular weights of 560 000 and 950 000. When 0.1 M KCl (the optimum concentration for enzyme activity) is included, the sedimentation coefficients determined from sucrose gradients (9.3 and 13.1 S) and from ultracentrifugation experiments (9.1, 12.1 and 15.1 S) suggest that the principal species present in both systems have molecular weights of approx. 185 000 and 300 000, *i.e.*, one-third the values in 0.02 M buffer. The shoulder present in the protein and activity profiles at the leading edge of the sucrose gradients (Fig. 4B) may correspond to traces of the faster (15.1 S) sedimenting material seen with the ultracentrifuge. No change in sedimentation coefficients resulted when 0.1 M NaCl replaced 0.1 M KCl in sucrose gradient experiments, or when ultracentrifugation was carried out with 0.1 M Tris–citrate buffer instead of 0.1 M KCl. With 0.38 M Tris–HCl buffer (the concentration present in the polyacrylamide separating gels) almost all the enzyme activity sedimented in sucrose gradients as two approximately equal protein-activity peaks (5.6 and 8.8 S) of mol. wt. 90 000 and 180 000, respectively.

Urea produced a loss of enzyme activity (Fig. 6) which was rapidly and fully reversible. At the minimum urea level (3 M) required to remove catalytic activity, IMP dehydrogenase showed a single peak in the ultracentrifuge with a molecular weight of approx. 100 000. This suggests that the molecular species of 9.1, 12.1 and 15.1 S seen in the ultracentrifuge in the absence of urea (Fig. 5) may be dimers, trimers and tetramers, respectively, of the species formed in 3 M urea. Titration of the enzyme with the 6-chloro analog of IMP has shown[1] that the enzyme possesses one IMP site per 100 000 molecular weight. The present findings do not eliminate the possibility that some of the 100 000-molecular-weight particles may have more than one IMP site and some may have none. Disc electrophoresis in 3 M urea (Fig. 3B) revealed a single protein component which lacked IMP dehydrogenase activity. Detection of this activity in the polyacrylamide gel columns entails extensive diffusion of IMP and NAD into the periphery of the gel and under these conditions outward diffusion of the relatively small urea molecule should be no less facile. Since 3 M urea is barely sufficient to suppress enzymatic activity, it is not clear from the urea studies alone whether the lack of enzymatic activity is an intrinsic property of the 100 000-molecular-weight species or whether the urea converts that species to a conformationally inactive form and that the gel matrix subsequently hinders restoration of the active conformation when urea diffuses away. However the four electrophoretic components seen in the absence of urea were capable of exerting IMP dehydrogenase activity while occluded in the gel matrix and under these conditions approximately half the dehydrogenase has a molecular weight of approx. 100 000. This indicates that protein of molecular weight as low as 100 000 does in fact possess the enzyme activity, because it is highly probable that protein association with the specificity required to induce

enzymatic activity would be effectively impeded by the matrix during the 10-min period of the activity test.

If the 100 000-molecular-weight species were all identical, certain of the four electrophoretic components which possess enzyme activity could be assembled from them either by additive association to yield a dimer, trimer, and tetramer, or, less probably, by combination of the 100 000-molecular-weight unit with itself in various ways to yield, for example, a series of dimers. The first possibility is excluded by the sucrose gradient centrifugation data in 0.38 M buffer and the second is rendered less likely by the widely differing electrophoretic mobilities of the four components (Fig. 3A). A more probable alternative, therefore, is that two or more types of 100 000-molecular-weight species exist and that 3 M urea causes conformational changes which mask their charge differences to produce a single protein band upon electrophoresis. Titration of the enzyme with Cl-IMP has given a non-linear plot of available IMP sites against enzyme activity from which it was concluded that the catalytic sites may not all possess the same degree of activity[1]. Such variability is in harmony with the present conclusion that the 100 000-molecular-weight particles of IMP dehydrogenase are catalytically active and the tentative suggestion that they are of more than one type.

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