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STUDIES ON INOSINE-5'-PHOSPHATE DEHYDROGENASE OF *BACILLUS SUBTILIS*

PURIFICATION AND GENERAL PROPERTIES

HIDEYOSHI YOKOSAWA*, TOHRU TOBITA** AND TOSHIRO YAMADA

Department of Biophysics and Biochemistry, Faculty of Science, the University of Tokyo, Hongo, Bunkyo-ku, Tokyo (Japan)

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SUMMARY

1. IMP dehydrogenase (IMP:NAD$^+$ oxidoreductase, EC 1.2.1.14) of *Bacillus subtilis* has been purified approximately 30-fold with a 60% yield, making use of the experimental result that effective stabilization of enzymatic activity is achieved by adding *p*-chloromercuribenzoate (PCMB). The purified enzyme is homogeneous with respect to chromatographic, disc electrophoretic and sedimentation criteria. The $s_{20,w}$ of this enzyme preparation is 7.6 S. A molecular weight of 165 000 has been calculated for the protein from the sedimentation equilibrium.

2. The enzyme activity is inhibited completely with either $2 \cdot 10^{-6}$ M PCMB or $1 \cdot 10^{-3}$ M *N*-ethylmaleimide. While the PCMB inhibition is completely reversed by 1.6 mM GSH, the *N*-ethylmaleimide inhibition is hardly reversed under similar conditions.

3. The pH optimum is 8.4. The Michaelis constant of IMP is $2.3 \cdot 10^{-5}$ M, independent of the concentration of NAD$^+$, and that of NAD$^+$ is $3.0 \cdot 10^{-4}$ M, independent of IMP concentration.

4. The amino acid composition of the enzyme is presented. Dinitrophenylation of the performic acid-oxidized enzyme reveals the existence of four NH_2-terminal amino acid residues per enzyme molecule; that is, one residue of threonine, one residue of tyrosine and two residues of histidine.

5. The reaction product formed by maleylation is examined by disc electrophoresis as well as in the ultracentrifuge. Three bands are visible on the gels and the Schlieren pattern shows two peaks. The subunit structure of this enzyme is discussed.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

* Present address, Department of Chemistry, The Institute of Medical Science, The University of Tokyo, Shiba Shirokane-daimachi, Minato-ku, Tokyo, Japan.

** Present address, Department of Chemistry, Faculty of Science, Chiba University, 1-33, Yayoicho, Chiba-shi, Japan.

INTRODUCTION

Since the first enzymological work of MAGASANIK *et al.*[1] on IMP dehydrogenase (IMP:NAD^+ oxidoreductase, EC 1.2.1.14) which occurs subsequent to a branching point in the pathway of purine nucleotide biosynthesis and is therefore the first enzyme in the biosynthetic pathway specific for GMP, several investigations[1–8] on this enzyme in microorganisms have been carried out. It is generally agreed that, as described by MAGER AND MAGASANIK[2] in *Aerobacter aerogenes* for the first time, its inhibition by purine nucleotides, particularly GMP, plays a role in the feedback control of this pathway in a similar way to many other enzymes participating in biosynthetic pathways[2,6,9–15].

While the inhibition of IMP dehydrogenase by GMP has been reported to be of the classical competitive type with a 150-fold purified enzyme of *A. aerogenes*[4], the partially purified enzyme obtained from both *Bacillus subtilis*[6] and *Salmonella typhimurium*[8] has been shown to be an allosteric protein, for which Lineweaver–Burk plots against IMP curve upward in the presence of GMP and XMP and are linear in their absence[6]. Although it has been widely accepted that IMP dehydrogenase is subject to negative feedback control by GMP *in vivo*, information about the mechanism of the inhibition at the enzyme level is far more uncertain.

In order to examine the regulation of the enzymatic activity and the correlation between function and molecular structure, it is desirable to use a highly purified enzyme. The present paper deals with a preparation of homogeneous IMP dehydrogenase from *B. subtilis* and some of its properties.

MATERIALS AND METHODS

Crude extract

The crude extract[6], which was prepared from derepressed cells of a mutant of *B. subtilis* Marburg G-6 (try$^-$ gua$^-$ reductase$^-$) by lysozyme digestion, was kindly donated to us by Dr. I. Shiio, Central Research Laboratories of Ajinomoto Co., Inc.

Enzyme assay

IMP dehydrogenase activity was assayed according to the method of MAGASANIK *et al.*[1]. The standard enzyme assay was as follows: The mixture containing 33.3 mM Tris–HCl (pH 7.8), 33.3 mM KCl, 1.6 mM GSH, 1.0 mM IMP and an appropriate amount of enzyme in a total volume of 2.8 ml, was preincubated for 10 min at 25° in a test tube and transferred to a quartz cuvette. 0.2 ml of 12.5 mM NAD^+ was then added to initiate the reaction and the increase in absorbance at 340 nm was followed by a Hitachi spectrophotometer (Type 139) at 25°. A unit of activity was defined according to MAGASANIK *et al.*[1].

Polyacrylamide disc gel electrophoresis

Electrophoresis of the enzyme was performed in polyacrylamide gels (pH 9.4), essentially as described by DAVIS[16]. Riboflavin was used instead of $(NH_4)_2S_2O_8$ to catalyze the polymerization of the running gel. The acrylamide concentration of the running gel and the sample gel were 7.5% and 2.5%, respectively.

Ultracentrifugal analysis

Sedimentation velocity and molecular weight analysis were performed in a Spinco Model E analytical centrifuge equipped with Schlieren optics. A Nippon Kōgaku comparator (Type 6) was used to analyze the photographic plates.

Amino acid analysis

The purified enzyme (1.2 mg) was hydrolysed in 1.5 ml of constant-boiling hydrochloric acid at 110° for 24 h or 32 h in a sealed tube under nitrogen. The hydrolysate was evaporated to dryness, and a quantitative amino acid analysis was carried out using a Hitachi automatic amino acid analyzer. Half-cystine was determined as cysteic acid for the oxidized enzyme protein. The performic oxidation of the enzyme was carried out at 0° by the method of MOORE[17]. The oxidized protein was recovered by lyophilization and hydrolysed for 32 h under the same conditions as before.

Tryptophan content was determined from the absorption spectrum of the enzyme in 0.1 M NaOH according to the method of BENCZE AND SCHMIDT[18].

N-terminal analysis

The oxidized protein was dinitrophenylated by the procedure of FRAENKEL-CONRAT *et al.*[19]. A hydrolysate of the DNP-protein was prepared by hydrolysis in 6 M HCl at 110° for 16 h under nitrogen. Upon hydrolysis the by-product dinitrophenol was eliminated by sublimation and the ether-soluble DNP-amino acids were separated by one-dimensional paper chromatography in the solvent system: toluene, 2-chloroethanol, pyridine, 0.8 M NH_4OH (100:60:30:60, by vol.) (Solvent I). Each spot was extracted with 4 ml of 1% $NaHCO_3$ and the quantity was determined spectrophotometrically in a Hitachi spectrophotometer.

The DNP-amino acids were identified by paper chromatography in the solvent system: *tert.*-amyl alcohol–phthalate (Solvent II), and by thin-layer chromatography of Silica Gel G in the solvent systems: chloroform, methanol, acetic acid (70:3:3, by vol.) (Solvent III); chloroform, methanol, acetic acid (95:5:1, by vol.) (Solvent IV); and benzene, pyridine, acetic acid (80:20:2, by vol.) (Solvent V).

The water-soluble DNP-amino acids in the aqueous phase which were separated from inorganic salts and free amino acids by adsorption chromatography on talcum ($Mg_3Si_4O_{10}(OH)_2$), were separated from each other by high-voltage paper electrophoresis. The DNP-derivative was determined spectrophotometrically after extraction with 1% $NaHCO_3$. The identification of the DNP-amino acid was further performed by paper chromatography in the Solvent II system and thin-layer chromatography on Silica Gel G in the Solvent IV system.

High-voltage paper electrophoresis was performed at 250–300 V for the first 15 min and at 1600 V for the following 60 min on Whatman 3 MM filter paper in borate–NaOH buffer (pH 10.0)[20].

Maleylation

Maleylation of IMP dehydrogenase was performed according to the method of BUTLER *et al.*[21].

Determination of sugar content

The phenol-concentrated H_2SO_4 method[22] was used to determine the sugar content.

Protein determination

Protein concentration was determined by the method of LOWRY *et al.*[23] with human serum albumin as a standard.

Reagents

IMP, GMP and GSH were purchased from Boehringer Co., NAD^+ from Sigma Chemical Co.

RESULTS

Enzyme purification

A typical procedure for the purification of IMP dehydrogenase is described below and summarized in Table I. All steps were carried out at 0–5°.

TABLE I

PURIFICATION OF IMP DEHYDROGENASE

Fraction	*Vol. (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg) protein)*	*Recovery (%)*
Crude extract	160	2290	1580	0.7	100
Sephadex G-25	220	1100	1845	1.7	117
First gel filtration	64	117	1740	14.9	110
Second gel filtration	15	43	1030	24.4	61

Step 1. PCMB treatment: On the basis of the finding that the stability of the enzyme was markedly improved by reaction with a limited amount of PCMB, as described later, the crude enzyme was treated with PCMB prior to the other steps, and the buffers employed for the following steps contained $1 \cdot 10^{-6}$ M PCMB. A 20-ml portion of the crude extract was mixed with 2 ml of $1 \cdot 10^{-3}$ M PCMB, kept for 10 min and passed through a Sephadex G-25 column (2.0 cm × 32 cm) equilibrated with 0.03 M phosphate buffer (pH 7.0). After the first fraction of 32 ml had been discarded, the following fraction of 50 ml was collected. This procedure was performed for three additional portions of the crude enzyme solution.

Step 2. Protamine treatment: To the combined fractions, 200 ml in total, 0.05 vol. of a 2% solution of protamine sulfate was added. The mixture was allowed to stand for about 10 min with occasional stirring and centrifuged at 10 000 × *g* for 15 min. The pellet material was discarded and 0.1 vol. of $1 \cdot 10^{-3}$ M PCMB solution was added to the clear supernatant.

Step 3. First $(NH_4)_2SO_4$ fractionation: To the supernatant, solid $(NH_4)_2SO_4$ was slowly added to give a 60% saturation and stirred constantly for several hours. The precipitate formed was collected by centrifugation for 15 min at 10 000 × *g* and re-

suspended in a 40% saturated $(NH_4)_2SO_4$ solution containing $7.5 \cdot 10^{-3}$ M KH_2PO_4 and $7.5 \cdot 10^{-3}$ M K_2HPO_4. The suspension was stirred for several hours and centrifuged. The pellet obtained was dissolved in 5 ml of 0.5 M K_2HPO_4. The insoluble material was removed by centrifugation for 5 min at 10 000 $\times$ *g*.

Step 4. First gel filtration on Sephadex G-200: The clear supernatant was applied to a Sephadex G-200 column. Gel filtration on Sephadex G-200 was carried out on a Pharmacia Laboratory column (5.0 cm $\times$ 35 cm) with an upwards flow adaptor. In order to increase the flow rate, 5% (v/v) cellulose powder was mixed with the wet Sephadex G-200 particles. The same buffer as in Step 1 was used. The flow rate was 25–30 ml/h. A typical elution profile is shown in Fig. 1. At this point, the above steps were repeated on three additional batches of crude enzyme solution. All fractions having lower activity than 20 units/ml at this stage were pooled, precipitated by $(NH_4)_2SO_4$ and rechromatographed using the same procedures as described above.

Step 5. Second $(NH_4)_2SO_4$ fractionation: All the fractions with higher activity than 20 units/ml obtained in Step 4 were combined, and the enzyme was precipitated by adding solid $(NH_4)_2SO_4$ up to a 55% saturation. After several hours standing, the precipitate collected by centrifugation was dissolved in 20 ml of 0.03 M phosphate buffer (pH 8.3) and fractionated further by precipitating with 30% $(NH_4)_2SO_4$ then

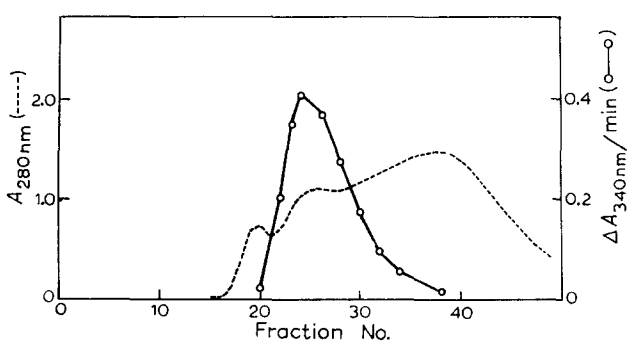


Fig. 1. Elution profile of the first Sephadex G-200 gel filtration. 5 ml of enzyme solution were applied to a Sephadex column (5.0 cm $\times$ 35 cm). The column was eluted with 0.03 M phosphate buffer, containing $1 \cdot 10^{-6}$ M PCMB (pH 7.0), and each 3-ml fraction was collected and assayed for IMP dehydrogenase activity using a 0.1-ml aliquot and measured absorbance at 280 nm.

by dissolving the precipitate in 20% saturated $(NH_4)_2SO_4$ (pH 6.0). Both operations were carried out by dipping the enzyme solution sealed in dialysis tubing into an excess amount of $(NH_4)_2SO_4$ solution with the concentration specified above.

Step 6. Second gel filtration on Sephadex G-200: The enzyme solution obtained in Step 5 was once more precipitated with 40% saturated $(NH_4)_2SO_4$ solution before being redissolved in 3 ml of the phosphate buffer and subjected to a second downwards gel filtration on Sephadex G-200 using a smaller column (2.0 cm $\times$ 32 cm). An elution pattern was obtained as shown in Fig. 2; both the activity and the protein content came out as a single peak with a symmetrical profile. Throughout the whole procedure the specific activity of the enzyme increased by a factor of 30, the recovery of the total activity being about 65%.

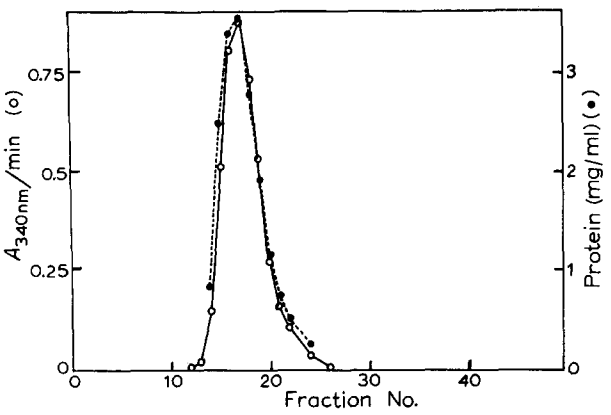


Fig. 2. Elution profile of the second Sephadex G-200 gel filtration. 3 ml of enzyme solution were placed on a 100 ml Sephadex G-200 column. The eluent buffer was the same as that of the first Sephadex G-200 chromatography. Each 3-ml fraction collected was assayed for IMP dehydrogenase activity using a 0.1-ml aliquot. Protein content was determined according to the method of LOWRY *et al.*[23].

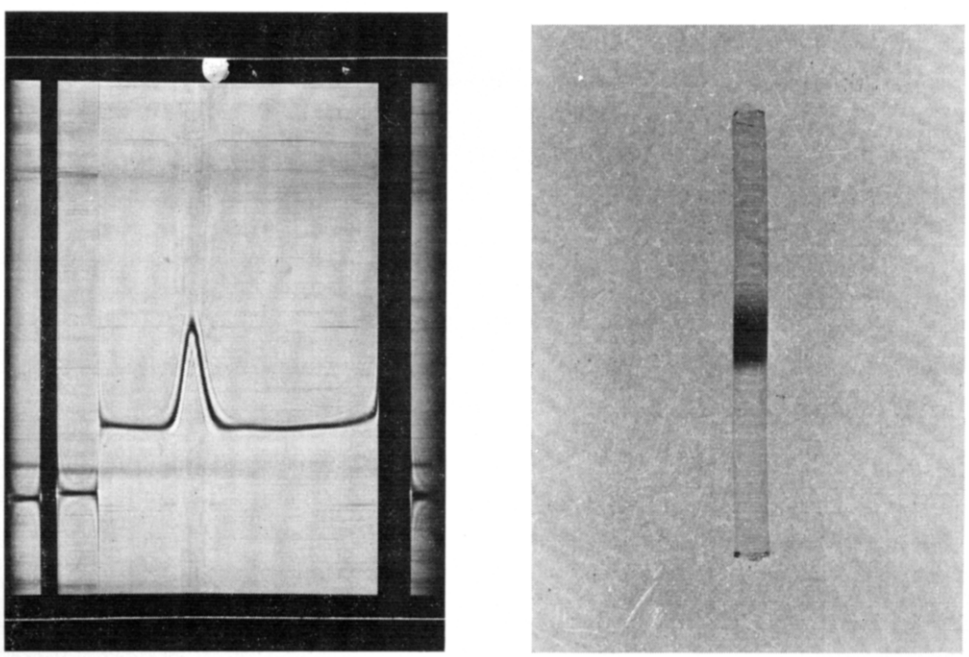


Fig. 3. Sedimentation velocity pattern of purified enzyme. The enzyme, at a concentration of about 10 mg/ml in 0.03 M potassium phosphate buffer (pH 7.0), was sedimented at 20.6° and 59 780 rev./min. A sedimentation constant, $s_{20,w}$, of 7.6 S was calculated from the data. The above picture was taken approximately 42 min after reaching full speed.

Fig. 4. Disc gel electrophoresis of purified enzyme. Riboflavin was used as a catalyst of the gel polymerization instead of $(NH_4)_2S_2O_8$. Gels were stained for protein with Amido schwarz.

Sedimentation velocity

The sedimentation profile of the purified enzyme preparation revealed that the protein migrated as a single boundary with an $s_{20,w}$ of 7.6 S at concentration of about 10 mg/ml (Fig. 3).

Disc gel electrophoresis

When the polymerization of the running gel was catalyzed with $(NH_4)_2S_2O_8$, a major band was accompanied by a faster moving minor band. It was suspected, however, that the latter band might be due to a secondary product brought about by the action of $(NH_4)_2S_2O_8$ as reported previously[24,25]. When the catalyst was replaced by riboflavin, the minor band disappeared, as illustrated in Fig. 4, and a single but rather diffuse band appeared. The diffuse nature of the band might originate from a tendency of the enzyme to form aggregates during electrophoretic migration.

The properties of the purified enzyme preparation thus far examined strongly suggest that the purified enzyme preparation consists of an homogeneous protein. The fact that complete purity was attained after only 30-fold purification was mainly due to a high enzyme content in the crude extract.

TABLE II

EFFECT OF PCMB

The effect of PCMB was examined with use of the enzyme which was partially purified by the procedures from Step 1 to Step 3 without PCMB treatment. Activity was assayed without activation by GSH. Activity was represented by $\Delta A_{340\ nm}$ per min.

PCMB concn. (M)	*Activity*	*Inhibition (%)*
None	0.072	0
$2.1 \cdot 10^{-8}$	0.071	2
$2.1 \cdot 10^{-7}$	0.040	45
$2.1 \cdot 10^{-6}$	0.000	100
$2.1 \cdot 10^{-5}$	0.000	100

Effect of sulfhydryl reagents on enzyme activity

The effect of PCMB on the enzyme activity is presented in Table II. The experiment was performed using enzyme which had been partially purified by the procedures through Step 1 to Step 3 without PCMB treatment. All inhibition experiments with sulfhydryl reagents, including the control experiment, were conducted with the omission of GSH under the standard assay conditions. Under these conditions, this particular enzyme preparation showed 70% of the full activity. Each inhibitor was added to the preincubation mixture described before. The enzyme activity was inhibited completely with either $2 \cdot 10^{-6}$ M PCMB or $1 \cdot 10^{-3}$ M *N*-ethylmaleimide. While the PCMB inhibition was readily reversed by 1.6 mM GSH, the *N*-ethylmaleimide inhibition was hardly reversed under the same conditions. These results suggest that the enzyme molecule contains free sulfhydryl residues essential for its catalytic activity.

TABLE III

STABILIZATION OF IMP DEHYDROGENASE BY PCMB TREATMENT

The enzyme was kept at 5° for 2 days either with or without any addition. The activity was assayed after activation by 1.6 mM GSH. The reaction mixture was the same as described in MATERIALS AND METHODS. Activity was represented by $\Delta A_{340\ nm}$ per min.

Addition	*Concn. (M)*	*Activity*		*Recovery*
		Before treatment	*After treatment*	
None		0.128	0.040	31.3
GSH	$1.6 \cdot 10^{-3}$	0.128	0.048	47.5
PCMB	$1.0 \cdot 10^{-6}$	0.128	0.117	91.4

Stability of the enzyme

The effect of PCMB on the stability of the enzyme is presented in Table III. The enzyme, which was kept at 5° for 2 days either with or without any addition, was activated by 1.6 mM GSH and assayed. Whereas the residual activity was about 30% of the original value for the sample kept without any addition, and about 50% for that with 1.6 mM GSH, the activity was almost completely retained when the enzyme was treated with $1 \cdot 10^{-6}$ M PCMB. In fact, the recovery of the total activity at the first gel filtration on Sephadex G-200 was 100% with PCMB treatment, but it did not exceed 60% without the treatment. The purified enzyme with PCMB treatment can be stored at 0° for several weeks without any appreciable loss of activity. However, longer storage causes gradual inactivation and finally formation of insoluble material.

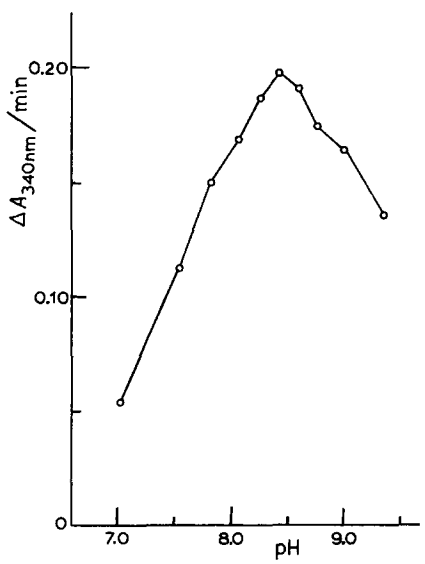


Fig. 5. pH dependence of IMP dehydrogenase. pH was measured using a Toa-Denpa pH-meter (Type HM-5A). The enzyme assay was done as described in MATERIALS AND METHODS.

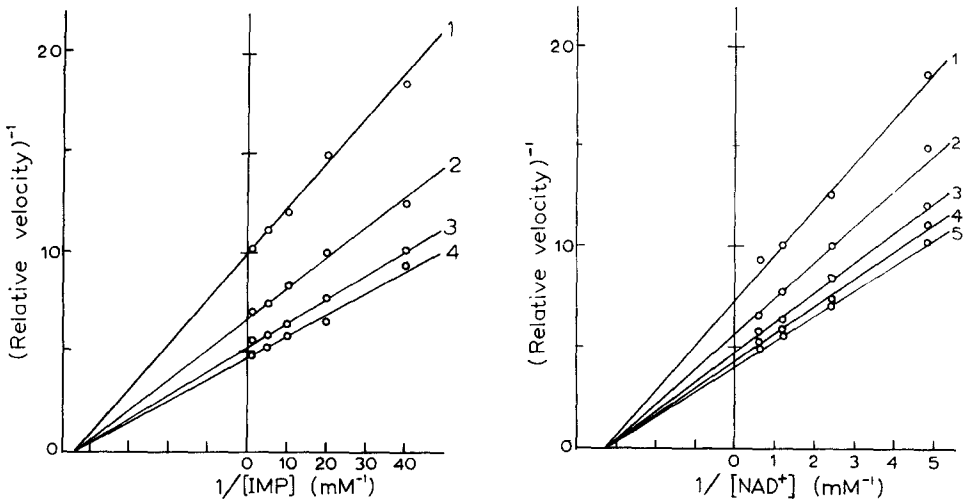


Fig. 6. Lineweaver–Burk plot of the initial velocity *vs.* IMP concentration. The four constant NAD^+ concentrations were as follows: 1, $2.08 \cdot 10^{-4}$ M; 2, $4.17 \cdot 10^{-4}$ M; 3, $8.33 \cdot 10^{-4}$ M; 4, $1.67 \cdot 10^{-3}$ M. The concentration of KCl was 33.3 mM.

Fig. 7. Lineweaver–Burk plot of the initial velocity *vs.* NAD^+ concentration. The following five constant concentrations of IMP were: 1, $2.5 \cdot 10^{-5}$ M; 2, $5.0 \cdot 10^{-5}$ M; 3, $1.0 \cdot 10^{-4}$ M; 4, $2.0 \cdot 10^{-4}$ M; 5, $1.0 \cdot 10^{-3}$ M. The KCl concentration of 33.3 mM was used.

Effect of pH on enzyme activity

Fig. 5 illustrates the pH-dependence of the enzyme activity. The buffer used was 33.3 mM Tris–HCl. The pH optimum of IMP dehydrogenase was observed to be around pH 8.4.

Substrate kinetics

Figs. 6 and 7 are Lineweaver–Burk plots of the initial velocity of the IMP dehydrogenase-catalysed reaction as functions of the concentrations of IMP and NAD^+ at pH 7.8. With four different concentrations of NAD^+, a single K_m value of $2.3 \cdot 10^{-5}$ M for IMP was obtained. A K_m value of $3.0 \cdot 10^{-4}$ M was obtained for NAD^+, independent of the concentration of IMP.

Determination of sugar content

When the phenol-concentrated H_2SO_4 method was used, no appreciable amount of sugar was found in the purified enzyme preparation.

Molecular weight

The results of the sedimentation equilibrium of IMP dehydrogenase are shown in Fig. 8 as the radial distribution of the enzyme concentration[26]. The derivative $d\ln c/dx$ of 0.260 was obtained from a linear least-squares analysis over the linear segments of the radial distribution. The empirical weight-average molecular weight calculated from the slope of this plot was 165 000. The partial specific volume of the protein was calculated to be 0.737 from the amino acid composition[27]. In the plots in Fig. 8, an upward deviation from the straight line was obvious towards the bottom.

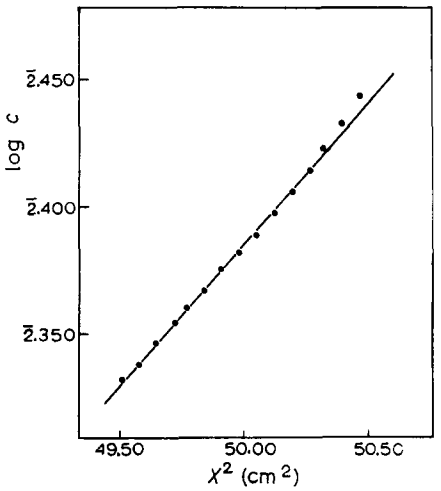


Fig. 8. Equilibrium centrifugation analysis of purified enzyme. Centrifugation was done at 5227 rev./min and 23° for 2.5 h in 0.03 M potassium phosphate buffer (pH 7.0). Log c represents the natural logarithm of the concentration of the enzymes, and X represents the distance from the center of rotation.

TABLE IV

AMINO ACID COMPOSITION OF IMP DEHYDROGENASE

Amino acid	*% by weight of residue*	*Residues/mol. wt. of 165 000*	
		Calc.	*Nearest integer*
Lysine	8.24	93.2	93
Histidine	2.39	25.4	25
Arginine	6.66	63.2	63
Cysteic acid*	1.05	14.3	14
Aspartic acid	8.45	104.8	105
Threonine	6.95	96.4	96
Serine	4.72	74.2	74
Glutamic acid	12.35	138.6	139
Proline	4.31	61.8	62
Glycine	6.48	142.5	143
Alanine	6.28	116.4	116
Valine	7.89	111.2	111
Methionine	4.23	46.8	47
Isoleucine	6.82	85.9	86
Leucine	6.41	80.8	81
Tyrosine	2.71	24.7	25
Phenylalanine	3.19	31.9	32
Tryptophan**	0.88	7.1	7
Total	100.01		1319

* Cysteic acid determined after performic acid oxidation.
** Tryptophan determined independently.

This may be a reflection of the fact that the enzyme tends to form aggregates as described before.

Amino acid composition

The results of amino acid analysis are presented in Table IV. Except for cysteic acid and tryptophan, the figures listed on the table are the higher values among those obtained after 24 h and 36 h hydrolysis. Tryptophan content was determined independently from the absorption spectrum of the enzyme in 0.1 M NaOH. The spectrum in Fig. 9 had a peak at about 291 nm and a shoulder at about 289 nm.

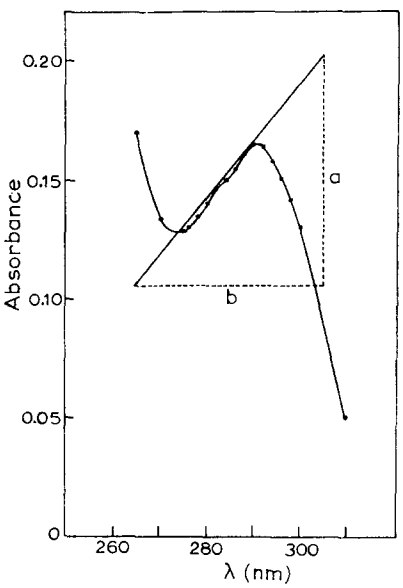


Fig. 9. Determination of tyrosine–tryptophan molar ratio from slope of tangent to absorption curve. The slope of the line drawn tangent to absorption curve in 0.1 M NaOH was $2.4 \cdot 10^{-3}$ (ΔA per Δnm). Tyrosine–tryptophan molar ratio of 3.4 was obtained according to the method of BENCZE AND SCHMID[27].

The slope of the tangent to the two maxima in the absorption curve was $2.14 \cdot 10^{-3}$ (ΔA per Δnm), and the molar ratio of tyrosine to tryptophan was calculated to be 3.4 according to the method of BENCZE AND SCHMIDT[18].

The number of residues of each amino acid calculated per mole of protein is given in Table IV, assuming the molecular weight of the enzyme to be 165 000. The values in the last column represented the nearest integer of each residue.

Determination of the N-terminal amino acid

The N-terminal groups of the native enzyme were analyzed by the DNP-method and the dansyl-method. In each case only a small amount of the amino acid derivatives derived from the N-terminal ends could be detected. The possibility that the free α-amino groups of the globular protein of high molecular weight did not react completely with the reagent must be considered, since the N-terminal ends

are buried in the protein molecule. Therefore, the denatured protein oxidized with performic acid was used for further analysis. From 30 mg of the oxidized protein 16.6 mg of the DNP-oxidized protein was obtained and hydrolyzed as described before.

The DNP-amino acids of the hydrolysate were identified on the basis of their R_F value on chromatography and R value of the DNP-derivatives. The R value is referred to as the ratio of absorbance of the DNP-derivatives at 390 nm to that at 360 nm; this value, which was approximately 0.7, was used as the criterion for the DNP-amino acids except for the proline derivative.

The DNP-amino acids of the ether extracts were resolved into one minor and two major spots by one-dimensional paper chromatography with the Solvent I system. One of the two major spots corresponded to DNP-serine or DNP-threonine and the other to di-DNP-lysine or di-DNP-tyrosine with respect to the R_F values. These spots were identified to be DNP-threonine and di-DNP-tyrosine by further analysis using one-dimensional paper chromatography with Solvent II and thin-layer chromatography with the Solvent III and IV systems. The minor spot located near the origin of the paper chromatogram in Solvent I might be considered to be DNP-glutamic acid or DNP-aspartic acid and was subjected to quantitative analysis by use of paper chromatography with Solvent II and thin-layer chromatography with Solvent III. Together with the R_F values of the DNP-derivatives, the results led to the conclusion that this spot is probably not a DNP-amino acid but an artefact of hydrolysis.

In the case of the water-soluble DNP-amino acids, the excess amount of ε-DNP-lysine derived from the intrapeptidic lysine compared to those from the N-terminal ends invariably precluded clear results when the usual chromatographic procedures were used.

High-voltage paper electrophoresis at pH 10.0 was carried out on the aqueous phase after talcum chromatography revealed only one DNP-amino acid besides ε-DNP-lysine (Fig. 10).

The amino acid derivative was identified to be di-DNP-histidine by paper chromatography and thin-layer chromatography, performed along with authentic samples as well as by qualitative tests, such as extractability in ethyl acetate and negative reactivity with Sakaguchi's reagent.

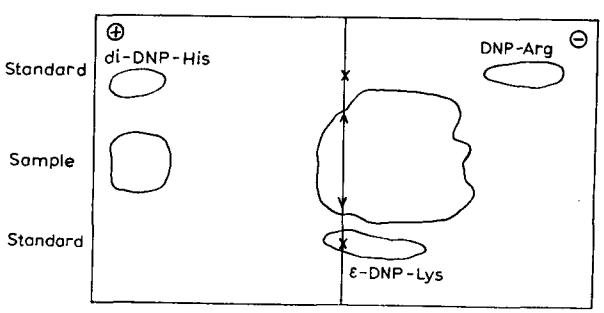


Fig. 10. High-voltage paper electrophoresis of the aqueous fraction. Electrophoresis on Whatman 3MM paper with the borate–NaOH buffer (pH 10.0) was done at 250–300 V for 15 min and then at 1600 V for 60 min.

TABLE V

N-TERMINAL AMINO ACID ANALYSIS OF IMP DEHYDROGENASE BY 2,4-DINITROFLUOROBENZENE PROCEDURE

The performic acid oxidized enzyme was used for the sample. The details are represented in MATERIALS AND METHODS and RESULTS.

DNP-amino acid	$\frac{A_{390\ nm}}{A_{360\ nm}}$	*Amount** (*μmole*)	*Recovery*** (%)	*Corrected* (*μmole*)
Threonine	0.592	0.035	51.5	0.068
Tyrosine(di-)	0.551	0.038	59.0	0.065
Histidine(di-)	0.566	0.080	59.0	0.136

* From 16.58 mg DNP-protein.
** Determined from standard DNP-amino acid.

The molar concentration of the sample was calculated using the absorption coefficients of 17.2 $mM^{-1} \cdot cm^{-1}$ for DNP-threonine, 16.7 $mM^{-1} \cdot cm^{-1}$ for di-DNP-tyrosine, and 21.5 $mM^{-1} \cdot cm^{-1}$ for di-DNP-histidine[29].

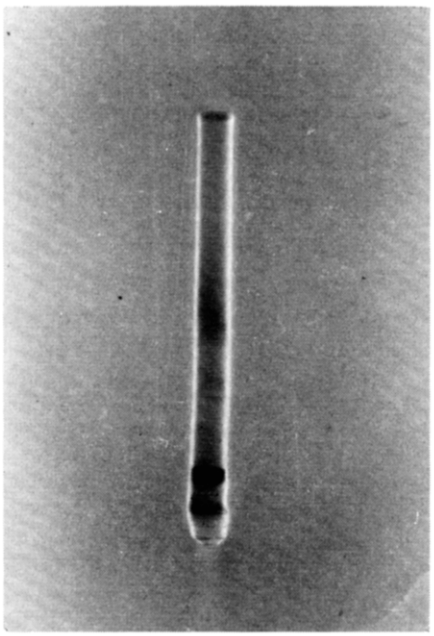
The results of the N-terminal analysis of IMP dehydrogenase are summarized in Table V. The loss of DNP-amino acids during these procedures was estimated from recovery of authentic DNP-amino acids treated by the same procedures. The amounts of DNP-amino acids, corrected for the loss, are 0.068 μmole DNP-threonine, 0.065 μmole di-DNP-tyrosine and 0.136 μmole di-DNP-histidine, in 16.58 mg DNP-protein, as shown in the last column. If one enzyme molecule is assumed to have one residue of threonine, one residue of tyrosine and two residues of histidine as the N-terminal amino acids, the molecular weight of the enzyme is estimated to be about 200 000. When it is taken into account that the recovery of the DNP-amino acid for large molecules is lower than that for a free amino acid, this value is in good agreement with that obtained by the other method.

Subunit structure

The results of the N-terminal amino acid analysis suggest that the enzyme may have more than a single polypeptide chain. This possibility was pursued by attempting to dissociate the whole molecule into its subunits by maleylation, which has been claimed to be a relatively mild treatment for this purpose[21].

The preparation obtained after maleylation was examined by polyacrylamide gel electrophoresis as well as by ultracentrifugation. As seen in Fig. 11, three bands, among which two major bands ran faster than the other minor band, were visible in the gel in contrast to that of the native enzyme. In the Schlieren pattern (Fig. 12) two peaks, divided sharply from each other, were observed. The $s_{20,w}$ of the peaks were found to be 5.6 S and 1.2 S, respectively, and the area occupied by the latter peak was much larger than that of the former.

From a careful comparison of these results it is assumed that the minor Schlieren peak corresponds to the minor band slower running in the electrophoresis which originates from the undissociated enzyme, although the undissociated protein molecule was probably unfolded by this treatment rendering its s value lower. If this is accepted, the 1.2-S component can be related to the fast-running bands which would



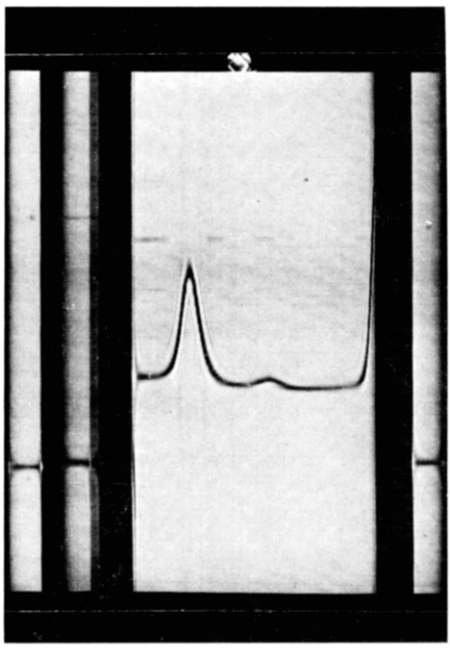


Fig. 11. Disc electrophoresis of maleylated enzyme. Riboflavin was used as a catalyst of gel polymerization. Gels were stained for protein with Amido schwarz.

Fig. 12. Sedimentation velocity pattern of maleylated enzyme. The enzyme of about 7 mg/ml in 0.03 M potassium phosphate buffer (pH 7.0) was sedimented at 17.6° and 59 780 rev./min. The above picture was taken approximately 48 min after reaching full speed.

represent the subunit fragment formed after maleylation. It is also deduced that, as the result of maleylation, the enzyme dissociates into subunit fragments which are of similar size under ultracentrifugation but are distinguishable on electrophoresis because of their varied electrostatic charges.

DISCUSSION

Purification of the enzyme, IMP dehydrogenase, has been hindered by the instability of the enzyme. Although the enzyme of *B. subtilis* apparently contains free sulfhydryl residues essential for its catalytic activity, which is inhibited reversibly by PCMB and irreversibly by *N*-ethylmaleimide, an effective stabilization of the enzymatic activity was achieved by adding such an amount of PCMB that the catalytic activity was just abolished.

In order to prevent inactivation of enzymes which are unstable and contain free sulfhydryl groups essential for their activity, compounds containing free sulfhydryl groups, such as β-mercaptoethanol, are widely employed as stabilizers. It is of significance that in the present experiment the use of an –SH blocking agent rather than –SH compounds gave better results. This finding suggests that PCMB treatment may be applicable to similar enzymes for the purpose of purification.

On the other hand, it must be borne in mind that treatment with mercurials

may alter some functions of enzymes as is already known to be the case in desensitization of allosteric enzymes. As far as the enzymatic properties presented in this report are concerned, however, the above possibility has not been realized, since the cofactor requirements as well as the kinetic constants do not differ markedly from those reported previously[1,4,6].

From the results of the sedimentation velocity experiments, the subunit fragments formed by maleylation are found to be of similar sizes, and $s_{20,w}$ was found to be around 1.2 S. Since maleylation by introducing negative charges on the protein results in disruption of noncovalent bonds in the molecules[28], the modified enzyme possibly does not behave as a compact globular molecule. In fact, KAWAHARA AND TANFORD[29] obtained randomly coiled subunits by treating rabbit muscle aldolase with 6 M guanidine hydrochloride and β-mercaptoethanol. If a similar situation occurs in the maleylated enzyme, the s value of 1.2 observed at a relatively low ionic strength in 0.06 I phosphate buffer and at a finite protein concentration (5 mg/ml) could correspond to a molecular weight close to 40 000[30]. This estimation is compatible with the finding that four N-terminal amino acids per single molecule of molecular weight 160 000 were detected. It is deduced from these considerations that the enzyme molecule contains four subunits.

The observed heterogeneity at the NH_2-terminus does not necessarily mean that the four subunits are nonidentical. It could well be explained by proteolytic action or by chemical modification at or near the NH_2-terminus of the originally identical polypeptide chains during the experimental procedure. The sharp separation of the maleylated subunits into two bands of nearly equal concentration upon disc electrophoresis, however, favors the possibility that the polypeptide chains are not identical for two reasons. Firstly, the addition of maleic anhydride in 10-fold excess over all the lysyl residues is expected to complete the reaction to well over 90% of the theoretical extent[31]. Secondly, since the acylation of lysyl residues of the dissociated subunits may proceed in an all-or-none fashion as suggested by MEIGHEN AND SCHACHMAN[32], the band separation does not stem from the difference in the extent of maleylation but is a reflection of the essential differences of the polypeptide chains. Taking into account further that the molar ratio of the three DNP-amino acids was calculated to be close to simple integers 2:1:1, it is tempting to conclude that the subunit structure of the enzyme molecule is of an A_2BC type, although it is possible that the analysed sample possesses a modified A_2B_2 structure or that the present preparation is a mixture of isozymes.

Recently BROX AND HAMPTON[33] obtained a highly purified preparation of IMP dehydrogenase from *A. aerogenes*. The sedimentation patterns of the native enzyme in an ultracentrifuge show several peaks, and the sedimentation coefficients change depending on the KCl concentrations in the media. The smallest sedimentation coefficient of 9.1 S observed in higher KCl concentrations may correspond to the s value of 7.6 for IMP dehydrogenase of *B. subtilis* calculated from the single Schlieren peak obtained during sedimentation in a medium containing only 0.03 M potassium phosphate buffer. BROX AND HAMPTON's suggestion that protein of a molecular weight as low as 100 000 possesses enzyme activity, might not contradict our model if it is assumed that both AB and AC would act as active catalytic subunits although it is still premature to draw a definite conclusion.

The significance of the existence of a subunit structure in protein with respect

to the regulation of enzymatic activity has been well documented[34]. It must await further investigation to elucidate whether this general principle is also applicable to the present enzyme, and if so, what kind of role is played by the individual subunits.

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