Inosine 5'-Monophosphate Dehydrogenase of *Escherichia coli*

PURIFICATION BY AFFINITY CHROMATOGRAPHY, SUBUNIT STRUCTURE AND INHIBITION BY GUANOSINE 5'-MONOPHOSPHATE

By Harry J. GILBERT, Christopher R. LOWE and William T. DRABBLE*
*Department of Biochemistry, School of Biochemical and Physiological Sciences,
University of Southampton, Bassett Crescent East, Southampton SO9 3TU, U.K.*

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Escherichia coli IMP dehydrogenase (EC 1.2.1.14) was purified by affinity chromatography on immobilized nucleotides. The enzyme binds to agarose-bound 8-(6-amino-hexyl)-AMP, *N*⁶-(6-amino-hexyl)-AMP and 8-(8-amino-octyl)-IMP but not to immobilized NAD⁺ or Cibacron Blue F3G-A. AMP proved to be an effective eluent. A large-scale purification scheme in which 8-(6-amino-hexyl)-AMP-agarose was used resulted in a homogeneous preparation of IMP dehydrogenase. The enzyme was also purified by immunoprecipitation with monospecific antisera. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, *N*-terminal amino acid analysis and tryptic 'finger-printing' demonstrated that IMP dehydrogenase comprises identical subunits of mol.wt. 58000. Trypsin and Pronase cleave the 58000-mol.wt. subunit into peptides of mol.wts. 42000 and 14000, with a concomitant decrease in enzyme activity. These observations rationalize much of the contradictory data on the subunit composition of the enzyme found in the literature. GMP appears to be a competitive inhibitor with respect to IMP, with no evidence for regulatory behaviour being found. The two purification procedures were also used to purify inactive mutant enzymes from *guaB* mutant strains of *E. coli*.

The conversion of IMP to GMP via XMP is catalysed by two enzymes under the control of the *gua* operon. The first, IMP dehydrogenase (IMP-NAD⁺ oxidoreductase, EC 1.2.1.14), catalyses the interconversion of IMP and XMP and is specified by the *guaB* structural gene, and the second, GMP synthetase (XMP-ammonia ligase, EC 6.3.4.1), is specified by the *guaA* gene and catalyses the conversion of XMP to GMP. In bacterial systems the synthesis of both enzymes is repressed by a guanine derivative (Nijkamp, 1969; Lambden & Drabble, 1973*a*) and stimulated by an adenine derivative (Mehra, 1978).

IMP dehydrogenase is a key enzyme in the synthesis of guanine nucleotides, since it catalyses the first reaction unique to GMP biosynthesis. Thus a number of workers have investigated the structure of the enzyme and the inhibitory properties of GMP. Two reports have shown that the enzyme from *Escherichia coli* does not possess regulatory properties in the presence of GMP (Powell *et al.*, 1969; Krishnaiah, 1975). However, Lambden & Drabble (1973*b*) presented evidence for allosteric behaviour

of IMP dehydrogenase from *E. coli* in the presence of GMP. The enzyme from *Aerobacter aerogenes* (Hampton & Nomura, 1967; Heyde *et al.*, 1976) is inhibited competitively by GMP with respect to IMP. This is not the case for the IMP dehydrogenases from *Bacillus subtilis* (Ishii & Shiio, 1968) or *Salmonella typhimurium* (Buzzeo & Levin, 1968), which possess allosteric properties in the presence of GMP. It appears that regulatory properties of the enzyme from *B. subtilis* can be removed by harsh treatment during purification (Wu & Scrimgeour, 1973*a*).

The subunit structure of IMP dehydrogenase from bacterial sources is also a matter of some controversy, Brox & Hampton (1970) suggest that the enzyme from *A. aerogenes* consists of subunits of mol.wt. 100000, whereas Heyde & Morrison (1976) have shown that this enzyme consists of identical subunits of mol.wt. 46000. IMP dehydrogenase from *S. typhimurium* appears to consist of identical subunits of mol.wt. approx. 60000 (Hanon & Levin, 1971). The subunit structure of IMP dehydrogenase from *B. subtilis* has been investigated by Yokosawa *et al.* (1971). The mol.wt. of the enzyme is 160000 and the subunits are non-identical, although they do have the same mol.wt. of 40000. However, Wu & Scrimgeour (1973*b*) have shown that the enzyme from *B. subtilis* contains only one type of subunit of mol.wt. 96000.

Abbreviations used: GSH, reduced glutathione; SDS, sodium dodecyl sulphate; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

* To whom correspondence should be addressed.

IMP dehydrogenase from *E. coli* has been shown to consist of two non-identical polypeptides of mol.wt. 62000 and 44000 (Powell, 1973). Krishnaiah (1975), on the other hand, has shown that the enzyme consists of subunits of mol.wt. of 54000.

It is the purpose of the present report to establish the subunit structure and regulatory properties of purified *E. coli* IMP dehydrogenase in the light of this conflicting evidence. It therefore describes two purification procedures for IMP dehydrogenase from *E. coli*. One technique involves affinity chromatography on immobilized IMP and AMP adsorbents, and the second involves immunoprecipitation of the enzyme. By using these methods IMP dehydrogenase has been shown to consist of identical subunits of mol.wt. 58000, which could be cleaved by proteolytic enzymes to 42000- and 14000-mol.wt. peptides. No co-operativity was observed between the IMP-binding sites of the enzyme in the presence or absence of GMP, suggesting that GMP is a straightforward competitive inhibitor of the enzyme with respect to IMP.

The purification procedures have also been used to purify inactive mutant forms of IMP dehydrogenase from *guaB* mutants. This will enable further investigation of the unusual complementation pattern observed among inactive mutant forms of the enzyme (Drabble & Gilbert, 1977).

Materials and Methods

Bacterial strains

E. coli K12 strain W3110 was used as the prototrophic parental strain for the isolation of *gua* mutants. Stock cultures were maintained on Dorset egg slopes (Lambden, 1972) supplemented with guanine (20 µg/ml). The *gua* mutants used in this study were PL1068 (*guaA48*), PL1063 (*guaB43*), PL1105 (*guaB85*), PL1138 (*guaB105*), HG1005 (*guaB1005*) and HG1018 (*guaB1018*).

Chemicals

NAD⁺, pig heart lactate dehydrogenase (400 units/mg) and rabbit muscle pyruvate kinase (200 units/mg) were obtained from the Boehringer Corp. (Lewes, Sussex, U.K.). Freund's complete adjuvant was obtained from Difco Laboratories (Detroit, MI, U.S.A.). Acrylamide (pure), butyl-PBD [5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] and toluene (sulphur-free) were obtained from Koch-Light Laboratories (Colnbrook, Bucks., U.K.). NCS solubilizer was obtained from Hopkin & Williams (Chadwell Heath, Essex, U.K.). GSH, guanine hydrochloride, riboflavin, Tris and trypsin (bovine pancreas, diphenylcarbamoyl chloride-treated, dialysed salt-free and freeze-dried) were obtained from Sigma (London) Chemical Co. Blue

Dextran 2000, Sepharose 4B, Sepharose 6B and Sephadex G-200 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). L-[4,5-³H]-Leucine (specific radioactivity 59 Ci/mmol) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Scintillation fluid contained 8 g of butyl-PBD/litre of toluene (sulphur-free). All other chemicals were supplied by BDH, Poole, Dorset, U.K.

Protein assay

The protein content of preparations was determined by the method of Lowry *et al.* (1951) unless otherwise stated, with crystalline bovine serum albumin (fraction V) as standard.

Assay for IMP dehydrogenase

The assay system for IMP dehydrogenase was based on the method of Magasanik *et al.* (1957). The reaction mixture contained the following in a total volume of 1 ml: Tris/HCl, pH 8.0, 50 µmol; KCl, 50 µmol; GSH, 5 µmol; NAD⁺, 1.25 µmol; IMP, 1.5 µmol; IMP dehydrogenase, 0.001–0.030 unit. The reaction was initiated by the addition of IMP to the reaction cuvette (10 mm pathlength), whereas the blank contained all components of the assay system except IMP. The reaction was followed continuously at 23°C in either a Unicam SP.1800 spectrophotometer (Pye-Unicam) or, for kinetic studies, a Cary 118 spectrophotometer (Varian Instruments) by measuring the production of NADH at 340 nm or by the increase in A_{290} due to XMP formation. XMP production was measured in crude enzyme preparations, such as cell-free extracts, because of interference by high concentrations of NADH oxidase. One unit of enzyme activity is defined as the amount of enzyme required to transform 1 µmol of substrate/min at 23°C. Specific activity is expressed as µmol of XMP or NADH produced/min per mg of protein. The XMP produced was calculated from a molar absorption coefficient of 4.6×10^3 litre·mol⁻¹·cm⁻¹ at 290 nm and pH 8.0 (Magasanik *et al.*, 1957). The NADH produced was calculated from a molar absorption coefficient of 6.22×10^3 litre·mol⁻¹·cm⁻¹ at 340 nm and pH 8.0.

Immunological procedures

Two methods were used to raise antibodies to *E. coli* IMP dehydrogenase. Firstly, antibodies were raised to a partially purified preparation of IMP dehydrogenase by the method of Lambden & Drabble (1973a). A monospecific anti-(IMP dehydrogenase) preparation was obtained by treating the serum with a crude extract (10 mg/ml) of a *guaB* mutant [PL1063, which contains no cross-reacting material to anti-(IMP dehydrogenase) antibodies (CRM⁻)] in a ratio of 9:1 (serum/extract, v/v). The mixture was

left overnight at 4°C, and precipitated protein was removed by centrifugation. This procedure was repeated until no further precipitation occurred.

Secondly, antibodies to purified *E. coli* IMP dehydrogenase were raised in a 3 kg New Zealand White male rabbit from the departmental colony. A solution of IMP dehydrogenase (1 ml; 1 mg/ml) purified to homogeneity by affinity chromatography, as described in this report, was emulsified with Freund's complete adjuvant and injected subcutaneously in 0.05 ml amounts into the back region of the animal by the multisite intradermal injection technique of Vaitukaitis *et al.* (1971). After 6 weeks a booster injection (1 mg) of purified IMP dehydrogenase was given intravenously and 2 weeks later the animal was killed by bleeding from the heart. Serum was obtained by leaving the blood for 1 h at 4°C, followed by centrifugation at 10000g to remove the clot. The serum was then fractionated with $(\text{NH}_4)_2\text{SO}_4$ (50% saturation, final concentration) and precipitated antibody, collected by centrifugation at 40000g, was redissolved in 0.05 M-potassium phosphate buffer, pH 7.4, containing 0.14 M-NaCl. The antibody preparation was dialysed against 2×1 litre of the same phosphate-buffered saline. The dialysed antibody fraction was then divided into portions (1 ml) and stored frozen at -15°C .

The antibody preparations were adjudged monospecific by Ouchterlony double-diffusion tests (Ouchterlony, 1949); a single precipitation line was obtained with crude cell-free extracts containing IMP dehydrogenase or with purified enzyme. In contrast, *guaB* mutants that were CRM⁻, as determined by the enzyme assay method (Lambden, 1972), gave no precipitation line, nor did control rabbit serum with cell-free extracts of *E. coli*. From these observations it appears that both antibody preparations are monospecific for IMP dehydrogenase.

Purification of IMP dehydrogenase by immunoprecipitation with monospecific antibodies

A cell-free extract of the *guaA* mutant PL1068 was prepared as described by Lambden & Drabble (1973a), except that the final culture was supplemented with L-[4,5-³H]leucine at 2 µg/ml (final specific radioactivity 1 µCi/mg). The specific activity of IMP dehydrogenase in this mutant extract was 50-fold greater than in cell-free extracts of wild-type *E. coli*. The cell-free extract (2 ml) was mixed with the 1 ml of monospecific antibody (prepared by either method) in the presence of the serine proteinase inhibitor phenylmethanesulphonyl fluoride (0.03 M final concentration in the 3 ml mixture). After 1 h the ³H-labelled immunoprecipitate was centrifuged at 30000g for 20 min and washed exhaustively with 0.015 M-Tris/HCl (pH 7.5) containing 0.15 M-NaCl until the radioactivity in the washings fell below 500 c.p.m./ml (five washes). The precipitate was then

redissolved in 2% (w/v) SDS (2 ml) containing 10% (v/v) 2-mercaptoethanol and 50 mM-Na₂CO₃ (pH 8.5).

Synthesis of ligands for affinity chromatography

(a) *N*⁶-(6-Aminohexyl)-AMP. The synthesis and characterization of *N*⁶-substituted AMP was performed as described by Guilford *et al.* (1972).

(b) 8-(6-Aminohexyl)-AMP and 8-(8-amino-octyl)-AMP. The synthesis of AMP ligands substituted in the 8-position of the adenine nucleus involved bromination of AMP to yield 8-bromo-AMP and subsequent nucleophilic displacement with a suitable ω -diaminoalkane (Scheme 1). 8-Bromo-AMP (II) and 8-(6-aminohexyl)-AMP were synthesized and characterized as described by Lowe (1977a). 8-(8-Amino-octyl)-AMP (III, Scheme 1) was synthesized in an analogous fashion, except that an equivalent solution of 1,8-diamino-octane was used in place of the 1,6-diaminohexane.

(c) 8-(8-Amino-octyl)-IMP (VI, Scheme 1). 8-(8-Amino-octyl)-AMP (III) (100 mg, 0.2 mmol) was dissolved in ice-cold water (5 ml) and the pH adjusted to 9.5 with 5 M-NaOH. *S*-Ethyltrifluorothiol acetate (0.2 ml) was added in two equal portions and the pH continuously adjusted to 9.5 with 1 M-NaOH over a period of 1 h at 0°C. A further portion (0.4 ml) of *S*-ethyltrifluorothiol acetate was added and titration to pH 9.5 continued for a further 1 h until reaction was adjudged complete by t.l.c. (Table 1). The solution was evaporated to dryness several times from ethanol, and the solid, 8-(8-trifluoroacetamido-octyl)-AMP (IV), was dissolved in acetic acid (10 ml) at 0°C. Solid NaNO₂ (0.5 g) was added in small portions over a period of 1 h and allowed to react for a further 30 min. The u.v.-absorption maximum ($\lambda_{\text{max}}^{\text{H}^+}$) decreased from 278 nm to 259 nm throughout this period. The solution was diluted to 500 ml with water, the pH adjusted to 8.0 with 4 M-NaOH, and applied to a column (14 mm $\times$ 95 mm) of Dowex 1 (X2; formate cycle; 200–400 mesh) equilibrated with water. The column was washed with water (250 ml) and developed with a linear gradient of formic acid (0–4 M; 300 ml total volume). Fractions (1.5 ml) were collected at a flow rate of 30 ml/h. A minor peak, representing 5–10% of the applied u.v.-absorbing material and presumably unchanged starting material, was eluted immediately after application of the gradient. Fractions comprising the major peak (90–95%) of the u.v.-absorbing species ($\lambda_{\text{max}}^{\text{H}^+}$, 259 nm) eluted at approx. 2 M-formic acid were pooled and freeze-dried. The resulting white solid 8-(8-trifluoroacetamido-octyl)-IMP (V) was homogeneous on t.l.c. and was ninhydrin-negative. The product was dissolved in water (5 ml) and the pH adjusted to 12.0 with 2 M-NaOH. The solution was left for 3–4 h at room temperature, diluted to 250 ml with water, the pH adjusted to 11.5, and applied to a

Scheme 1. Synthesis of the 8-substituted AMP and IMP analogues for affinity chromatography
Abbreviations: NaAc, sodium acetate; HAc, acetic acid.

column (14mm×95mm) of Dowex 1 (X2; formate cycle; 200–400 mesh). The column was washed with water (100ml), and nucleotide was eluted with a linear gradient of formic acid (0–0.1M; 300ml total volume) at 30ml/h. Fractions (4.6ml) containing the major peak of u.v.-absorbing material were pooled and freeze-dried to yield approx. 30mg of pure product, 8-(8-amino-octyl)-IMP (VI).

(d) *N*⁶-(6-Aminohexyl)-carbamoylmethyl-NAD⁺. The *N*⁶-substituted NAD⁺ analogue was synthesized essentially as described by Larsson *et al.* (1976).

Analytical procedures

T.l.c. was performed on the media detailed in Table 1, and plates were developed in the solvent systems listed in the legend. Nucleotide derivatives were detected by viewing under u.v. light (254nm), and free amino groups by spraying with ninhydrin (Stepka, 1957). *N*-Trifluoroacetylated nucleotides were detected by spraying with 10mM-KOH in aq. 50% (v/v) acetone, drying the thin-layer plates in an oven at 120°C, and respraying with ninhydrin (Stepka, 1957).

Phosphate was determined by a modification of the Fiske and Subbarow technique (Bartlett, 1959), and amino group determinations were performed spectrophotometrically with 2,4,6-trinitrobenzene-1-sulphonic acid, with 6-aminohexanoic acid as standard (Satake *et al.*, 1960).

Spectral data were recorded on a Unicam SP.1800 u.v. spectrophotometer.

Preparation of agarose-bound affinity ligands

Agarose (Sephacrose 4B) was thoroughly washed with water and activated by the CNBr technique (Larsson *et al.*, 1976) by using 100mg of CNBr/g of moist gel for 10–12min at pH10.8–11.0 and 20°C. The activated gel was exhaustively washed with ice-cold 0.1M-Na₂CO₃/NaHCO₃ buffer, pH9.0, and allowed to react with the ligand (10μmol/g of moist gel) for 16h at 0–4°C. The gel was successively washed with 0.1M-NaHCO₃, 0.001M-HCl, 1M-NaCl and water. The concentrations of bound nucleotides were determined by direct u.v. measurements in ethylene glycol or by phosphate analysis (Bartlett, 1959) after oxidation of the agarose–ligand conjugate (Lowe, 1977a). The concentration of bound nucleotides was approx. 3.4μmol/g of moist gel.

Hexyl-agarose and 8-amino-octyl-agarose were obtained from Miles Laboratories (Stoke Poges, Bucks., U.K.).

Preliminary assessment of affinity adsorbents

Columns (7mm×150mm) containing agarose-bound nucleotide analogue (2g) were equilibrated with 50mM-potassium phosphate buffer, pH7.8. Cell-free extract (0.2ml; protein, 10mg/ml) of a *guaA* mutant (PL1068) was applied to the column,

Table 1. *T.l.c. and spectral properties of 8-substituted nucleotide analogues*
The solvent systems used were: A, ethanol/0.5 M-ammonium acetate (9:4, v/v); B, butan-1-ol/acetic acid/water (5:2:3, by vol.); C, propan-1-ol/0.25 M-NH₄HCO₃ (9:5, v/v); D, isobutyric acid/aq. 1 M-NH₃ (5:3, v/v); E, 0.05 M-LiCl; F, 0.10 M-LiCl; G, 0.25 M-LiCl; H, 0.50 M-LiCl. λ_{max} values were determined by using 0.1 M-HCl (pH 1), 0.1 M-potassium phosphate buffer (pH 7) and 0.1 M-NaOH (pH 13). The structures of the compounds are shown in Scheme 1.

Compounds	<i>R_F</i> values in chromatography systems										Detected by:		$\lambda_{\text{max.}}$ (nm) at pH:		
	Cellulose A	Silica			Poly (ethyleneimine) cellulose										
		B	C	D	E	F	G	H							
	U.v. light	Nin- hydrin	1.0	7.0	13.0										
AMP (I)	0.46	0.24	0.24	0.54	0	0.10	0.28	0.43	+	—	259.0	260.0	261.0		
8-Bromo-AMP (II)	0.59	—	—	—	—	—	—	—	+	—	262.5	—	267.0		
8-(8-Amino-octyl)-AMP (III)	0.51	0.30	0.15	0.55	0.8	—	—	—	+	+	277.5	281.0	282.0		
8-(8-Trifluoroacetamido-octyl)-AMP (IV)	0.76	0.68	0.58	0.59	0.8	—	—	—	+	—	275.5	—	—		
8-(8-Trifluoroacetamido-octyl)-IMP (V)	0.82	—	—	—	0.8	—	—	—	+	—	260.0*	—	—		
8-(8-Amino-octyl)-IMP (VI)	0.56	0.28	0.12	0.54	0.8	—	—	—	+	+	259.0*	271.0†	273.0		
IMP (VII)	0.62	0.21	0.21	0.33	0.12	0.16	0.38	0.51	—	—	250.0	251.0	254.0		

* Shoulder at 280 nm.
† Molar absorption coefficient 15 670 litre·mol⁻¹·cm⁻¹ at 271 nm.

allowed to run in and the flow stopped for 15 min. Non-adsorbed protein was washed through with 50 mM-potassium phosphate buffer, pH 7.8 (10 ml), after which elution was effected with an appropriate ligand (10 mM; 10 ml). Fractions (1.0 ml) were collected at a flow rate of 20 ml/h. IMP dehydrogenase activity in the fractions was assayed as described above and protein was determined by measuring *A*₂₈₀.

Large-scale purification of IMP dehydrogenase

An overnight culture (10 ml) of a *guaA* mutant (PL1068) in minimal medium (Vogel & Bonner, 1956) supplemented with guanine (20 µg/ml) was transferred to minimal medium (500 ml) supplemented with guanine (10 µg/ml). The culture was shaken for 8 h at 37°C, after which samples (200 ml) were transferred to each of two aspirators containing 20 litres of minimal medium supplemented with casein hydrolysate (1 g/litre) and guanine (5 mg/litre). The cultures were incubated at 37°C for 12 h under continuous aeration with filtered air at a flow rate of 15 litres/min. The cells were harvested in a flow-through Sharples centrifuge at 50 000*g*. The combined yield of cells from two 20-litre cultures (40 g of cell paste) was used to prepare IMP dehydrogenase. All stages in the following purification scheme were performed at 0–4°C. The bacteria were washed with 50 mM-potassium phosphate buffer, pH 7.8, and resuspended in 200 ml of the same phosphate buffer. The cells, in two separate 100 ml portions, were then ultrasonically disintegrated in bursts of 2 min, in a 100 W MSE ultrasonic disintegrator operating at 20 kHz for a total of 10 min. Cell debris was removed by centrifugation at 30 000*g* for 40 min, and then 50 ml of protamine sulphate (2%, w/v, solution in 50 mM-potassium phosphate buffer, pH 7.8) was added over a 10 min period, with stirring, to the combined cell-free extract. The precipitated nucleic acid was removed by centrifugation at 30 000*g* for 15 min. Solid (NH₄)₂SO₄ (25 g/100 ml) was added over a 10 min period with constant stirring; the mixture was then stirred for a further 15 min after which the precipitated protein was collected by centrifugation at 30 000*g* for 30 min. The pellet was redissolved in 10 ml of potassium phosphate buffer, pH 7.8, and dialysed overnight against 100 vol. of the same buffer.

The dialysed protein solution was applied to a column (15 mm × 120 mm) containing 8-(6-amino-hexyl)-AMP-agarose (30 g of moist gel; 120 µmol of AMP), allowed to equilibrate for 30 min, and then unbound protein was washed through with 50 mM-potassium phosphate buffer, pH 7.8, until no more protein was detected by *A*₂₈₀ in the washings. The enzyme was eluted with a linear gradient of AMP (0–40 mM; total volume 200 ml). Fractions (10 ml) were collected at a flow rate of 70 ml/h. The fractions

containing IMP dehydrogenase activity (or for *guaB* mutants, cross-reacting material) were pooled and concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation. The progress of the gradient was determined by following A_{295} .

Polyacrylamide-disc-gel electrophoresis

Non-denaturing gel electrophoresis was performed as described by Davis (1964) by using running-gel and sample-gel acrylamide concentrations of 5% (w/v) and 2% (w/v) respectively.

In an alternative procedure the stacking gel was omitted and the acrylamide concentration of the running gel was 5% (w/v). The reservoir and running-gel buffers were 50mM-Tris/HCl, pH 8.0. Polymerization of the gel was catalysed by ammonium persulphate. The stock solutions were mixed in the following proportions: gel solution buffer (0.5M-Tris/HCl, pH 8.0)/acrylamide solution (acrylamide, 0.3g/ml; *NN'*-methylenebisacrylamide 8mg/ml)/initiator solution (ammonium persulphate, 2.8mg/ml)/water (1.0:1.6:4.0:3.4, by vol.). Polymerization was initiated by the addition of 22 μl of *NNN'*-tetramethylethylenediamine (TEMED) to 10ml of gel solution.

SDS/polyacrylamide-gel electrophoresis was based on the method of Neville (1971). Samples were prepared for electrophoresis by the addition of an equal volume of a 2% (w/v) SDS solution containing 10% (v/v) 2-mercaptoethanol and 50mM- Na_2CO_3 , pH 8.5, to enzyme preparations (0.1–2.0mg/ml) followed by heating for 5 min at 100°C. The samples (10–100 μl) were applied through the upper reservoir to 11% and 7.5% gels and subsequently stained for protein with Coomassie Blue.

Detection of IMP dehydrogenase activity in polyacrylamide gels

Polyacrylamide-gel electrophoresis, under non-denaturing conditions, was performed at 4°C with three gels in parallel. One gel was stained for protein, and another was placed in 10ml of freshly prepared enzyme assay solution containing the following: IMP, 15 μmol ; NAD^+ , 12.5 μmol ; GSH, 50 μmol ; KCl, 500 μmol ; Tris/HCl, pH 7.0, 500 μmol ; Nitro Blue Tetrazolium, 2.5mg; phenazine methosulphate, 2.5mg. The gel was incubated in the dark for 1 h at 37°C, whence enzyme activity was indicated by the formation of a Blue Formazan in the gel. The third gel was incubated in the same enzyme assay solution minus IMP for 1 h in the dark at 37°C to act as a control. After staining, the gels were washed in water and stored in 7% (v/v) acetic acid.

Determination of radioactivity in polyacrylamide gels

Destained polyacrylamide gels were frozen in a Petri dish containing crushed solid CO_2 . The frozen gel was then placed on a bank of razor blades and

cut into 2mm slices. The slices were placed into scintillation vials containing NCS solution (0.6ml) and heated overnight at 55°C. After cooling, 15ml of toluene/butyl-PBD scintillator was added to each sample and radioactivity was counted in a Philips liquid-scintillation analyser.

Amino acid analysis

Amino acid analysis was performed on a JEOL JLC 6AH amino acid analyser by using a 0.3M-sodium citrate buffer system in the pH range 3.25–5.25. Samples (1mg) were hydrolysed in 1ml of N_2 -flushed 6M-HCl containing 0.01M-mercaptoacetic acid (anti-oxidant). The samples were sealed in ampoules under vacuum after successive flushing with N_2 , and heated for 16 or 48 h at 110°C.

N-Terminal amino acid analysis

The method is based on that described by Weiner *et al.* (1972). Purified IMP dehydrogenase (1mg, approx. 15nmol) was precipitated with 10% (w/v) trichloroacetic acid. The precipitate was then taken up in 0.4ml of 0.5M- Na_2CO_3 , adjusted to pH 9.8 with 2M-NaOH, containing 1% (w/v) SDS. Dansyl chloride (0.2ml, 5mg/ml) was added and the mixture was incubated in the dark at 37°C for 1 h. The dansylated protein was then precipitated by the cautious addition of ice-cold 20% (w/v) trichloroacetic acid. The solution was added dropwise with continuous vortex-mixing to avoid uncontrollable foaming caused by CO_2 production.

The precipitated protein was collected by centrifugation at 10000g, and hydrolysed with 6M-HCl as described above. The hydrolysate was dried down in a vacuum desiccator and the residue was dissolved in approx. 1–7 μl of aq. 50% (v/v) pyridine. The hydrolysate was continuously spotted on to a 75mm $\times$ 75mm polyamide sheet. A standard amino acid mixture (arginine, glutamic acid, glycine, isoleucine, methionine, phenylalanine, proline and serine) containing about 10nmol of each amino acid was spotted on the back of the plate opposite the test sample.

Ascending chromatography, in three solvents, was performed in succession. Solvents II [benzene/acetic acid (9:1, v/v)] and III [ethyl acetate/acetic acid/methanol (20:1:1, by vol.)] were run in the same direction, perpendicular to solvent I [1.5% (v/v) formic acid]. Amino acids on the plates were detected by fluorescence under u.v. light.

Tryptic 'fingerprinting'

IMP dehydrogenase purified by affinity chromatography was carboxymethylated as follows: freeze-dried enzyme (7mg) was incubated for 2 h at 37°C in 0.5M-Tris/HCl buffer, pH 8.0 (1ml), containing 6M-guanidine hydrochloride to denature the protein fully. Dithiothreitol was added to a final

concentration of 10 mM and the solution was left for a further 30 min at 37°C. Iodoacetic acid was added to a final concentration of 40 mM over a 30 min period and the solution was left for 1 h. The solution was then dialysed against distilled water for 2 days, after which time the precipitated protein was removed by centrifugation and resuspended in 50 mM-NH₄HCO₃ (1 ml). Trypsin, treated with diphenylcarbamoyl chloride, was added in a ratio of 100:1 (w/w) enzyme/trypsin, and the suspension was incubated for 6 h at 37°C. At the end of 6 h remaining solids (faint precipitate) were removed by centrifugation and the supernatant fraction was freeze-dried. The resulting white solid was dissolved in distilled water (50–100 µl) and spotted on Whatman 3MM chromatography paper (460 mm × 570 mm). Descending chromatography was performed for 15 h in the upper phase of butanol/water/acetic acid (4:1:5, by vol.). Electrophoresis was performed in the second dimension in pyridine/water/acetic acid (25:225:1, by vol.), pH 6.4, for 90 min in Gilson High-Voltage Electrophorator, model D. The paper was dried, dipped in a ninhydrin/collidine stain (600 ml of ethanol, 200 ml of acetic acid, 80 ml of collidine, 1 g of ninhydrin) and heated at 60°C for 30 min to develop the colour. The paper was inspected the next day when the colour had fully developed.

Molecular-weight determination of IMP dehydrogenase

IMP dehydrogenase was purified by affinity chromatography both from a *guaA* mutant of *E. coli* and from *guaB* mutants. Enzyme (0.5 ml; 1 mg/ml) in 50 mM-potassium phosphate buffer, pH 7.8, was applied to the top of a column (500 mm × 25 mm) of Sepharose 6B equilibrated with the same buffer. The column was eluted at a flow rate of 10 ml/h. Fractions (3 ml) were collected. The void volume was determined by using Blue Dextran 2000, and the protein standards (bovine heart cytochrome *c*, bovine serum albumin, pig heart lactate dehydrogenase, rabbit muscle pyruvate kinase and Jack-bean urease of approx. mol.wts. 12 000, 68 000, 144 000, 228 000 and 484 000 respectively) (1 mg of each) used were applied to the column individually in the same volume of buffer as the sample tested.

Hybrid IMP dehydrogenase from *guaB* mutants

Cell-free extracts were prepared as described by Lambden & Drabble (1973a). Before dialysis the extracts (2 ml) were denatured by adding 2-mercaptoethanol to a final concentration of 0.1 M followed by 1.14 g of solid guanidine hydrochloride over a 5 min period. The extracts were then left for 30 min at 4°C. The denatured extracts from two different *guaB* mutants were mixed and then dialysed twice over a 16 h period against 100 vol. of 0.02 M-sodium phos-

phate buffer, pH 7.4, containing 0.01 M-2-mercaptoethanol. After any precipitated protein had been spun down, the supernatant was assayed for IMP dehydrogenase activity.

Kinetic studies on IMP dehydrogenase

IMP dehydrogenase was assayed as described previously in a Cary 118 spectrophotometer. Kinetic data were analysed graphically by the method of Lineweaver & Burk (1934). The competitive inhibition constant (K_i) was calculated from the equation:

$$K_p = K_m \left(1 + \frac{[\text{inhibitor}]}{K_i} \right)$$

where K_p is the apparent value of K_m obtained in the presence of inhibitor.

Results

Purification of IMP dehydrogenase by immunoprecipitation

Native IMP dehydrogenase, purified by immunoprecipitation, when subjected to SDS/polyacrylamide-gel electrophoresis on 7.5 and 11% gels gave three protein bands corresponding to mol.wts. of 58 000, 50 000 and 25 000 (Fig. 1). The densitometer trace depicted in Fig. 1(a) demonstrates two sharp

Fig. 1. SDS/polyacrylamide-gel electrophoresis of an IMP dehydrogenase-anti-(IMP dehydrogenase) antibody complex

Electrophoresis was performed in a 7.5% gel, with 70 µg of the antigen-antibody complex prepared as described in the Materials and Methods section. The gel was stained with Coomassie Blue and scanned at 550 nm (a), and then sectioned into 2 mm slices for measurement of radioactivity (b).

slower moving bands and a fast moving fainter and more diffuse band. The corresponding radioactive profile (Fig. 1b) shows that almost all of the ^3H was located in the 58000-mol.wt. protein band. Fig. 1 therefore indicates that the high-molecular-weight (58000) polypeptide is *E. coli* IMP dehydrogenase, and that the subunits of the enzyme have the same molecular weight and are possibly identical. On some occasions a minor radioactive protein band, corresponding to a mol.wt. of 42000 and representing up to 4% of the total radioactivity was observed. This minor component is probably a degradation product, as its proportion increases to 4–8% when the serine proteinase inhibitor (phenylmethanesulphonyl fluoride) is omitted from the purification procedure. The two non-radioactive bands are the heavy (mol.wt. 50000) and light (mol.wt. 25000) chains of immunoglobulin G.

Three enzymically inactive mutant forms of IMP dehydrogenase (from HG1005, HG1018 and PL1138) were also purified by immunoprecipitation. The dissolved antigen-antibody precipitates were run individually, or mixed with antigen-antibody preparations of the native enzyme on 7.5% gels containing SDS. The electrophoretic mobility of the mutant proteins was identical with that of the native enzyme, and only three protein bands were observed when a mixture of each mutant and native protein was used. The observation that the mutant forms of IMP dehydrogenase have subunits of the same molecular weight as the native enzyme suggests that the three strains tested carry missense rather than nonsense mutations in *guaB*.

Structural assignment of the affinity ligands

The synthetic routes leading to the 8-substituted AMP and IMP analogues are depicted in Scheme 1. The method is relatively facile and, in general, proceeds with an overall yield of about 25% based on the parent nucleotide AMP. The method of synthesis (Scheme 1) and the properties of the intermediates (Table 1) all support the structures assigned. Thus the shift in absorption maximum ($\lambda_{\text{max}}^{\text{H}^+}$) from 262.5 nm for 8-bromo-AMP to 277.5 nm on reaction with diaminoalkanes is characteristic for 8-aminoalkyl-substituted adenine derivatives (Ikehara & Uesugi, 1969; Trayer *et al.*, 1974; Lowe, 1977a). Reaction with *S*-ethyltrifluorothiol acetate produced a ninhydrin-negative derivative (IV) without a concomitant shift in $\lambda_{\text{max}}^{\text{H}^+}$. However, deamination by treatment with HNO_2 followed by removal of the protecting trifluoroacetyl group produced an 8-substituted IMP analogue, 8-(8-amino-octyl)-IMP, which was ninhydrin-positive and displayed λ_{max} values at pH 1.0, 7.0 and 13.0 typical of 8-aminoalkyl-substituted inosine derivatives (Michal *et al.*, 1974). The molar absorption coefficient in 0.1 M-potassium phosphate buffer, pH 7.0, was

15670 litre·mol $^{-1}$ ·cm $^{-1}$ at 271 nm, lower than that for the corresponding AMP analogue (Michal *et al.*, 1974; Lowe, 1977a). Phosphate analysis (Bartlett, 1959), amino group analysis (Satake *et al.*, 1960) and u.v. absorption revealed approx. 1 mol of phosphate/mol of amino group per mol of purine base for 8-(8-amino-octyl)-IMP.

Cyanoethylation of IMP and 8-(8-amino-octyl)-IMP with acrylonitrile generated a shoulder at 280–290 nm in the u.v.-absorption spectrum in 0.1 M-potassium phosphate buffer, pH 7.5, which is characteristic of 1-cyanoethyl-IMP and its analogues (Ofengand, 1967). In contrast, under the same conditions no effect of acrylonitrile was observed on AMP, 8-bromo-AMP or 8-(8-amino-octyl)-AMP. However, the adenosine nucleotides reacted with aq. 40% (v/v) chloroacetaldehyde to generate highly fluorescent 1,*N*⁶-ethenoadenosine derivatives. In contrast, IMP and the product, 8-(8-amino-octyl)-IMP, produced no fluorescent product on incubation with chloroacetaldehyde (Barrio *et al.*, 1972).

*N*⁶-(6-Aminoheptyl)-AMP and *N*⁶-(6-aminoheptyl)-carbamoylmethyl-NAD⁺ were characterized as described by Guilford *et al.* (1972) and Larsson *et al.* (1976).

Chromatographic behaviour of IMP dehydrogenase

E. coli IMP dehydrogenase bound to agarose-bound *N*⁶-(6-aminoheptyl)-AMP, 8-(6-aminoheptyl)-AMP and 8-(8-amino-octyl)-IMP but not to columns of Cibacron Blue-agarose or immobilized NAD⁺. Elution of adsorbed enzyme from the above columns could be effected by 10 mM-AMP (Figs. 2a, 2b and 2d), but not by 10 mM-GMP or 10 mM-XMP. Furthermore, although IMP at a concentration 1000-fold higher than K_M^{IMP} (Krishnaiah, 1975), i.e. 10 mM, was ineffectual in eluting the enzyme from immobilized-AMP adsorbents, a slow release of the enzyme from the immobilized-8-(8-amino-octyl)-IMP adsorbent was possible (Fig. 2c).

As a consequence, the use of immobilized-AMP or -IMP affinity adsorbents resulted in very substantial purification of the enzyme from cell-free extracts. However, IMP dehydrogenase also bound to both control adsorbents 8-amino-octyl-agarose and hexyl-agarose, although the enzyme was not eluted by 10 mM-AMP. Elution from these adsorbents was possible, however, with 0.5 M-KCl (Figs. 2e and 2f). The enzyme from the 8-(6-aminoheptyl)-AMP adsorbent displayed significantly enhanced purity on SDS/polyacrylamide-gel electrophoresis compared with that from the 8-amino-octyl-agarose column. Clearly only columns comprising matrix-bound nucleotide can provide substantial purification of IMP dehydrogenase.

Large-scale purification of IMP dehydrogenase

A 40-litre culture of mutant PL1068 (*guaA* mutant)

Fig. 2. Elution of IMP dehydrogenase from nucleotide affinity columns

The columns were as follows: (a) 8-(6-aminohexyl)-AMP-agarose; (b) 8-(8-amino-octyl)-IMP-agarose; (c) 8-(8-amino-octyl)-IMP-agarose; (d) N^6 -(6-aminohexyl)-AMP-agarose; (e) 8-amino-octyl-agarose; (f) hexyl-agarose. Enzyme was eluted by AMP or IMP (10 mM, 10 ml) or by KCl (0.5 M) as indicated ($\downarrow$). The chromatographic procedures are given in the Materials and Methods section. $\bullet$, Enzyme activity; $\circ$, A_{280} .

yielded approx. 10 mg of pure IMP dehydrogenase after elution from the 8-(6-aminohexyl)-AMP-agarose affinity adsorbent with 25 mM-AMP (Fig. 3). The specific activity of the purified enzyme varied between batches but was typically 9–12 units/mg of protein (Table 2). Other published procedures resulted in purified enzymes with specific activities of 15 units/mg of protein (Powell *et al.*, 1969) and 19 units/mg of protein (Krishnaiah, 1975). In the latter

Fig. 3. Elution of IMP dehydrogenase from an 8-(6-aminohexyl)-AMP-agarose column

The preparation of the affinity column and the elution of IMP dehydrogenase from it are described in the Materials and Methods section. AMP in the eluate was detected by measuring the A_{259} . $\bullet$, Enzyme activity; $\circ$, A_{280} .

case, however, the enzyme was assayed at 37°C and not 23°C as used in the assay system described here.

Purified IMP dehydrogenase was very unstable, with 50% activity loss after 3 days storage at -15°C in 50 mM-potassium phosphate buffer, pH 7.8. Consequently enzyme was routinely used within 2 days of purification.

Gel electrophoresis of purified IMP dehydrogenase

Polyacrylamide-gel electrophoresis (Davis, 1964) demonstrated approximately seven ill-defined protein bands staining for IMP dehydrogenase activity. This result confirms observations by other workers (Powell *et al.*, 1969; Powell, 1973; Krishnaiah, 1975). The various protein bands appear to be different aggregated forms of the enzyme rather than isoenzymes, as the same pattern is observed on analytical ultracentrifugation and sucrose-density sedimentation (Powell *et al.*, 1969). The alternative method of polyacrylamide-gel electrophoresis (see the Materials and Methods section) revealed only one protein band, which also stained for IMP dehydrogenase activity. Possible explanations why the enzyme does not split into several protein fractions when the second method is used are as follows. Firstly, in the buffer system used only one aggregated form of the enzyme predominates. This is supported by the findings of Powell (1973) that IMP dehydrogenase from *E. coli* is eluted from gel-

Table 2. *Large-scale purification of IMP dehydrogenase from E. coli*
Full details of the purification scheme are given in the Materials and Methods section.

Purification stage	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
1. Cell-free extract	230	2070	400	0.19	100	1.0
2. Protamine sulphate precipitation	260	1530	347	0.23	87	1.2
3. 0–40% satd. $(\text{NH}_4)_2\text{SO}_4$	22	110	233	2.12	58	10.9
4. 8-(6-Aminohexyl)-AMP-agarose	4	10	92	9.20	23	47.5

Fig. 4. *SDS/polyacrylamide-gel electrophoresis of purified IMP dehydrogenase on an 11% gel*

IMP dehydrogenase was purified by affinity chromatography on an 8-(6-aminohexyl)-AMP-agarose column, as described in the Materials and Methods section; 50 μg of protein was applied to the gel. The gel was scanned at 550nm after staining with Coomassie Blue.

filtration columns by 50mM-Tris/HCl, pH8.0, as a sharp peak, indicating the presence of a single aggregated form of the enzyme. However, at higher or lower ionic strengths the enzyme is eluted as a broad peak, suggesting the presence of a number of different aggregated forms. Another explanation for the single protein band is that as the protein has not moved very far into the gel the different aggregated forms of the enzyme may not have run far enough along the gel to effect separations. In both methods of polyacrylamide-gel electrophoresis all the protein bands stained for IMP dehydrogenase activity, suggesting that the purified enzyme is virtually homogeneous.

SDS/polyacrylamide-gel electrophoresis (Fig. 4) of IMP dehydrogenase purified by affinity chromatography revealed one major protein band of mol.wt. 58000, a value in good agreement with that obtained on purification of the enzyme by immunoprecipitation. On some occasions a minor protein band of mol.wt. 42000 was observed. This protein was never more than 4% of the total protein.

To ensure that no proteins were present in the enzyme preparation that did not stain with Coomassie Blue, radioactive IMP dehydrogenase was purified by

Fig. 5. *Effect of trypsin on the structure and activity of IMP dehydrogenase*

Affinity-purified IMP dehydrogenase (1mg) was treated with trypsin in a ratio of 500:1 (w/w, IMP dehydrogenase/trypsin) in 50mM-Tris/HCl buffer (pH8.0) at 0°C for 1 h. At various time intervals two samples were removed. One, containing 40 μg of protein, was denatured in SDS and applied to SDS/polyacrylamide gels, and the other sample (1.5 μl) was assayed for enzyme activity (●). The proportion of each peptide in the total protein was determined from 550nm scans of the destained gels; ○, 58000-mol.wt. polypeptide; △, 42000-mol.wt. polypeptide; □, 14000-mol.wt. polypeptide.

affinity chromatography [PL1068 was grown in 500ml of minimal medium supplemented with guanine (4 $\mu\text{g}/\text{ml}$) and L-[4,5- ^3H]leucine (2 $\mu\text{g}/\text{ml}$; final specific radioactivity 500 $\mu\text{Ci}/\text{mg}$] and subjected to SDS/polyacrylamide-gel electrophoresis. Radioactivity was detected only in the 58000-mol.wt. protein band.

Partial proteolysis of IMP dehydrogenase

IMP dehydrogenase was susceptible to cleavage with Pronase and trypsin. SDS/polyacrylamide-gel electrophoresis revealed that there was a progressive cleavage of the IMP dehydrogenase poly-

Table 3. Kinetic constants of IMP dehydrogenase

The assay was as described in the Materials and Methods section unless otherwise stated. Enzyme activity was determined by the formation of NADH in purified preparations or by XMP formation at 290nm in cell-free extracts. Kinetic constants are quoted as means $\pm$ S.E.M., with the numbers of determinations in parentheses.

Enzyme preparation	Assay conditions	Kinetic constants (μ M)		
		K_m^{IMP}	$K_m^{NAD^+}$	K_i^{GMP}
Purified	33 mM-KCl; pH 8.0	11.1 ± 0.6 (6)	330 ± 10 (4)	56 ± 3 (3)
Purified	33 mM-KCl; pH 7.5	11.5 ± 0.4 (3)	334 ± 21 (3)	55 ± 4 (3)
Purified	500 mM-KCl; pH 7.5	12.2 ± 0.9 (4)	315 ± 37 (2)	52 ± 2 (3)
Cell-free extract*	33 mM-KCl; pH 7.5	10.1 ± 0.5 (3)	287 ± 25 (4)	61 ± 3 (3)
Cell-free extract†	33 mM-KCl; pH 7.5	12.7 ± 1.1 (3)	362 ± 1.1 (3)	53 ± 7 (3)

* Prepared by sonication (Lambden & Drabble, 1973a).
† Prepared by lysozyme treatment of bacteria (Kaback, 1971).

Fig. 6. Inhibition of IMP dehydrogenase by GMP at different concentrations of IMP

The reaction mixture contained in a total volume of 1 ml: IMP, 0.01–0.20 mM; NAD⁺, 1.25 mM; Tris/HCl (pH 7.5), 50 mM; KCl, 33 mM; GSH, 5 mM; purified IMP dehydrogenase, 0.2 μ g. The velocity (*v*) is quoted in μ mol of NADH/min. $\circ$, Enzyme assayed in the absence of GMP; Δ , enzyme assayed in the presence of 0.1 mM-GMP; $\square$, enzyme assayed in the presence of 0.2 mM-GMP.

peptide chain by trypsin into two smaller polypeptides of mol.wts. 42000 and approx. 14000. Quantitative conversion to the smaller polypeptides was achieved in 1 h (Fig. 5) and was accompanied by a parallel decrease in enzyme activity. These observations provide an explanation for the presence of peptides of mol.wt. 42000 in some preparations of IMP dehydrogenase.

Amino acid composition of IMP dehydrogenase

There appear to be no major differences between the amino acid composition of the enzyme purified by affinity chromatography described in this report with that previously published (Krishnaiah, 1975). The

analysis revealed a total of 55 lysine and arginine residues per subunit of mol.wt. 58000.

Tryptic 'fingerprint' of IMP dehydrogenase

Complete tryptic digestion of four different preparations of carboxymethylated IMP dehydrogenase released 48–50 peptides. An inactive mutant IMP dehydrogenase from PL1138 (*guaB105*), when purified by the same affinity method as was used for the native enzyme and detected by immuno-gel diffusion against monospecific antibody to the native enzyme, displayed a similar tryptic 'map' to that of the native enzyme.

N-Terminal amino acid analysis

Methionine was the only N-terminal amino acid observed.

Molecular weight of native, mutant and hybrid forms of IMP dehydrogenase

A Sepharose 6B column was calibrated with a number of standard proteins as described in the Materials and Methods section. Native (PL1068), denatured and renatured native, mutant [PL1138 (*guaB105*), PL1105 (*guaB85*) and HG1005 (*guaB1005*)] and hybrid (HG1005 with PL1138) forms of IMP dehydrogenase were all eluted in a symmetrical peak in the same volume as pyruvate kinase (mol.wt. 228000), suggesting a tetramer of mol.wt. 232000 containing four subunits of mol.wt. 58000 under the conditions used.

Kinetics

K_m^{IMP} , $K_m^{NAD^+}$ and K_i^{GMP} were determined under several conditions (Table 3). Linear double-reciprocal plots of $1/v$ against either $1/[IMP]$ or $1/[NAD^+]$ both in the presence and absence of GMP were obtained under the conditions used (Fig. 6). These observations indicate that GMP is a competitive inhibitor with respect to IMP.

Discussion

The objective of this study was to develop a rapid and facile purification scheme for IMP dehydrogenase in order to study the subunit structure of the enzyme and the effect of GMP on the enzyme. The final specific activity of the homogeneous protein (9–12 units/mg) was comparable with other published values (Powell *et al.*, 1969; Krishnaiah, 1975), and the overall recovery of enzyme activity was approx. 20%. The main advantages of using affinity chromatography on immobilized nucleotides are: the purification is rapid, taking only 36 h after the cells have been harvested; the affinity column can be used repeatedly, provided that it is washed with 2M-KCl between uses; the enzyme is not subjected to harsh conditions, such as heat-treatment (Powell *et al.*, 1969; Powell, 1973) or denaturing agents (Krishnaiah, 1975); finally the technique can also be applied to the purification of some inactive mutant forms of the enzyme, such as those derived from *E. coli* strains HG1005 and PL1138. It is doubtful, however, whether the interaction between the enzyme and immobilized nucleotides represents 'true' biospecific affinity adsorption (Lowe & Dean, 1974; Lowe & Mosbach, 1975; Lowe, 1977a,b), since it has been shown that the ability of 8-substituted AMP affinity columns to bind a number of dehydrogenases depends on the hydrophobicity of the spacer arm (Lowe, 1977a). It is possible that hydrophobic interactions of this type represent a component part of the binding of IMP dehydrogenase to the affinity columns described here. Nevertheless, IMP dehydrogenase prepared by affinity chromatography was adjudged homogeneous by polyacrylamide-gel electrophoresis.

SDS/polyacrylamide-gel electrophoresis of IMP dehydrogenase purified by affinity chromatography revealed one major protein band of mol.wt. 58000. Likewise, IMP dehydrogenase purified to homogeneity by immunoprecipitation displayed only one subunit of the same molecular weight. These results suggest that the subunits of IMP dehydrogenase have identical molecular weights, an observation in agreement with Krishnaiah (1975), but not with Powell (1973). A minor protein band was sometimes present, but it was never more than 4% of the total protein. Several observations suggest that this minor protein and the smaller polypeptide chain (mol.wt. 44000) observed by Powell (1973) are breakdown products of the major 58000-mol.wt. protein. Thus the minor protein band was not removed by passing the purified enzyme through Sephadex G-200 and the proportion of the minor protein was reduced when the enzyme was purified, by immunoprecipitation, in the presence of a proteinase inhibitor.

Compelling evidence suggesting breakdown of the

native enzyme monomers comes from the action of proteinases on the purified enzyme. Trypsin, or the non-specific proteinase Pronase, cleaves the 58000-mol.wt. peptide exclusively into a 42000-mol.wt. peptide and a peptide of approx. 14000-mol.wt. (Fig. 5). This indicates the presence of a region of the protein particularly susceptible to proteinase action, a phenomenon that is common (Reichenbecker & Grass, 1978).

Three observations suggest that the subunits of IMP dehydrogenase not only have the same molecular weight but are in fact identical. Firstly, as all protein mol.wt. 58000 is converted into two smaller polypeptide chains, the subunits are likely to be identical, because non-identical subunits of the same molecular weight are unlikely to be cleaved into two polypeptides of exactly the same size by proteinases with different specificities. Secondly, the number of peptides released on complete tryptic digestion is similar to the predicted value of 56 for identical subunits containing 55 lysine and arginine residues. Thirdly, only methionine is detected by N-terminal analysis.

Previous work has been shown that IMP dehydrogenase from *E. coli* and other bacterial sources exists in a number of different aggregated forms, depending on the pH and ionic strength of the buffer used (Powell *et al.*, 1969; Brox & Hampton, 1970; Hanon & Levin, 1971; Powell, 1973). Evidence presented here and elsewhere (Powell, 1973) has shown that in 50mM-potassium phosphate buffer, pH7.8, the *E. coli* enzyme exists predominantly in the tetrameric form. Krishnaiah (1975), however, suggests that the enzyme is active in a dimeric form. It is difficult to reconcile this difference, as no details of the buffer system were given by Krishnaiah (1975). However, as 3M-urea was used to elute the enzyme from an ill-defined immobilized-GMP affinity column, it is possible that the enzyme only partially renatured on dilution into 20mM-phosphate buffer.

There have been conflicting reports on the inhibition of IMP dehydrogenase by GMP. Lambden & Drabble (1973b) have shown co-operativity between IMP-binding sites in the presence of GMP, suggesting that the enzyme is inhibited by GMP at an allosteric site. However, other workers (Powell *et al.*, 1969; Krishnaiah, 1975) have shown GMP to be a competitive inhibitor with respect to IMP. Wu & Scrimgeour (1973a) have shown that IMP dehydrogenase from *B. subtilis* is inhibited by GMP at an allosteric site, but it can be easily desensitized to GMP inhibition by heat-treatment, denaturation, sonication or assaying the enzyme at pH 8.0 or above. IMP dehydrogenase from *S. typhimurium* has also been reported to possess 'allosteric' kinetics in the presence of GMP, although the degree of co-operativity depends upon the ionic strength of the assay mixture (Hanon & Levin, 1971). The purification

procedure of Powell *et al.* (1969) involved heat-treatment and sonication, and Krishnaiah (1975) likewise used sonication and then denaturing conditions to elute the enzyme from the affinity column. It is possible therefore that these preparations had been desensitized to GMP. As no cooperativity was observed between the IMP sites of the enzyme in the presence or absence of GMP under the conditions used in the present report (Fig. 6), it appears that GMP inhibits IMP dehydrogenase competitively with respect to IMP. It is difficult to explain the conflicting observations by Lambden & Drabble (1973b), although the enzyme used by these workers was only partially purified. Lambden & Drabble (1973b) monitored enzyme activity by the increase in A_{340} due to NADH formation, but NADH oxidase contaminants in the preparation would reoxidize NADH. At low concentrations of IMP in the presence of GMP, the enzyme displays a submaximum velocity, and under these conditions the rate of reoxidation of the reduced coenzyme may be sufficiently large to underestimate the real rate and thus generate an upward-curving double-reciprocal plot.

The scheme for the purification of the native enzyme by affinity chromatography may be extended to the purification of mutant forms of IMP dehydrogenase present in *E. coli* strains carrying *guaB* mutations. More detailed investigation of the structure of these mutant proteins, their affinity for the natural substrate IMP and how this relates to the position of the amino acid substitution in the primary sequence of the mutant enzyme may lead to a more detailed knowledge of the relationship between structure and function of IMP dehydrogenase (Gilbert & Drabble, 1978). Also, molecular-weight determinations of mutant protein subunits after rapid purification by immunoprecipitation followed by SDS/polyacrylamide-gel electrophoresis will enable evaluation of whether a missense or nonsense mutation has occurred in the bacteria that gave rise to the mutant proteins. Finally immunoprecipitation can be used to determine the quantity of IMP dehydrogenase produced by the bacteria under different growth conditions and so distinguish *de novo*.

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