Active-site modification of native and mutant forms of inosine 5'-monophosphate dehydrogenase from *Escherichia coli* K12

Harry J. GILBERT* and William T. DRABBLE†

Department of Biochemistry, University of Southampton, Southampton SO9 3TU, U.K.

(Received 8 April 1980/Accepted 25 July 1980)

IMP dehydrogenase of *Escherichia coli* was irreversibly inactivated by Cl-IMP (6-chloro-9- β -D-ribofuranosylpurine 5'-phosphate, 6-chloropurine ribotide). The inactivation reaction showed saturation kinetics. 6-Chloropurine riboside did not inactivate the enzyme. Inactivation by Cl-IMP was retarded by ligands that bind at the IMP-binding site. Their effectiveness was $\text{IMP} > \text{XMP} > \text{GMP} \gg \text{AMP}$. NAD^+ did not protect the enzyme from modification. Inactivation of IMP dehydrogenase was accompanied by a change in λ_{max} of Cl-IMP from 263 to 290 nm, indicating formation of a 6-alkylmercaptapurine nucleotide. The spectrum of 6-chloropurine riboside was not changed by IMP dehydrogenase. With excess Cl-IMP the increase in A_{290} with time was first-order. Thus it appears that Cl-IMP reacts with only one species of thiol at the IMP-binding site of the enzyme: 2–3 mol of Cl-IMP were bound per mol of IMP dehydrogenase tetramer. Of ten mutant enzymes from *guaB* strains, six reacted with Cl-IMP at a rate similar to that for the native enzyme. The interaction was retarded by IMP. None of the mutant enzymes reacted with 6-chloropurine riboside. 5,5'-Dithiobis-(2-nitrobenzoic acid), iodoacetate, iodoacetamide and methyl methanethiosulphonate also inactivated IMP dehydrogenase. Reduced glutathione re-activated the methanethiolated enzyme, and 2-mercaptoethanol re-activated the enzyme modified by Cl-IMP. IMP did not affect the rate of re-activation of methanethiolated enzyme. Protective modification indicates that Cl-IMP, methyl methanethiosulphonate and iodoacetamide react with the same thiol groups in the enzyme. This is also suggested by the low incorporation of iodo[^{14}C]acetamide into Cl-IMP-modified enzyme. Hydrolysis of enzyme inactivated by iodo[^{14}C]acetamide revealed radioactivity only in S-carboxymethylcysteine. The use of Cl-IMP as a probe for the IMP-binding site of enzymes from *guaB* mutants is discussed, together with the possible function of the essential thiol groups.

IMP dehydrogenase (IMP– NAD^+ oxidoreductase, EC 1.2.1.14) of *Escherichia coli* consists of identical subunits, with the active form of the enzyme being predominantly tetrameric (Gilbert *et al.*, 1979). Mutant (*guaB*) strains of *E. coli*, lacking functional IMP dehydrogenase, have been isolated; several of these produce material (CRM) cross-reacting to anti-(IMP dehydrogenase) antiserum. Complementation *in vitro* between CRM⁺ *guaB*

strains reveals the presence of active enzyme in mixed cell-free extracts of certain pairs of mutants (Gilbert & Drabble, 1980). Cl-IMP has been shown to inactivate the IMP dehydrogenase from *Aerobacter aerogenes*, possibly by interacting with a thiol group at the IMP-binding site of the enzyme (Hampton & Nomura, 1967; Brox & Hampton, 1968). In the present paper we describe the use of Cl-IMP to investigate the integrity of the IMP-binding site of enzymes isolated from several complementing *guaB* strains of *E. coli*.

Other agents known to modify thiols are found to inactivate IMP dehydrogenase. Mutual protection against inactivation by these agents indicates that Cl-IMP, methyl methanethiosulphonate and iodoacetamide modify the same thiol group at the IMP-binding site. IMP does not retard re-activation

Abbreviations used: Cl-IMP, 6-chloro-9- β -D-ribofuranosylpurine 5'-phosphate (6-chloropurine ribotide); GSH, reduced glutathione.

* Present address: Centre for Applied Microbiological Research, Porton Down, Salisbury SP4 0JG, U.K.

† To whom reprint requests should be addressed.

of methanethiolated enzyme, suggesting an important function for this thiol group in the binding of IMP.

Materials and methods

Bacterial strains

The guanine auxotrophs used were PL1072 (*guaB52*), PL1096 (*guaB76*), PL1105 (*guaB85*), PL1138 (*guaB105*), MW1087 (*guaB792*), HG1001 (*guaB1001*), HG1005 (*guaB1005*), HG1017 (*guaB1017*), HG1018 (*guaB1018*), HG1019 (*guaB1019*). Native IMP dehydrogenase was isolated from the *guaA* mutant PL1068 (*guaA48*). All these mutants were derived from *E. coli* K12 strain W3110 (Gilbert & Drabble, 1980).

Chemicals

NAD⁺ was obtained from Boehringer Corp. (Lewes, Sussex, U.K.) and Cl-IMP from Calbiochem (Bishop's Stortford, Herts., U.K.). GSH, 6-chloropurine riboside (chloroinosine), guanine hydrochloride and IMP were from Sigma (London) Chemical Co. 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.) and Hyamine hydroxide (1M solution in methanol) was from Nuclear Enterprises (Reading, Berks., U.K.) Iodo-[1-¹⁴C]acetamide (sp. radioactivity 40mCi/mmol) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.) Methyl methanethiosulphonate was prepared by Dr. D. P. Bloxham of this Department. All other chemicals were supplied by BDH, Poole, Dorset, U.K.

Purification of IMP dehydrogenase

The enzyme was purified to homogeneity from *guaA* and *guaB* mutants by affinity chromatography as described by Gilbert *et al.* (1979).

Assay for IMP dehydrogenase

The enzyme-assay system used was as described by Gilbert *et al.* (1979) unless otherwise stated.

Protein assay

The protein content of preparations was routinely determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin (fraction V) as standard. The protein content of two samples of purified IMP dehydrogenase was also estimated by acid hydrolysis followed by amino acid analysis (Gilbert *et al.*, 1979); 627 µg of enzyme protein estimated by this method corresponded to 1 mg of enzyme protein estimated by the Lowry *et al.* (1951) method with the bovine serum albumin standard.

Spectrophotometry

Kinetic measurements and absorption spectra were recorded with a Cary 118 spectrophotometer (Varian Instruments) unless otherwise stated.

Radioactivity measurements

Protein samples, dissolved in 1M-Hyamine hydroxide, were added to 15 ml of butyl-PBD scintillation solution (8 g of butyl-PBD in 1 litre of toluene) together with 0.25 ml of acetic acid (to neutralize the Hyamine hydroxide). Radioactivity was measured in a Philips liquid-scintillation analyser (wide ¹⁴C channel).

Electrophoresis

Protein hydrolysate (0.1 ml) was spotted on to Whatman 3MM chromatography paper (460 mm × 570 mm). ϵ -Carboxymethyl-lysine, *S*-carboxymethylcysteine, *N*¹-carboxymethylhistidine and *N*³-carboxymethylhistidine were also applied (carboxymethylated standards were used because acid hydrolysis of the modified enzyme converts amide derivatives into free acids). The amino acids were separated by electrophoresis in pyridine/acetic acid/water (1:10:289, by vol.) at pH 3.6 and 3000 V for 1 h by using a Gilson high-voltage Electrophorator model D. The paper was dried, cut into 15 mm squares, and counted for radioactivity in scintillation solution [8 g of butyl-PBD in 1 litre of toluene/methanol (3:1, v/v)]. The carboxymethyl-amino acid standards were detected by spraying with ninhydrin reagent.

Results

Inactivation of IMP dehydrogenase by Cl-IMP

Cl-IMP inactivated IMP dehydrogenase with apparent first-order kinetics (Fig. 1). The inactivation was not dependent on the presence of GSH and was not reversed by extensive dialysis nor by increasing the concentration of substrates in the assay system. Unlike the enzyme from *A. aerogenes* (Hampton & Nomura, 1967), no enzyme activity was recovered by including GSH in the assay of enzyme inactivated in the absence of GSH (however, the activity of unmodified enzyme was increased by GSH by 40–70%; Table 3). These results suggest covalent modification of the enzyme by Cl-IMP, even enzyme that is not fully activated (omission of GSH). A linear relationship was given by plotting $1/k_{app}$ (reciprocal of observed first-order rate constants for inactivation; Fig. 1) versus $1/[Cl-IMP]$, indicating a 'rate saturation effect' (Baker *et al.*, 1962) and confirming that inactivation by Cl-IMP is preceded by reversible binding to the enzyme.

Fig. 1. Inactivation of IMP dehydrogenase at different concentrations of Cl-IMP

IMP dehydrogenase (30 $\mu\text{g}/\text{ml}$) was incubated at 23°C in 20 mM-potassium phosphate buffer, pH 7.0, containing 25 mM-KCl and 5 mM-GSH with Cl-IMP at the following concentrations: 11 μM (○), 18 μM (△), 24 μM (□), 48 μM (●), 60 μM (▲). Samples (50 μl) were removed at the times indicated and assayed for enzyme activity.

Fig. 2. Inactivation of IMP dehydrogenases by Cl-IMP in the presence of nucleotides

IMP dehydrogenase (100 $\mu\text{g}/\text{ml}$) was treated at 23°C with Cl-IMP (65 μM) in 20 mM-potassium phosphate buffer, pH 7.4, containing 25 mM-KCl and 5 mM-GSH. Samples (20 μl) were removed at the times indicated and assayed for enzyme activity. Nucleotides (200 μM) present during the incubation were IMP (○), GMP (△), XMP (□), AMP (●), none (▲).

Although IMP binds tightly to IMP dehydrogenase, the corresponding nucleoside, inosine, does not (Nichol *et al.*, 1967). 6-Chloropurine riboside (200 μM) did not inactivate the enzyme (0.1 mg/ml) within 2 h, whereas Cl-IMP (at one-tenth the concentration) caused total inactivation after 15 min under the same conditions. This supports the conclusion that initial binding to the active site precedes the covalent inactivation of the enzyme by Cl-IMP.

Protection by nucleotides against inactivation by Cl-IMP

A further indication of chemical modification at the active site of an enzyme is the protection afforded by ligands which bind to or affect the active site. The inactivation of IMP dehydrogenase by Cl-IMP was retarded by nucleotides (Fig. 2). IMP provided the best protection, followed by GMP and XMP, with AMP only slightly retarding the rate of inactivation. This order of effectiveness is good evidence that Cl-IMP is acting at the IMP-binding site, as the protection provided by the nucleotides correlates with their respective K_m and K_i values (Powell *et al.*, 1969; Gilbert *et al.*, 1979). NAD⁺ did not protect the enzyme; this indicates that Cl-IMP is not interacting with the NAD⁺-binding site.

Changes in the absorption spectrum of Cl-IMP

As Cl-IMP reacted with IMP dehydrogenase, there was a progressive development of a new

absorption peak at 290 nm (Cl-IMP has λ_{max} at 263 nm), accompanied by loss of enzyme activity. These changes in the absorption spectrum of Cl-IMP were similar to those described by Brox & Hampton (1968) for the enzyme from *A. aerogenes*. No spectral changes were observed in the absence of enzyme, and a 25-fold excess of 6-chloropurine riboside produced neither a change in absorbance nor loss of enzyme activity over the same time period. IMP retarded the appearance of the 290 nm peak, half-maximum absorbance at 290 nm occurring after 5 and 120 min respectively in the absence and presence of IMP (Table 1). However, the same relationship between A_{290} and enzyme activity was demonstrated in both the absence and presence of IMP. Measurement of increases in A_{290} can therefore be used to follow enzyme inactivation.

Enzyme inactivation was pH-dependent (Fig. 3) in a simple manner that suggests the involvement of an ionizable group with a $\text{p}K_a$ of 8.4. As Cl-IMP contains no group with a $\text{p}K_a$ of this value, the group must be part of the enzyme, being possibly a thiol (these have $\text{p}K_a$ values around 8–9). In addition, the

Table 1. Covalent modification of native and mutant forms of IMP dehydrogenase

IMP dehydrogenase, purified from *gua* mutants (Gilbert *et al.*, 1979), was concentrated by (NH₄)₂SO₄ precipitation and redissolved in 20 mM-potassium phosphate buffer, pH 7.4, containing 25 mM-KCl and 5 mM-GSH. The enzyme solution was then dialysed for 18 h at 4°C against 100 vol. of the same buffer solution. The enzyme concentration was adjusted to 1 mg/ml. Cl-IMP (32 nmol) was then added to 1 ml of enzyme solution with and without IMP (200 nmol) and the *A*₂₉₀ recorded continuously at 10°C. —, No reaction. The fine-structure map of the *guaB* gene (shown below) is modified from Gilbert & Drabble (1980). + or — shown above the allele numbers indicates that the IMP dehydrogenase isolated from the mutant strain does or does not react with Cl-IMP.

Diagram illustrating the structure of the *guaB* gene region, showing the locations of various alleles (1005, 76, 1001, 52, 1018, 1017, 792, 85, 105) relative to the *guaO*, *guaB*, and *guaA* genes. The alleles are indicated by lines pointing to specific positions on the gene map. The *guaB* gene is flanked by *guaO* and *guaA* genes.

Source of enzyme	Time (min) to reach half-maximum A_{290}	
	IMP absent	IMP (0.2 mM) present
PL1068 <i>guaA48*</i>	5.0	120
HG1005 <i>guaB1005</i>	4.0	102
HG1019 <i>guaB1019</i>	6.5	91
MW1087 <i>guaB792</i>	7.0	101
PL1072 <i>guaB52</i>	4.0	52
PL1105 <i>guaB85</i>	7.5	92
PL1138 <i>guaB105</i>	7.5	133
HG1001 <i>guaB1001</i>	—	—
HG1007 <i>guaB1017</i>	—	—
HG1018 <i>guaB1018</i>	—	—
PL1096 <i>guaB76</i>	—	—

* Native enzyme.

appearance of a new λ_{max} at 290 nm suggests the formation of a 6-alkylmercaptapurine nucleotide derivative. A molar absorption coefficient of 16 800 at 290 nm and pH 7.4 has been given for 6-alkylmercaptapurine nucleotides (Brox & Hampton, 1968). From this value, the absorbance at 290 nm associated with 97% inactivation of the enzyme corresponds to the formation of about 2 mol of 6-alkylmercaptapurine nucleotide/mol of tetrameric enzyme. This ratio was established with three enzyme preparations (Table 4).

Enzyme was incubated with a 50-fold excess of

Fig. 3. Rate of inactivation of IMP dehydrogenase by Cl-IMP at different pH values

IMP dehydrogenase (100 μ g/ml) in 50 mM-Tris/HCl buffer, pH 7.1–9.5, containing 25 mM-KCl was incubated at 23°C with Cl-IMP (30 μ M). Samples (20 μ l) were removed at intervals and assayed for enzyme activity in the absence of GSH. *k*_{app} at each pH was calculated from the slopes of the lines ($\times 2.303/60$) obtained by plotting the first-order kinetic equations (as in Fig. 1).

Fig. 4. Changes in *A*₂₉₀ when excess Cl-IMP is incubated with IMP dehydrogenase

IMP dehydrogenase (1 mg/ml, 17 μ M) was incubated at 10°C with excess Cl-IMP (850 μ M) in 20 mM-potassium phosphate buffer, pH 7.4, containing 25 mM-KCl and 5 mM-GSH.

Cl-IMP and the reaction (A_{290}) was followed at 10°C with a Unicam SP.1800 spectrophotometer. The linear relationship obtained (Fig. 4) denotes

first-order kinetics and therefore the modification of only one species of thiol group.

Covalent modification by Cl-IMP of IMP dehydrogenase from *guaB* mutants

IMP dehydrogenase from ten complementing CRM⁺ *guaB* mutants (Gilbert & Drabble, 1980) was treated with Cl-IMP. The enzyme purified from six of the mutants and the native enzyme (PL1068) react with Cl-IMP at a similar rate. The times taken to reach 50% of maximum absorbance at 290nm are presented in Table 1. Each reaction resulted in a shift of λ_{max} from 263 to 290nm and gave the same maximum absorbance at 290nm as the native enzyme treated identically. These reactions were considerably retarded by IMP (Table 1). No reaction occurred between 6-chloropurine riboside and any enzyme. IMP dehydrogenase from the remaining four *guaB* mutants did not react with Cl-IMP. Those mutant enzymes that react with Cl-IMP may have an unimpaired IMP-binding site, as modification is preceded by an initial reversible binding between reagent and enzyme. The retardation of the reaction by IMP (competes with Cl-IMP at the active site), together with the absence of any reaction with 6-chloropurine riboside (sug-

Table 2. Inactivation of IMP dehydrogenase by chemical modification

IMP dehydrogenase (100 µg/ml) was incubated at 0°C with the agents listed in 20mm-potassium phosphate buffer, pH 7.4, containing 25mm-KCl. At timed intervals, samples (3 µl) were removed and assayed (in the absence of GSH) for enzyme activity.

Modifying agent	Time (min) for 50% decrease in enzyme activity	
	IMP absent	IMP (0.2 mM) present
Methyl methanethio-sulphonate (1.7 µM)	—*	—*
5,5'-Dithiobis-(2-nitro-benzoate) (10 µM)	0.5	7
Iodoacetate (1 mM)	4.0	20
Iodoacetamide (100 µM)	3.0	15
Cl-IMP (20 µM)	1.5	25

* Too rapid to determine.

Table 3. Protective modification of IMP dehydrogenase

IMP dehydrogenase (100 µg), previously inactivated in the absence of a thiol reagent by either Cl-IMP or methyl methanethiosulphonate (MTS), was treated at 0°C in a final volume of 1 ml with 0.1 mM-iodoacetamide (Cl-IMP- and MMTS-treated enzymes) or 0.1 mM-Cl-IMP (MTS-treated enzyme) in 20mm-potassium phosphate buffer, pH 7.4, containing 25mm-KCl. Unmodified enzyme was used as a control. When the control enzyme was fully inactivated, IMP dehydrogenase that had been pre-treated with Cl-IMP or MMTS was assayed for enzyme activity. The enzyme assay was as described by Gilbert *et al.* (1979), except that the thiol reagent (5 mM) was omitted unless stated otherwise. Enzyme activity is expressed relative to that of unmodified enzyme assayed in the presence of 5 mM-GSH (line 2).

	Pretreatment of enzyme	Alkylating agent added to pre-treated enzyme	Additions to assay system	Enzyme activity (%)
1	—	—	—	70
2	—	—	GSH	100
3	—	—	2-Mercaptoethanol	100
4	—	—	2-Mercaptoethanol + GSH	100
5	Cl-IMP	—	—	0
6	Cl-IMP	—	2-Mercaptoethanol	100
7	Cl-IMP	Iodoacetamide	—	0
8	Cl-IMP	Iodoacetamide	2-Mercaptoethanol	86
9	—	Iodoacetamide	—	0
10	—	Iodoacetamide	2-Mercaptoethanol	0
11	MTS	—	—	0
12	MTS	—	GSH	100
13	MTS	Cl-IMP	—	0
14	MTS	Cl-IMP	GSH	88
15	—	Cl-IMP	—	0
16	—	Cl-IMP	GSH	0
17	MTS	Iodoacetamide	—	0
18	MTS	Iodoacetamide	GSH	94
19	—	Iodoacetamide	—	0
20	—	Iodoacetamide	GSH	0

gests an initial specific binding), are evidence for an intact IMP-binding site in these mutant enzyme. Those mutations producing Cl-IMP-insensitive enzymes are not closely grouped on the gene map (Table 1), therefore it is not possible to locate any one region of the *guaB* gene that codes for the IMP-binding site of the enzyme.

Inactivation of IMP dehydrogenase by other modifying agents

The inactivation of IMP dehydrogenase by protein-modifying agents that are not substrate analogues was investigated. The times taken for a 50% decrease in enzyme activity are given in Table 2. Reactivity towards the enzyme is predictable from the relative reactivities of the agents towards thiols. The exception is Cl-IMP; this does not react with thiols such as 2-mercaptoethanol and GSH at pH 7.4 (Brox & Hampton, 1968). An initial specific binding of Cl-IMP to the enzyme may be responsible for the

rapid reaction with the enzyme at pH 7.4. IMP protects the enzyme from inactivation by all the agents except methyl methanethiosulphonate. However, that compound reacts so rapidly (Table 2) that an accurate assessment of protection by IMP was not possible. Nevertheless methyl methanethiosulphonate and Cl-IMP probably modify the same thiol groups, because both protect the enzyme against iodoacetamide (see the next section).

Protective modification of IMP dehydrogenase

IMP dehydrogenase, after complete inactivation by Cl-IMP, was assayed in either the presence or absence of 5 mM-2-mercaptoethanol (Tables 3, lines 5 and 6). The modified enzyme was fully re-activated by 2-mercaptoethanol after approx. 5 min. Addition of 5 mM-2-mercaptoethanol did not affect the activity of native enzyme assayed in the presence of 5 mM-GSH (Table 3, lines 2 and 4). Similarly, enzyme that had been fully inactivated by methyl methanethiosulphonate (methanethiolated) could be re-activated, with full recovery of activity, by 5 mM-GSH (Table 3, lines 11 and 12).

The protective effect of one agent against inactivation of IMP dehydrogenase by a second agent was investigated. Treatment of Cl-IMP-modified enzyme with iodoacetamide, under conditions that inactivate native enzyme, did not prevent re-activation by 2-mercaptoethanol (Table 3, lines 7–10). Methanethiolated enzyme, after further treatment with Cl-IMP under conditions that completely inactivate native enzyme, was re-activated by GSH (Table 3, lines 13–16). Finally methanethiolated enzyme was active after treatment with iodoacetamide provided that the assay mixture contained GSH (Table 3, lines 17–20). Therefore Cl-IMP protects the enzyme from alkylation by iodoacetamide, and methyl methanethiosulphonate protects the enzyme from alkylation by iodoacetamide and Cl-IMP. This indicates that all three agents modify the same thiol groups of IMP dehydrogenase.

The rate of re-activation by GSH of methanethiolated IMP dehydrogenase was determined in the presence and absence of IMP (Fig. 5). The enzyme was fully re-activated after 40 min, with no observable retardation by IMP. These data suggest modification of an active-site thiol group by methyl methanethiosulphonate such that binding of IMP is precluded. The thiol group modified by methyl methanethiosulphonate may therefore be important in the binding of IMP to the active site of the enzyme.

Incorporation of iodoacetamide into native and Cl-IMP-modified IMP dehydrogenase

IMP dehydrogenase was treated with iodo[1-¹⁴C]-acetamide (Table 4). The alkylation was accom-

Fig. 5. Re-activation of methanethiolated IMP dehydrogenase by GSH in the presence and absence of IMP. IMP dehydrogenase (1 mg, 0.5 mg/ml) in 50 mM-potassium phosphate buffer, pH 7.4, was inactivated at 0°C by successive additions of methyl methanethiosulphonate. The enzyme was then diluted 20-fold into the same buffer solution containing 1 mM-GSH. Samples (2 µg of protein) removed at timed intervals were assayed in the absence of GSH for enzyme activity. For the control, unmodified enzyme was treated in the same way; this enzyme had constant activity over the course of the experiment. IMP (200 µM) was either present in (O) or absent from (●) the dilution buffer.

Table 4. Incorporation of iodo[1-¹⁴C]acetamide into native and Cl-IMP-modified IMP dehydrogenase
Purified IMP dehydrogenase (4mg/ml, 68μM) in 20mM-potassium phosphate buffer, pH 7.4, containing 25mM-KCl was fully inactivated at 23°C by Cl-IMP. The modified enzyme (4mg), and native enzyme used as a control, were treated with 0.5μmol of iodo[1-¹⁴C]acetamide (sp. radioactivity 75μCi/mmol). Samples (0.2ml) were placed at intervals into 1ml of ice-cold 10% (w/v) trichloroacetic acid. As a control, 0.2ml of iodo[1-¹⁴C]acetamide (0.1μmol) in the same buffer was placed in 1ml of 10% trichloroacetic acid. After 30min, cell-free extract (Lambden & Drabble, 1973) of strain PL1068 (10mg of protein) was added to each acidified sample to act as a carrier. After a further 20min. the precipitated protein was washed five times with 5% trichloroacetic acid, twice with acetone, and then dissolved in 0.5ml of 1M-Hyamine hydroxide by heating at 60°C for 1h. Radioactivity was measured as described in the Materials and methods section. Samples (2μg of protein) were also removed from the reaction and assayed for enzyme activity. Results from three separate experiments are shown. Protein concentration was determined by the method of Lowry *et al.* (1951), and molarity of tetrameric enzyme was calculated by using 232000 for the mol.wt. (Gilbert *et al.*, 1979). Values shown for mol of modifying agent incorporated per mol of tetrameric enzyme are increased by a factor of 1.6 when the protein concentration estimated from amino acid analysis is substituted (see the Materials and methods section).

Enzyme preparation	Incorporation (mol/mol of tetrameric enzyme) required for complete inactivation	
	Cl-IMP	Iodoacetamide
Native	2.05, 1.92, 2.13	2.13, 2.30, 2.20
Modified by Cl-IMP	—	0.25, 0.16, 0.27

panied by a progressive decrease in enzyme activity. For each of three preparations, the amount of iodoacetamide bound per tetramer that caused total inactivation of the enzyme was similar to the amount of bound Cl-IMP required for total inactivation (Table 4). IMP dehydrogenase, previously modified with Cl-IMP, incorporated only about 10% of the iodoacetamide incorporated into native enzyme. These results give further support to the proposal that iodoacetamide and Cl-IMP modify the same groups on the enzyme.

The amino acid modified by iodoacetamide was identified as described in the Materials and methods section. After hydrolysis of the enzyme, most of the radioactivity coincided with *S*-carboxymethylcysteine (Fig. 6). A small peak of radioactivity at the

Fig. 6. Electrophoresis of acid-hydrolysed IMP dehydrogenase after treatment with iodo[1-¹⁴C]acetamide
Purified IMP dehydrogenase (2mg) that had been fully inactivated at 23°C by iodo[1-¹⁴C]acetamide (sp. radioactivity 75μCi/mmol) was hydrolysed under nitrogen with 6M-HCl at 110°C for 24 h. The hydrolysate was dissolved in 0.1 ml of water before electrophoresis as described in the Materials and methods section. Carboxymethylated amino acids used as markers were ϵ -carboxymethyl-lysine (CM-Lys), *S*-carboxymethylcysteine (CM-Cys), *N*¹-carboxymethylhistidine (*N*¹-CM-His) and *N*³-carboxymethylhistidine (*N*³-CM-His).

origin probably corresponded to precipitated protein. No radioactivity moved to the cathode, therefore cysteine is the only amino acid modified by iodoacetamide.

Discussion

The inactivation of IMP dehydrogenase of *E. coli* by Cl-IMP is consistent with covalent modification at the active site of the enzyme. Thus the kinetics of the reaction display the rate-saturation effect of a two-stage inactivation (Baker *et al.*, 1962), and purine nucleotides protect against modification to an extent predictable from their affinities for the enzyme. NAD⁺ does not protect, however, even at the high concentration of 2mM; this implies a specific action of Cl-IMP at the IMP-binding site. NAD⁺ binds to the K⁺-enzyme complex in a non-compulsory order with respect to IMP (Heyde & Morrison, 1976), so presumably NAD⁺ binds under the experimental conditions described in the present paper. Additional evidence for initial specific and reversible binding of Cl-IMP to the enzyme is the inertness of 6-chloropurine riboside. It should be

noted that inosine does not bind to IMP dehydrogenase (Nichol *et al.*, 1967).

Apparently 2 mol of Cl-IMP are required to inactivate 1 mol of IMP dehydrogenase tetramer, an indication of half-site reactivity (Levitzki & Koshland, 1976). However, this calculation is based on a determination of protein concentration by the method of Lowry *et al.* (1951), with a bovine serum albumin standard, and is therefore not wholly reliable. In addition, only half the enzyme units were recovered from the affinity column during purification (Gilbert *et al.*, 1979), so contaminating inactive enzyme may be present in the preparation. Inactivation of 1 mol of tetrameric enzyme by approx. 3 mol of Cl-IMP is indicated by using an estimate of enzyme concentration based on the total quantity of amino acids released by complete hydrolysis of the protein. The linear increase in log A_{290} with time also argues against half-site reactivity; a non-linear relationship would be expected for groups with different reactivities (unless two subunits of the tetramer are modified very rapidly to inactivate the enzyme before the other two subunits have reacted). Sites with different degrees of catalytic activity and reactivity towards Cl-IMP have been proposed for IMP dehydrogenase of *A. aerogenes* (Brox & Hampton, 1968), an enzyme containing non-identical subunits, albeit of the same molecular weight. The enzyme of *E. coli* has identical subunits (Gilbert *et al.*, 1979; Gilbert & Drabble, 1980). No enzyme activity was detectable after denaturing and renaturing enzyme modified by Cl-IMP (Gilbert & Drabble, 1980), but some activity would be expected from the combination of four unmodified subunits from half-reacted enzyme. However, the preferred conformation of tetrameric enzyme could be two modified plus two native subunits. In summary, the data do not permit unambiguous assignment of stoichiometry to the reaction, but the results are consistent with a specific modification, be it full or half-site, at the active site of the enzyme.

Cl-IMP provides a useful probe for the IMP-binding site of IMP dehydrogenase from *guaB* mutants because the interaction can be easily followed spectrophotometrically. A mutant enzyme was considered to have an intact IMP-binding site when it (a) reacted with Cl-IMP at a rate comparable with that for native enzyme, (b) was protected from modification by IMP, and (c) was not modified by 6-chloropurine riboside. On this basis, six of the ten CRM⁺ *guaB* strains with mutations of known map position produce enzymes with intact IMP-binding sites. The positions of the four mutations that abolish IMP binding are widely spaced on the gene map, therefore several regions of the protein, well separated in the primary sequence, are involved in binding IMP. It should be possible to

extend this experimental approach to the NAD⁺-binding site by using affinity-labelling analogues of that substrate.

The shift in the $\lambda_{\max}$ of Cl-IMP from 263 to 290 nm on addition of IMP dehydrogenase indicates formation of a 6-alkylmercaptapurine nucleotide derivative (Brox & Hampton, 1968) by reaction of C-6 of the purine with a thiol group at the IMP-binding site. As 6-alkylaminopurines and 6-alkoxypurines have absorption maxima at 248 nm and 266–267 nm respectively (Johnson *et al.*, 1958), an amino or hydroxy group is not modified by Cl-IMP. However, the spectra of modifying agents bound to protein can be quite different from spectra in free solution, because of the influence of the local environment around the protein-bound ligand. Nevertheless, the following considerations suggest modification of a thiol by Cl-IMP. Variations in the rate of modification by Cl-IMP at different pH values suggest the presence of a reactive group in IMP dehydrogenase with a pK_a of 8.4, i.e. within the range expected for thiols. In addition to Cl-IMP, agents that are known to modify thiol groups in enzymes also inactivate IMP dehydrogenase, and labelling with iodo[¹⁴C]acetamide gives rise only to radioactive carboxymethylcysteine on hydrolysis of the enzyme. The mutual protection against inactivation provided by these agents indicates the modification of the same thiol groups. This conclusion is supported by the observations that similar molar amounts of iodoacetamide and Cl-IMP are required to abolish enzyme activity and that pre-treatment of the enzyme with Cl-IMP decreases iodoacetamide binding by 10-fold.

The recovery of activity when methanethiolated enzyme is treated with GSH is unaffected by IMP. Therefore even a small modification of the active-site thiol group results in an enzyme unable to bind IMP. GSH, a bulky charged molecule, would be unable to re-activate a thiol group protected by a bound nucleotide. It should be noted that IMP protects the enzyme against inactivation by iodoacetate and iodoacetamide, both smaller molecules than GSH, and Cl-IMP, although removed from the enzyme by 2-mercaptoethanol, is not by GSH. Several dehydrogenases have a cysteine residue at the active site. As modification of this amino acid inactivates these enzymes, an essential function in catalysis has been proposed for this residue. However, methanethiolated lactate dehydrogenase retains full catalytic activity, but with a large increase in the K_m for pyruvate (Bloxham & Wilton, 1977; Bloxham *et al.*, 1979). The thiol is not involved, therefore, in any essential covalent step in the catalytic mechanism, but in the binding of substrate, possibly by stabilizing an ionic interaction. The reactive thiol groups of IMP dehydrogenase may have a similar function in binding an ionic species of IMP (Hampton, 1963).

H. J. G. thanks the Scientific Research Council for a research training studentship.

References

- Baker, B. R., Lee, W. W. & Tong, E. (1962) *J. Theor. Biol.* **3**, 459–483
- Bloxham, D. P. & Wilton, D. C. (1977) *Biochem. J.* **161**, 643–651
- Bloxham, D. P., Sharma, R. P. & Wilton, D. C. (1979) *Biochem. J.* **177**, 769–780
- Brox, L. W. & Hampton, A. (1968) *Biochemistry* **7**, 2589–2596
- Gilbert, H. J. & Drabble, W. T. (1980) *J. Gen. Microbiol.* **117**, 33–45
- Gilbert, H. J., Lowe, C. R. & Drabble, W. T. (1979) *Biochem. J.* **183**, 481–494
- Hampton, A. (1963) *J. Biol. Chem.* **238**, 3068–3074
- Hampton, A. & Nomura, A. (1967) *Biochemistry* **6**, 679–689
- Heyde, H. & Morrison, J. F. (1976) *Biochim. Biophys. Acta* **429**, 661–671
- Johnson, J. A., Thomas, H. J. & Schaeffer, H. J. (1958) *J. Am. Chem. Soc.* **80**, 699–702
- Lambden, P. R. & Drabble, W. T. (1973) *J. Bacteriol.* **115**, 992–1002
- Levitzki, A. & Koshland, D. E. (1976) *Curr. Top. Cell. Regul.* **10**, 1–40
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Nichol, A. W., Nomura, A. & Hampton, A. (1967) *Biochemistry* **6**, 1008–1015
- Powell, G. F., Rajagopalan, K. V. & Handler, P. (1969) *J. Biol. Chem.* **244**, 4793–4797