Inactivation of Inosine 5′-Monophosphate Dehydrogenase by the Antiviral Agent 5-Ethynyl-1-β-D-Ribofuranosylimidazole-4-Carboxamide 5′-Monophosphate†

Wen Wang,‡ Vladimir V. Papov,§ Noriaki Minakawa,|| Akira Matsuda,|| Klaus Biemann,*,§ and Lizbeth Hedstrom*,‡

Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254, Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-Ku, Sapporo 060, Japan

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ABSTRACT: Inosine 5′-monophosphate dehydrogenase (IMPDH) is the rate-limiting enzyme in *de novo* guanine nucleotide biosynthesis. IMPDH converts inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP) with concomitant conversion of NAD^+ to NADH. The antiviral agent 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (EICAR) is believed to inhibit IMPDH by forming an active metabolite, the 5′-monophosphate EICARMP. The experiments reported here demonstrate that EICARMP irreversibly inactivates both human type II and *Escherichia coli* IMPDH. IMPDH is protected from EICARMP inactivation by IMP, but not by NAD^+. Further, denaturation/renaturation of the EICARMP-inactivated enzyme did not restore enzyme activity, which indicates that EICARMP forms a covalent adduct with IMPDH. EICARMP was successfully used to titrate the active sites of IMPDH; these experiments demonstrate that four active sites are present in an IMPDH tetramer. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry of native *E. coli* IMPDH established that protein translation initiates at the third ATG of the DNA sequence. Thus, the *E. coli* IMPDH monomer is only 488 amino acids long and contains five instead of six cysteines. In addition, MALDI-TOF mass spectrometry showed that EICARMP is covalently bound to Cys-305 (Cys-331 in human type II IMPDH numbering), suggesting that Cys-305 functions as a nucleophile in the IMPDH reaction. The inactivation of the *E. coli* enzyme is a single-step reaction with $k_{on} = 1.94 \times 10^4\ M^{-1}\ s^{-1}$. In contrast, the inactivation of human type II IMPDH involves a two-step mechanism where $K_i = 16\ \mu M$, $k_2 = 2.7 \times 10^{-2}\ s^{-1}$ and $k_{on} = 1.7 \times 10^3\ M^{-1}\ s^{-1}$. These results demonstrate that significant differences exist between bacterial and human IMPDH and suggest that this enzyme may be a target for antibiotic drugs.

Inosine 5′-monophosphate dehydrogenase (IMPDH)[1] catalyzes the oxidation of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP) with the concomitant reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH. This reaction is the rate-limiting step in guanine nucleotide biosynthesis (Weber, 1983). IMPDH is highly expressed in tumor cells and rapidly growing tissues (Jackson et al., 1975; Nagai et al., 1991), and inhibitors of IMPDH have anticancer, antiviral, and immunosuppressive activity (Jayaram et al., 1986; Nelson et al., 1990; Robins, 1982; Malinoski & Stollar, 1981; Mizuno et al., 1974). One possible mechanism for the IMPDH reaction is shown in

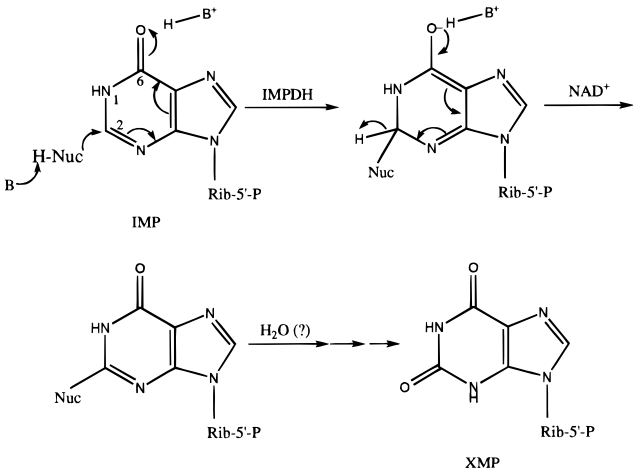

FIGURE 1: Mechanism of the IMPDH reaction.

Figure 1. Base activation of a nucleophile, either a water molecule or an enzyme residue, results in nucleophillic attack at position 2 of IMP. The resultant oxyanion is stabilized by an acidic residue from the enzyme. Collapse of the oxyanion expels a hydride, which is transferred to NAD^+. If the nucleophile in the first step is water, this reaction sequence produces XMP directly. If the nucleophile is an enzyme residue, an additional hydrolysis step is required to produce XMP.

Several observations indicate that a cysteine is located in the IMP binding site of IMPDH. IMPDH is inactivated by

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* To whom correspondence should be addressed.
‡ Brandeis University.
§ Massachusetts Institute of Technology.
|| Hokkaido University.

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[1] Abbreviations: IMPDH, inosine 5′-monophosphate dehydrogenase; IMP, inosine 5′-monophosphate; XMP, xanthosine 5′-monophosphate; EICARMP, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide 5′-monophosphate; EICAR, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide; 6-Cl-IMP, 6-chloropurine riboside 5′-monophosphate; NAD^+, nicotinamide adenine dinucleotide; BSA, bovine serum albumin; TFA, trifluoroacetic acid; DTT, dithiothreitol; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; MS, mass spectrometry; *m*/*z*, mass-to-charge ratio.

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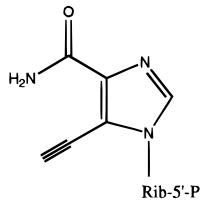

EICARMP

FIGURE 2: Structure of EICARMP.

sulfhydryl reagents such as iodoacetamide or methyl methanethiosulfonate. The enzyme is protected from this inactivation by IMP (Gilbert & Drabble, 1980; Antonino et al., 1994). In addition, 6-chloropurine riboside 5′-monophosphate (6-Cl-IMP) inactivates IMPDH by forming a covalent adduct with Cys-331 (human type II IMPDH numbering; Collart & Huberman, 1988). IMP also protects IMPDH from inactivation by 6-Cl-IMP (Antonino et al., 1994; Hampton & Nomura, 1967). These results indicate that Cys-331 is located near the IMP binding site, where it could act as the nucleophile, general acid, or general base. Recently, Wu and colleagues (1995) have reported that IMP forms a covalent adduct with Cys-331 during the IMPDH reaction, which suggests that Cys-331 is the catalytic nucleophile.

The enzyme residues located near position 2 of IMP are candidates for the nucleophile and/or general base required in the IMPDH reaction. 5-Ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide 5′-monophosphate (EICARMP, Figure 2) was designed to trap such residues (Minakawa et al., 1991). EICARMP is the active metabolite of nucleoside EICAR, a potent antiviral and antileukemic agent (De Clercq et al., 1991; Matsuda et al., 1988; Minakawa et al., 1991). Adenosine kinase converts the nucleoside to EICARMP, which inhibits IMPDH in murine leukemia L1210 and human lymphocyte CEM cells (Balzarini et al., 1993). In addition, previous work has shown that EICARMP is a time dependent inhibitor of IMPDH from L1210 cells (Balzarini et al., 1993). The results presented here demonstrate that EICARMP is a potent irreversible inactivator of human and *Escherichia coli* IMPDH and suggest that Cys-331 (human type II numbering) acts as a nucleophile in the active site. The nature of the enzyme−nucleotide adduct was identified by MALDI-TOF-MS (Karas & Hillenkamp, 1988) of both native and EICARMP-modified *E. coli* IMPDH. In addition, the amino acid sequence of native *E. coli* IMPDH was found to be shorter than that predicted from the DNA sequence (Teideman & Smith, 1985).

MATERIALS AND METHODS

Materials. IMP, NAD^+, Trizma base, and DTT were purchased from Sigma, glycerin and EDTA were from Fisher, 4-vinylpyridine and α-cyano-4-hydroxycinnamic acid were from Aldrich, a solution of 8 M guanidine hydrochloride was from Pierce, ultrapure-grade urea was from ICN, and endoproteinase-Lys-C (Endo-Lys-C) was from Boehringer Mannheim. The plasmid pJS49 containing the *E. coli guaB* gene was the generous gift of Dr. John M. Smith. Plasmid pHIMP containing human IMPDH type II cDNA was the generous gift of Dr. Frank Collart.

Expression of IMPDH. Human type II IMPDH was expressed in H712 cells (which lack *E. coli* IMPDH; Nijkamp & De Haan, 1967) containing the expression vector pHI. This vector was constructed by ligating the 1.6 kb fragment from a partial *Nco*I/*Hin*dIII digest of pHIMP into the *Nco*I/*Hin*dIII fragment of pKK223-2. *E. coli* IMPDH was expressed in H712 cells containing pJS49. The *guaB* gene was sequenced using a PRISM Dyedeoxy Terminator Cycle Sequencing kit (ABI) and an Applied BioSystems 373A DNA sequencer at the Brandeis Sequencing Facility.

Preparation of IMPDH. Both *E. coli* and human IMPDH were isolated as previously described in the purification of *Tritrichomonas foetus* IMPDH (Hedstrom & Wang, 1990). The cells were resuspended in buffer A (50 mM Tris, pH 7.5, 1 mM DTT, 10% glycerol) and disrupted by sonication. Cell debris was removed by centrifugation, and the crude extract was chromatographed on a Bio-Gel A-5m column pre-equilibrated with buffer A. The column was eluted with the same buffer. Fractions with enzymatic activity were pooled and applied to an IMP affinity column pre-equilibrated with buffer A (Ikegami et al., 1987). IMPDH was eluted with buffer A containing 0.5 mM IMP. The enzyme solution was dialyzed against buffer A to remove IMP immediately before each experiment.

EICARMP. EICAR was synthesized as previously reported (Matsuda et al., 1988). The nucleoside was phosphorylated with phosphorus oxychloride in trimethyl phosphate (Yoshikawa et al., 1967). The phosphorylation reaction mixture was desalted by charcoal treatment and further purified on a DEAE cellulose column. The eluate was chromatographed on a DIAION PK212 column (Mitsubishi Kasei Corp., Japan) followed by a DIAION WK20 column (Mitsubishi Kasei Corp., Japan), giving the disodium salt of EICARMP as a solid (49%): FAB-MS (negative) *m*/*z* 368 ($M^+ - Na$); UV λ_{max} (H_2O) 265 nm (ϵ 9400); ^{1}H-NMR (D_2O) 8.30 (s, 1 H), 5.98 (d, 1 H), 4.66 (dd, 1 H), 4.45 (m, 1 H), 4.31 (br s, 1 H), 3.99 (m, 2 H); ^{31}P-NMR (D_2O) 4.44. Anal. Calcd. for $C_{11}H_{12}N_3O_8PNa_2 \cdot 3H_2O$: C, 29.67; H, 4.08; N, 9.44. Found: C, 29.51; H, 3.54; N, 9.26.

Enzyme Assays. The standard assay solution contains 50 mM Tris, pH 8.0, 100 mM KCl, 1 mM DTT, 3 mM EDTA. The following substrate concentrations were used: (a) human IMPDH type II assay (0.125 mM IMP, 0.100 mM NAD) and (b) *E. coli* IMPDH assay (1.0 mM IMP, 2.5 mM NAD). The reaction was initiated by addition of enzyme, and the absorbance was recorded at 340 nm for 5 min at 25 °C. Protein concentration was determined with Bio-Rad protein assay using IgG as standard. A Hitachi U-2000 spectrophotometer was used for all spectrophotometric analysis.

Kinetics of IMPDH Inactivation by EICARMP. The appropriate amount of NAD, IMP, and EICARMP was added to the standard assay buffer, and the reaction was initiated by the addition of enzyme. The reaction was monitored by absorbance at 340 nm. Less than 10% of the substrate was consumed during data collection. The data were fit to eq 1

$$A - A_o = V_o[1 - \exp(-k_{obs}t)] \quad (1)$$

where k_{obs} is the observed rate constant, t is time, A is the absorbance at time t, A_o is the initial absorbance at time zero and V_o is the initial reaction rate. The k_{obs} values for inactivation of *E. coli* IMPDH were fit to eq 2

$$k_{obs} = k_{on}[I]/(1 + [S]/K_m) \quad (2)$$

where S is IMP, I is inhibitor, k_{on} is the apparent second-

order rate constant for the reaction of EICARMP and IMPDH, and K_m is the Michealis−Menten constant for IMP. The values of k_{obs} for inactivation of human IMPDH were fitted to eqs 3−5 to obtain K_i, the first-order rate constant k_2, and k_{on}.

$$k_{obs} = k_2[I]/(K_{i,app} + [I]) \quad (3)$$

$$K_i = K_{i,app}/(1 + [S]/K_m) \quad (4)$$

$$k_{on} = k_2/K_i \quad (5)$$

Inactivation, Denaturation, and Renaturation of E. coli IMPDH. *E. coli* IMPDH was incubated with 20 equiv of EICARMP for 1 h at room temperature. Urea was added to a final concentration of 8 M, and the solution was incubated for another hour at room temperature. The denatured enzyme was diluted 6-fold with a solution containing 50 mM Tris, 1 mM DTT, and 8 M urea and concentrated to its original volume with a Centricon 30. This dilution−concentration process was repeated four times, and the final concentrate was dialyzed overnight against a buffer containing 50 mM Tris and 1 mM DTT. A control sample of *E. coli* IMPDH was treated following the same procedure. Enzymatic activity for both control and treated enzyme was monitored at each step.

UV Difference Spectrum between the IMPDH−EICARMP Complex and EICARMP. The UV spectrum of *E. coli* IMPDH (7.7 μM in monomer concentration) in standard assay buffer solution under anaerobic conditions was measured. This spectrum was used as the base line. EICARMP (3.4 μM) was added to the enzyme solution, and the spectrum was recorded after 10 min. The spectrum of EICARMP in the same buffer was subtracted from this spectrum.

Reduction and Alkylation of E. coli IMPDH. An aqueous solution of *E. coli* IMPDH (approximately 30 pmol/μL) was mixed with an equal volume of denaturing buffer (8 M guanidine hydrochloride, 0.25 M Tris-HCl, pH 8.3, 0.002 M EDTA). The reducing agent, DTT (14 μg/μL), was dissolved in denaturing buffer and added to this solution (to give approximately a 2000-fold molar excess compared to IMPDH) and incubated at 37 °C. After 3 h, 4-vinylpyridine dissolved in *n*-propanol was added to result in a 5-fold molar excess over DTT, and the reaction mixture was kept at 37 °C overnight. For desalting it was injected onto a C_4 reverse-phase guard column and washed with 0.05% aqueous TFA. The alkylated protein was then eluted from the guard column with 0.035% TFA/acetonitrile, collected, and dried using a centrifugal evaporator.

Endo-Lys-C Digestion of E. coli IMPDH. Both EICARMP-modified and native IMPDH were first reduced with DTT and alkylated with 4-vinylpyridine (see above). About 15 μg of protein was dissolved in 2 M urea and 50 mM Tris-HCl (pH 9.0). Endo-Lys-C (0.2 μg) was then added, and the digest solution was kept at room temperature for 3 h. The digestion was stopped by the addition of glacial acetic acid.

MALDI Mass Spectrometry. Native IMPDH, EICARMP-modified IMPDH, and the Endo-Lys-C digests of IMPDH were analyzed by MALDI using a Voyager Elite TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA) operated in the linear mode. A saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in 30% acetonitrile was used as the matrix solution for MALDI analysis of the IMPDH protein. IMPDH was dissolved in this matrix solution to give a final protein concentration between 1 and 10 pmol/μL. About 1 μL of the analyte/matrix solution was applied to the target plate and was evaporated. The sample spots were irradiated using an N_2 laser (337 nm), which was operated at a repetition rate of about 4 Hz. The resulting ions were accelerated to 30 kV kinetic energy. The guide wire voltage was adjusted to 0.30% of the full accelerating potential. To increase mass accuracy, BSA was added as an internal standard to the IMPDH/matrix solution (final concentration between 1 and 10 pmol/μL). At least six separate mass spectra (sum of 128 laser shots) were obtained for each experiment to ensure good measurement precision ($\leq$0.1%). The *m*/*z* values for IMPDH reported in the Results and Discussion section are the averaged results.

For the mass spectra of Endo-Lys-C digests, α-cyano-4-hydroxycinnamic acid (10 mg/mL) in 50% acetonitrile was used as the matrix solution. Aliquots from the digests were taken directly and diluted with the matrix solution to give a final peptide concentration between 1 and 10 pmol/μL. The mass spectra of the Endo-Lys-C digests were initially calibrated by using bovine insulin as an external standard.

RESULTS AND DISCUSSION

Inactivation of IMPDH by EICARMP. Both human type II and *E. coli* IMPDH are inactivated by EICARMP in a time dependent manner as shown in Figure 3. Activity cannot be restored by dialysis of the inactivated enzyme. Urea denaturation/renaturation of the EICARMP-inactivated *E. coli* enzyme also did not restore the enzyme activity. A control containing active enzyme recovered 90% of its activity after urea treatment. These results suggest that a covalent bond is formed between EICARMP and IMPDH.

UV Difference Spectrum between the IMPDH−EICARMP Complex and EICARMP. The UV−Vis spectrum of EICAR displays an absorption maximum at 269 nm (ϵ = 9800 cm^{-1} M^{-1}). EICAR reacts with CH_3SH (Minakawa et al., 1991). The addition product, (*Z*)-5-[2-(methylthio)vinyl]-1-β-D-ribofuranosylimidazole-4-carboxamide, has an absorption maximum at 281 nm (ϵ = 4800 cm^{-1} M^{-1}). The UV difference spectrum between the IMPDH−EICARMP complex and EICARMP shows a maximum decrease in absorbance between 260 and 270 nm when EICARMP reacts with IMPDH (Figure 4). This observation is consistent with thio addition to the ethynyl group of EICARMP.

Stoichiometry of Inactivation. The enzyme was quantitated using a Bio-Rad protein assay with IgG as the standard. *E. coli* IMPDH was completely inactivated by EICARMP when the molar ratio of EICARMP/tetramer equalled 2.6, which suggests that two active sites exist per IMPDH tetramer. A similar stoichiometry has been reported for the inactivation of IMPDH by 6-Cl-IMP (Brox & Hampton, 1968; Gilbert & Drabble, 1980). Since the activity of the EICARMP-inactivated enzyme was not recovered after denaturation and renaturation, any unmodified subunits failed to recombine to regenerate active enzymes. As shown below, unmodified monomers cannot be detected in IMPDH inactivated with 2.6 equiv of EICARMP. This result suggests that four active sites are present per IMPDH tetramer and that the Bio-Rad assay overestimates the IMPDH concentra-

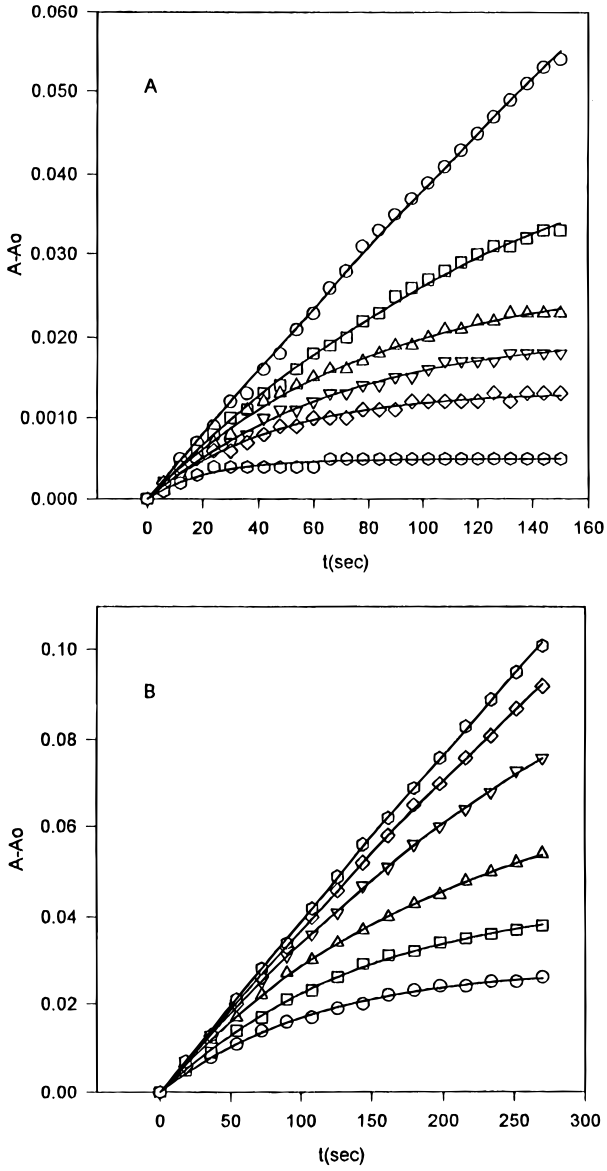

FIGURE 3: Progress curves of the inactivation of IMPDH by EICARMP. A: *E. coli* IMPDH, [IMP] = 1 mM, [NAD^+] = 2.5 mM, [EICARMP] = (○) 8.45; (□)16.9; (△) 25.4; (▽) 33.8; (◇) 42.3; (⬡) 50.7 mM. B: human IMPDH, [IMP] = 0.25 mM, [NAD^+] = 0.1 mM, [EICARMP] = (⬡) 6.67; (◇) 16.9; (▽) 33.8; (△) 67.6; (□) 101.4; (○) 169.0 mM.

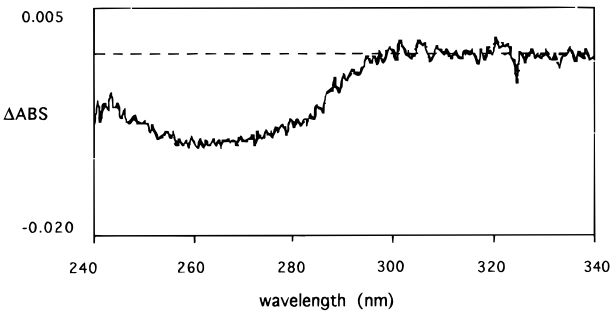

FIGURE 4: UV difference spectrum between the IMPDH−EICARMP complex and EICARMP.

tion. More importantly, EICARMP can be used to titrate the active sites.

Substrate Protection of the Inactivation of IMPDH by EICARMP. Table 1 shows that the rate of inactivation by EICARMP decreases as the concentration of IMP increases and that the inactivation rate does not change significantly when the concentration of NAD^+ changes. Thus IMP protects IMPDH against EICARMP inactivation while no protection was observed by NAD^+. Assuming that EICARMP and IMP compete for the same site, a 4.6-fold decrease in k_{obs} is expected over the range of IMP concentrations in Table 1, which is in reasonable agreement with the

Table 1: Substrate Protection of the Inactivation of *E. coli* IMPDH by EICARMP[a]

[IMP] mM	[NAD] mM	k_{obs} s^{-1}
0.3	2.5	0.030
0.5	2.5	0.019
1	2.5	0.013
1.5	2.5	0.0087
1	0.625	0.016
1	1.250	0.015
1	1.875	0.014
1	2.500	0.013
1	3.125	0.014

[a] k_{obs} was obtained from progress curves as described in Materials and Methods. The error in k_{obs} is less than 5% in all cases. Conditions as described in Materials and Methods, [EICARMP] = 25.6 μM.

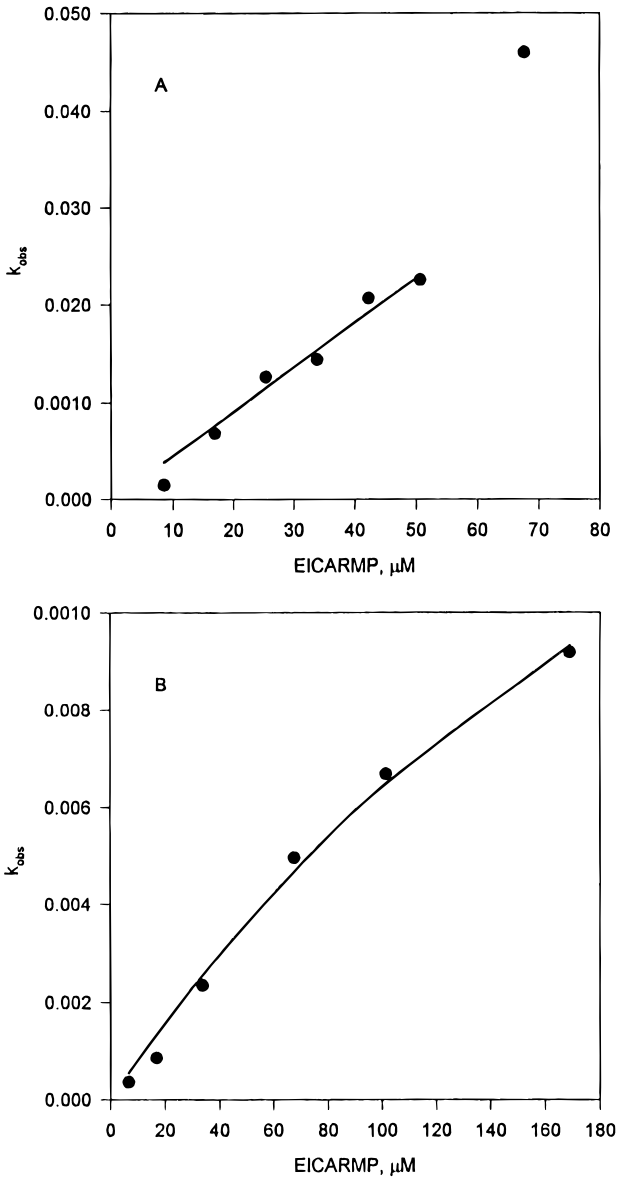

FIGURE 5: Replot of k_{obs} vs EICARMP concentration. k_{obs} is derived from the progress curves as described in Materials and Methods. A, *E. coli* IMPDH; B, human IMPDH.

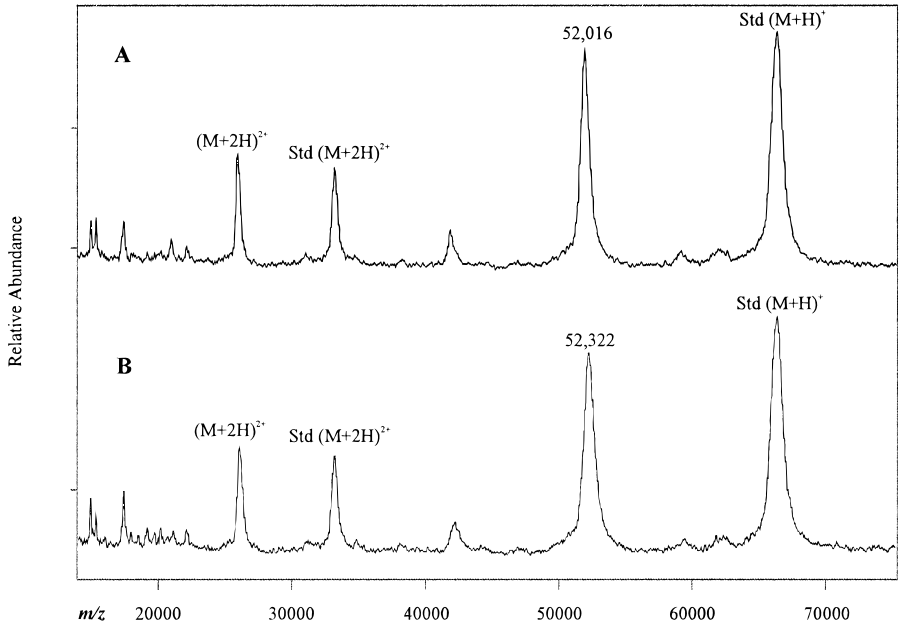

FIGURE 6: MALDI-TOF mass spectra containing singly and doubly charged ions of *E. coli* IMPDH. A, native; B, EICARMP-modified. The peaks labeled "Std $(M + H)^+$" at *m/z* 66 431.0 and "Std $(M + 2H)^{2+}$" at *m/z* 33 216.0 are due to the singly and doubly charged ions, respectively, of the internal calibrant bovine serum albumin (BSA).

Scheme 1

$$E + I \xrightarrow{k_{on}} E{\cdot}I^*$$

Scheme 2

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_2} E{\cdot}I^*$$

3.4-fold decrease that is observed. These results suggest that EICARMP reacts at the IMP binding site of IMPDH.

Kinetics of Enzyme Inactivation. The reactions of *E. coli* and human type II IMPDH with EICARMP are too fast to monitor in the absence of IMP. Therefore, progress curves were used to measure the kinetics of EICARMP inactivation. K_i and k_{on} were adjusted by a factor of $(1 + [IMP]/K_m)$ to account for the presence of IMP (see Materials and Methods), where $K_m = 24\ \mu M$ for *E. coli* and $K_m = 13\ \mu M$ for human type II IMPDH. These agree with the previously reported K_m values [18 μM for *E. coli* IMPDH (Streeter et al., 1973) and 9.3 μM for human type II (Carr et al., 1993)].

For *E. coli* IMPDH the dependence of k_{obs} on EICARMP concentration is linear up to 0.07 mM EICARMP (Figure 5A). This result is consistent with a single-step reaction mechanism as shown in Scheme 1. The second-order rate constant k_{on} is $2.33 \times 10^4\ M^{-1}\ s^{-1}$ from eq 2.

For human IMPDH, the values of k_{obs} display a hyperbolic dependence on EICARMP concentration (Figure 5B). This result suggests that the human IMPDH reacts with EICARMP through a two-step reaction mechanism as shown in Scheme 2, where $K_i = 16\ \mu M$, $k_2 = 2.7 \times 10^{-2}\ s^{-1}$, and $k_{on} = 1.7 \times 10^3\ M^{-1}\ s^{-1}$.

The failure to detect an E·I complex in the EICARMP inactivation of *E. coli* IMPDH probably results from experimental limitations. An E·I complex could only have been detected under the conditions of Figure 3 if $K_i < 1.6\ \mu M$; however, we expect K_i is about 20 μM by analogy to the human enzyme. Therefore, the EICARMP concentration was not high enough to show saturation kinetics. Unfortunately, the rate of inactivation is too fast to measure at higher concentrations of EICARMP. Thus, the mechanisms of inactivation of both enzymes by EICARMP probably involve initial formation of an E·I complex followed by a first-order inactivation step.

MALDI Mass Spectrometry. The MALDI mass spectrum of the isolated *E. coli* IMPDH (Figure 6A) exhibited singly $(M + H)^+$ and doubly charged $(M + 2H)^{2+}$ ions for both BSA and IMPDH (the ions due to BSA were used as an internal standard to calibrate the mass spectrum). The native *E. coli* IMPDH produced an $(M + H)^+$ ion at *m/z* 52 016 (M_r 52 015) rather than the expected value (54 576.7) based on the primary structure derived from the DNA sequence (Teideman & Smith, 1985). Furthermore, reduced and (*S*)-ethylpyridylated *E. coli* IMPDH produced an $(M + H)^+$ ion at *m/z* 52 541 corresponding to the addition of vinylpyridine to five rather than six cysteine residues. MALDI analysis of an aliquot from the Endo-Lys-C digest of (*S*)-ethylpyridylated protein (Figure 7A) provided sufficient additional information to explain these unexpected results. The N-terminal peptide MQSVTLCIMPRQYLLTTLVEILPML-RIAK [calculated $(M + H)^+ = m/z$ 3482.41] expected from the DNA sequence was not observed in this mass spectrum. However, an ion at *m/z* 731.4, whose mass did not correspond to any predicted Lys-C peptides, was in agreement with the calculated *m/z* value (731.98) for the protonated peptide MLRIAK. Subsequent Edman degradation (five cycles) provided further confirmation that the N-terminal sequence begins with MLRIA. This result indicates that protein synthesis for *E. coli* IMPDH begins at the third methionine of the original DNA-derived sequence (Teideman & Smith, 1985) and that the protein consists of only 488 amino acids and five instead of six cysteines per IMPDH monomer. Furthermore, an error in the published DNA sequence was discovered upon sequencing the *guaB* gene, which revealed that the sequence of nucleotides 924−925 is CG instead of GC and indicates that residue 206 is arginine and not alanine. This finding is corroborated by the presence of an ion at *m/z* 1122 in the MALDI mass spectrum (Table 2 and Figure

Table 2: Peptides Resulting from Endo-Lys-C Digest of *E. coli* IMPDH and EICARMP-Modified IMPDH

	$(M + H)^+$			
position	native[a]	modified[a]	calcd.	amino acid sequence
1−6	731.4	−	731.98	( )MLRIAK(E)
7−36	3213.0	3213.0	3211.64	(K)EALTFDDVLLVPAHSTVLPNTADLSTQLTK(T)
37−72	3825.0	3824.8	3824.55	(K)TIRLNIPMLSAAMDTVTEARLAIALAQEGGIGFIHK(N)
73−87	1846.12*	1846.12*	1846.12	(K)NMSIERQAEEVRRVK(K)
88−110	2537.3	2537.0	2536.90	(K)KHESGVVTDPQTVLPTTTLREVK(E)
89−110	2408.5	−	2408.72	(K)HESGVVTDPQTVLPTTTLREVK(E)
111−156	5157.7	5157.3	5156.83	(K)ELTERNGFAGYPVVTEENELVGIITGRDVRFVTDLNQPVSVYMTPK(E)
157−174	2055.4	2055.5	2055.39	(K)ERLVTVREGEAREVVLAK(M)
183−199	1900.9	1901.9	1901.28	(K)ALVVDDEFHLIGMITVK(D)
204−212	1121.9	1122.3	1122.34	(K)AERKPNACK(D)
213−267	5708.33*	5708.33*	5708.33	(K)DEQGRLRVGAAVGAGAGNEERVDALVAAGVDVLLIDSSHGHSEGVLQRIRETRAK(Y)
268−296	2894.7	2894.3	2894.3	(K)YPDLQIIGGNVATAAGARALAEAGCSAVK(V)
297−349	5227.0	5470.3[d]	5227.05	(K)VGIGPGSICTTRIVTGVGVPQITAVADAVEALEGTGIPVIADGGIRFSGDIAK(A)
350−383	3446.6	3446.5	3445.90	(K)AIAAGASAVMVGSMLAGTEESPGEIELYQGRSYK(S)
384−396	1345.4	1345.1	1345.59	(K)SYRGMGSLGAMSK(G)
397−411	1661.6	−	1661.68	(K)GSSDRYFQSDNAADK(L)
412−424	1431.5	1431.4	1431.68	(K)LVPEGIEGRVAYK(G)
429−456	3260.3	3259.9	3259.87	(K)EIIHQQMGGLRSCMGLTGCGTIDELRTK(A)
457−479	2496.1	2496.0	2495.80	(K)AEFVRISGAGIQESHVHDVTITK(E)
480−488	1022.8	−	1023.09	(K)ESPNYRLGS( )

[a] $(M + H)^+$ ions marked with asterisks were used as internal standards for mass calibration. [b] Amino acids in parenthesis refer to those preceding and following the Endo-Lys-C cleavage sites. [c] Residue 88 and the regions of 175−178, 179−182, 200−203, and 425−428 IMPDH were not detected in either native or modified IMPDH. The signals for these tetrapeptides are probably obscured by those from the matrix. [d] Calculated 5469.14 for the addition of EICARMP (M_r 347.22) instead of 4-vinylpyridine (M_r 105.14).

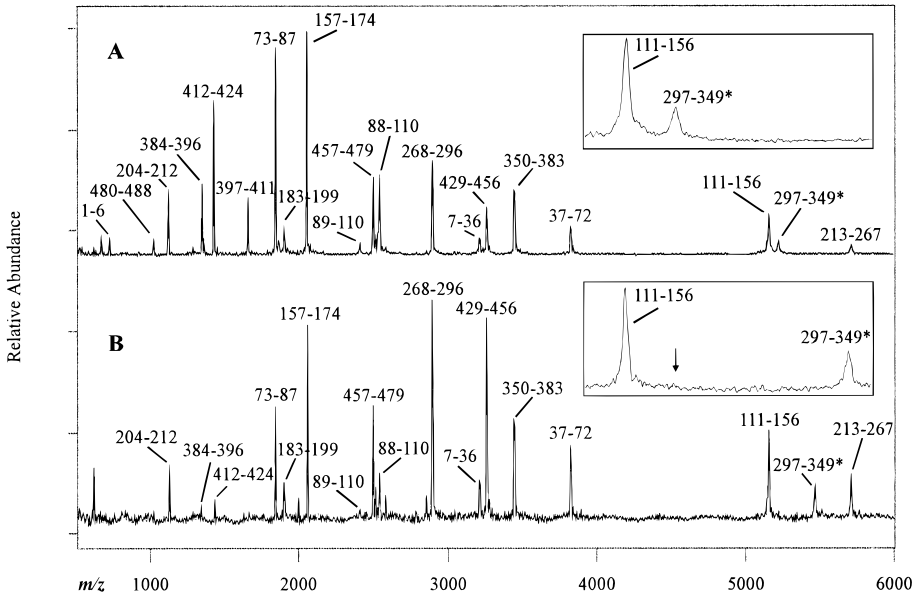

FIGURE 7: MALDI-TOF mass spectra of Endo-Lys-C digests of (*S*)-ethylpyridylated *E. coli* IMPDH. A, native; B, EICARMP-modified. The peak labels represent the positions of the peptides in the corrected amino acid sequence. The peptides containing Cys-305 are denoted by an asterisk. This region is expanded in the insets to indicate the absence of the peak at *m*/*z* 5227.0 (denoted by the arrow) in B.

7) of the Endo-Lys-C digests of both native and EICARMP-modified enzyme. It corresponds to peptide 204−212, AERKPNACK, $(M + H)^+$ calcd. *m*/*z* 1122.34, found *m*/*z* 1121.9 and 1122.3, respectively, in which the Lys−Pro bond had resisted cleavage by Endo-Lys-C. Taking these corrections into account, the $(M + H)^+$ ion of the (*S*)-ethylpyridylated IMPDH (lacking residues 1−23, i.e., beginning with MLRIAK ...) would be at *m*/*z* 52 549.3, which agrees quite well with the measured value (*m*/*z* 52 541).

The calculated *m*/*z* value for the $(M + H)^+$ ion from the native *E. coli* IMPDH using the corrected sequence is 52 023.6 (assuming no disulfide bonds) while the experimentally determined value (Figure 6A) is *m*/*z* 52 016. The MALDI mass spectrum of the EICARMP-modified *E. coli* enzyme (Figure 6B) exhibited an $(M + H)^+$ ion at *m*/*z* 52 322, which is within the MALDI-TOF-MS mass accuracy of ±0.1% (Beavis & Chait, 1990) for the calculated value of 52 370.8 for the addition of one molecule of EICARMP.

The MALDI mass spectrum of the Endo-Lys-C digest of EICARMP-modified (*S*)-ethylpyridylated *E. coli* IMPDH is shown in Figure 7B. Although the mass spectra in Figure 7A (unmodified but (*S*)-ethylpyridylated *E. coli* IMPDH digested with Endo-Lys-C) and B are very similar, they are not identical. Table 2 lists the measured and calculated masses as well as the sequences of the IMPDH peptides from these two digests. The major qualitative difference between the two spectra is the presence of a new ion at *m*/*z* 5470.3 and the absence of the ion at *m*/*z* 5227.0 in Figure 7B. The latter corresponds to peptide 297−349 (Table 2) in which Cys-305 (Cys-331 in human type II IMPDH numbering) is

(*S*)-ethylpyridylated while the former corresponds to the same peptide but in which EICARMP is attached instead. Since the ion at *m*/*z* 5470.3 is consistent only with the addition of one molecule of EICARMP (M_r 347.2) *instead* of vinylpyridine (M_r 105.1), the modification must be at position 305, the only cysteine in this peptide. Finally, the unmodified peptide of *m*/*z* 5227.0 was not detected in Figure 7B (compare enlarged insets in Figure 7), which suggests that more than 90% of the IMPDH monomers were modified by EICARMP.

Implications for IMPDH Mechanism. The observation that EICARMP alkylates Cys-305 strongly supports the hypothesis that Cys-305 is located near the 2 position of IMP in the IMP/IMPDH complex and that it is the nucleophile that attacks at position 2 of IMP (Figure 1). Such a mechanism has precedent in the oxidation of aldehydes by glyceraldehyde-3-phosphate dehydrogenase and aldehyde dehydrogenase (Trentham, 1971).

Conclusions. EICARMP irreversibly inactivates both human (type II) and *E. coli* IMPDH. The inactivation is time dependent and protected by IMP but not by NAD^+. MALDI-TOF mass spectrometry of native *E. coli* IMPDH established that protein translation initiates at the third ATG of the DNA sequence (Teideman & Smith, 1985). Thus, the *E. coli* IMPDH monomer has only 488 amino acid residues and five (instead of six) cysteines. EICARMP covalently alkylates Cys-305 of *E. coli* IMPDH (Cys-331 in human type II IMPDH numbering) as determined by MALDI-TOF-MS, which suggests that Cys-305 functions as the nucleophile in the IMPDH reaction. EICARMP can be used to titrate the active sites of IMPDH, and four active sites per IMPDH tetramer were determined by this method. Finally, these results demonstrate that significant differences exist between bacterial and human IMPDH and show promise for this enzyme as a target of antibiotic drugs.

NOTE ADDED IN PROOF

Huete-Pérez et al. (1995) report that the analogous Cys residue in *Tritrichomonas foetus* IMPDH forms a covalent adduct with IMP.

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