Probing the Active Site of Human IMP Dehydrogenase Using Halogenated Purine Riboside 5′-Monophosphates and Covalent Modification Reagents

Lisa C. Antonino,§ Kenneth Straub,‡ and John C. Wu*,§

Institute of Biochemistry and Cell Biology and Institute of Analytical Research, Syntex Discovery Research, Palo Alto, California 94304

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ABSTRACT: Active-site amino acid residues of human type II inosine 5′-monophosphate dehydrogenase (IMPDH) were investigated using the covalent modification reagents 6-chloroinosine 5′-monophosphate (6-Cl-IMP) and iodoacetamide. IMPDH was incubated with these reagents in the presence and absence of IMP, NAD, and NADH, and the activity of the enzyme for IMP dehydrogenation or 2-Cl-IMP dehalogenation was followed. IMPDH activity was rapidly lost when the enzyme was incubated with the IMP analog, 6-Cl-IMP, or with iodoacetamide. The enzyme was protected against inactivation in the presence of the substrate IMP. It was not protected against inactivation by NAD alone. Saturating concentrations of IMP and NADH reduced the inactivation rate by about the same amount as with IMP alone. IMPDH samples labeled with 6-Cl-IMP and an unlabeled control were alkylated with iodoacetamide, digested with trypsin, and analyzed by HPLC–mass spectrometry (HPLC–MS). All eight cysteines of human type II IMPDH were found to exist as free sulfhydryls on the active, unlabeled form of the enzyme. At an enzyme/inactivator ratio of 1:4, only one cysteine residue, Cys-331, was found to be covalently modified by 6-Cl-IMP. From the results of the substrate protection experiments and HPLC–MS data, it is concluded that 6-Cl-IMP binds in the IMP binding site of IMPDH and reacts covalently with Cys-331 to form a purine riboside 5′-monophosphate–enzyme adduct.

Inosine 5′-monophosphate dehydrogenase (IMPDH;[1] EC 1.2.1.205) catalyzes the NAD-dependent oxidation of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP). Its role in the *de novo* guanine nucleotide synthesis pathway makes it a useful target in the development of drugs for antitumor chemotherapy and immunosupressants (Jackson *et al.*, 1975; Weber, 1983; Eugui *et al.*, 1991). In this study, 6-chloropurine riboside 5′-monophosphate (6-Cl-IMP) is used to identify amino acids in the active site of human type II IMPDH by mass spectrometry. 6-Cl-IMP has been reported to inactivate the IMPDH from *Aerobacter aerogenes* and to form a covalent purine riboside 5′-monophosphate adduct with a cysteine residue in the IMP binding site of *Escherichia coli* IMPDH (Hampton & Nomura, 1967; Gilbert & Drabble, 1980); however, the location of the modified amino acid residue in the primary sequence was not determined. We also examine the inactivation of IMPDH by iodoacetamide, which is an alkylating reagent that reacts specifically with cysteine under the conditions tested. The activity of IMPDH incubated with these reagents is measured using the standard substrates, IMP and NAD, as well as with 2-Cl-IMP, which reacts with IMPDH to form XMP (Antonino & Wu, 1994). The dehalogenation reaction does not require NAD as a cofactor. Therefore, reagents which modify the NAD site without affecting the IMP site would not be expected to alter the activity of 2-Cl-IMP with IMPDH. In contrast, activity of IMPDH with IMP and NAD as substrates should be reduced by such a reagent. This potential differentiation of activity was investigated using the reagents 6-Cl-IMP and iodoacetamide. By comparing the rates of inactivation for the two substrates, we hope to gain insight into the amino acid residues which are important for catalysis and to distinguish the residues required for IMP binding from those associated with NAD binding.

MATERIALS AND METHODS

Human Type II IMPDH. The enzyme used for these experiments has been described previously (Antonino & Wu, 1994).

Preparation of 2-Cl-IMP. The synthesis and properties of this substrate have been described previously (Antonino & Wu, 1994).

Kinetics of Inactivation of IMPDH by 6-Cl-IMP. A concentrated aqueous solution of 6-chloropurine riboside 5′-monophosphate (Sigma) was diluted into 0.1 M Tris, pH 8.0, 0.1 M KCl, and 3 mM EDTA. The concentration of 6-Cl-IMP was determined by spectrophotometrically using an extinction coefficient, ϵ_{263}, of 8400 M^{-1} cm^{-1} (Brox & Hampton, 1968). Incubation of 6-Cl-IMP (0–200 μM) with IMPDH was at 23 °C at molar ratios ranging from 0–9.4 to 1. Enzyme activity (initial velocity, v) was measured by taking samples of the incubation mixture at various time intervals and assaying at 40 °C in a standard IMP/NAD assay mixture (200 μM IMP, 400 μM NAD in 0.1 M Tris, pH 8.0, 0.1 M KCl, and 3 mM EDTA). The reaction was followed spectrophotometrically at 340 nm (NADH formation). The fractional activity at various time intervals, (v_t/v_0), was calculated, and the rate of inactivation, k_i, and inactivation constant, K_i, were determined using iterative calculations with

* To whom correspondence should be addressed.
§ Institute of Biochemistry and Cell Biology.
‡ Institute of Analytical Research.
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[1] Abbreviations: IMPDH, inosine 5′-monophosphate dehydrogenase; IMP, inosine 5′-monophosphate; XMP, xanthosine 5′-monophosphate; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide, reduced form; 6-Cl-IMP, 6-chloroinosine 5′-monophosphate; MS, mass spectrometry; CMA, carboxymethanamido; PRMP-IMPDH, purine riboside 5′-monophosphate adduct of IMPDH with 6-Cl-IMP.

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the program, Sigma Plot, by fitting to the equation

$$\ln (v_t/v_0) = \frac{(-k_i t)}{[1 + (K_i/[I])]} \quad (1)$$

where [I] is the 6-Cl-IMP concentration, based on the following inactivation scheme:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_i} EI^* \quad (2)$$

where $K_i = k_{-1}/k_1$, E is IMPDH, EI is the reversibly bound enzyme–inhibitor complex, and EI* is the irreversibly inactivated enzyme. For this analysis, only data in the linear part of the plot of v_t/v_0 vs time were used.

Activity Assays for Incubation Studies. The standard assay mixture for the incubation studies consisted of 200 μM IMP and 400 μM NAD in 0.1 M Tris, pH 8.0, 0.1 M KCl, and 3 mM EDTA. The 2-Cl-IMP assay mixture contained 100 μM 2-Cl-IMP in the same buffer and no IMP or NAD. An enzyme concentration of 50 nM was used for the IMP/NAD assays and 300 nM for the 2-Cl-IMP assays. The assays were performed at 25 °C using a Hewlett-Packard HP8452 diode array spectrophotometer with a multicell transport carriage. The change in absorbance due to XMP formation (ϵ_{280}XMP = 8900, ϵ_{290}XMP = 4600) was measured, and the initial rates were calculated using the HP software.

Inactivation of IMPDH by 6-Cl-IMP—Protection by Substrates. Incubation of 6-Cl-IMP (100 μM) with IMPDH (5 μM) was performed at 23 °C. The enzyme was also incubated with 6-Cl-IMP in the presence of 200 μM IMP, 400 μM NAD, or 200 μM IMP + 400 μM NADH. NADH is a product of the reaction and is used here in combination with IMP to occupy binding sites without substrate turnover. Enzyme activity was measured by taking samples of the incubation mixture at various time intervals and assaying for XMP formation at 25 °C in both the standard assay mix and the 2-Cl-IMP mix.

Inactivation of IMPDH by Iodoacetamide—Protection by Substrates. A concentrated aqueous solution of iodoacetamide was diluted into 0.1 M Tris, pH 8.0, 0.1 M KCl and 3 mM EDTA. Incubation of iodoacetamide (2 mM) with IMPDH (5 μM) was done at 23 °C. The enzyme was also incubated with iodoacetamide in the presence of 200 μM IMP, 400 μM NAD, or 200 μM IMP + 400 μM NADH. Enzyme activity was measured by taking samples of the incubation mixture at various time intervals and assaying at 25 °C in both the standard and the 2-Cl-IMP assays.

Covalent Modification of IMPDH with 6-Cl-IMP, Digestion by Trypsin, and Analysis of Tryptic Fragments by Mass Spectrometry. Human type II IMPDH was treated with 6-Cl-IMP (20 μM) at an enzyme/reagent ratio of 1:4 for 2 h at 23 °C. The protein was precipitated with trichloroacetic acid and washed with acetone to remove noncovalently bound 6-Cl-IMP. The pellet was dissolved in 50 μL of 0.4 M NH_4HCO_3 and 8 M urea, pH 8.0, reduced with dithiothreitol (4.1 mM, 15 min, 50 °C), and alkylated with iodoacetamide (8.3 mM, 15 min, 23 °C). A sample of untreated IMPDH was reduced and alkylated as a control. The mixture was diluted with distilled, deionized water to give a final urea concentration of 2 M. Digestion with trypsin or chymotrypsin was done at an IMPDH/protease ratio of 25:1 overnight (22 h) at 37 °C. The samples (IMPDH/trypsin, PRMP-IMPDH/trypsin, IMPDH/chymotrypsin, and PRMP-IMPDH/chymotrypsin) were stored at −20 °C prior to analysis by HPLC–MS. One sample was also reacted with 6-Cl-IMP (3.2 mM) at a ratio of 1:1000 IMPDH/6-Cl-IMP followed by the same reduction, alkylation, and digestion steps.

HPLC was carried out using Vydac C18 and C4 columns (2.1 × 150 mm, 5-μm particle size), eluted at 100 μL/min with a gradient consisting of solvents A (0.1% TFA in H_2O) and B (0.1% TFA in 80:20 acetonitrile/H_2O), as follows: 0–10 min, 2% B; 10–140 min, linear gradient to 100% B. The gradient was formed using an ABI model 140B microsyringe pumping system equipped with a static mixer (3-μL internal volume). Approximately 300 pmol of the sample (60 μL) was injected onto the column. The column effluent was passed through a flow splitter, where *ca.* 1.8 μL/min was directed to the mass spectrometer through a 100-μm i.d. fused silica transfer line. This transfer line was attached to the inlet needle of a TSQ700 electrospray ion source (Finnigan-MAT, San Jose, CA). A sheathing flow of 2-methoxyethanol (2.1 μL/min) was used in combination with a nebulizing nitrogen gas flow to assist the formation of micro droplets via electrospray. Data were acquired in centroid mode using a scan range of m/z 300–1900 in 3 s. Typical operating conditions for the ESI source were as follows: electrode/capillary entrance voltage, −3000 V; capillary exit, +50 V; tube lens, +180 V; drying gas temperature, 255 °C.

The main portion of the column effluent was directed to an absorbance detector (ABI model 783A) equipped with a 1.8-μL flow cell and then to a fraction collector. Absorbance was monitored at 214 nm. Fractions were collected at 1-min intervals. In the subsequent tandem MS experiments, individual LC fractions were infused directly into the electrospray ion source using a syringe pump (Harvard model 22) at a flow rate of 1 μL/min together with a sheathing flow of 1 μL/min of 2-methoxyethanol. Collision-assisted dissociation (CAD) spectra were then acquired on either the +3, +2, or +1 charged ions at a collision pressure of ca. 2.5 mtorr argon and energies (E_{lab}) of between −9 and −15 eV (for the +2 ion) or −20 eV (for the +1 ion). Data were acquired in profile mode using signal averaging, and then converted to centroid data.

Identification of Free Sulfhydryls and Sulfur Atoms Contained in Disulfides by Mass Spectrometry. IMPDH (1.5 nmol in 100 μL of 0.1 M Tris, pH 8.0) was treated with iodoacetamide for 1 h at 23 °C. The protein was precipitated with trichloroacetic acid and washed with acetone to remove noncovalently bound reagent. The pellet was dissolved in 0.4 M NH_4HCO_3 and 8 M urea, pH 8.0, reduced with dithiothreitol (4.1 mM, 15 min, 56 °C) and alkylated with 4-vinylpyridine (1 h, 23 °C). Samples were also prepared without reduction and alkylation by 4-vinylpyridine in order to preserve any disulfides. Hydrolysis with trypsin or chymotrypsin was done at an IMPDH/protease ratio of 25:1 overnight (22 h) at 37 °C in 0.4 M NH_4HCO_3 and 2 M urea, pH 8.0. The samples were stored at −20 °C prior to analysis by HPLC–MS. (See above for details of MS analyses.)

RESULTS

Kinetics of Inactivation by 6-Cl-IMP. Incubation of IMPDH with 6-Cl-IMP caused a time-dependent decrease in v_t/v_0, indicating that the enzyme is inactivated by 6-Cl-IMP (Figure 1). The dissociation constant, K_i, and the first-order rate constant of inactivation, k_i, determined by fitting the data to eq 1 (see Methods), were 78 ± 10 μM and 0.21 ± 0.02 min^{-1}, respectively. Activity of IMPDH incubated with 100 μM 6-Cl-IMP in the presence and absence of IMP, NAD, and IMP + NADH, as measured by the IMP/NAD assay, is shown in Figure 2. The rate of inactivation is greatly reduced when the enzyme is incubated with 200 μM IMP or with IMP

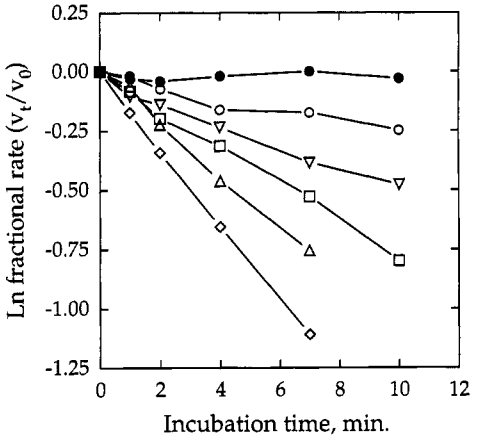

FIGURE 1: Irreversible inactivation of IMPDH as a function of 6-Cl-IMP concentration. Figure symbols are as follows: ○, 10 μM 6-Cl-IMP; ▽, 25 μM; □, 50 μM; △, 100 μM; ◇, 200 μM; ●, no 6-Cl-IMP.

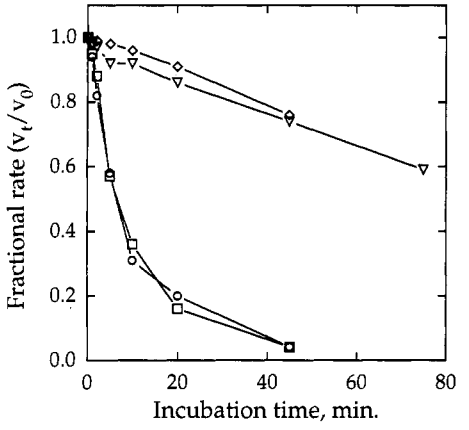

FIGURE 2: Inactivation of IMPDH by 6-Cl-IMP in the presence and absence of IMP, NAD, and IMP + NADH as measured by the IMP/NAD assay. Figure symbols are as follows: ○, 100 μM 6-Cl-IMP; ▽, 6-Cl-IMP + 200 μM IMP; □, 6-Cl-IMP + 200 μM NAD; ◇, 6-Cl-IMP + 200 μM IMP + 200 μM NADH.

Table 1: Summary and Inactivation Results ($k_{i(Obs)}$, min^{-1}) for 6-Cl-IMP and Iodoacetamide (See Text for Experimental Details)

reaction	incubation conditions	6-Cl-IMP	iodoacetamide
IMP/NAD		0.07[a]	0.86
dehydrogenation	+IMP	0.01	0.13
	+NAD	0.06	0.77
	+IMP + NADH	0.01	0.09
2-Cl-IMP		0.06	0.27
dehalogenation	+IMP	0.03	0.09
	+NAD	0.05	0.14
	+IMP + NADH	0.02	0.06

[a] $k_{i(Obs)}$, min^{-1}.

and NADH. NAD by itself did not protect against inactivation, and a mixture of IMP and NADH had approximately the same effect as IMP alone. The data suggest that 6-Cl-IMP does not bind at a site that overlaps with the NAD binding site. Inactivation as measured by both the IMP/NAD dehydrogenation and 2-Cl-IMP dehalogenation assays is summarized in Table 1. The inactivation rates in the presence and absence of IMP, NAD, and IMP + NADH for the 2-Cl-IMP assay are similar to those of the IMP/NAD assay described above.

Kinetics of Inactivation by Iodoacetamide. Iodoacetamide reacts with free cysteine residues in proteins to form carboxymethanamido (CMA) derivatives. Incubation of IMPDH with 5 mM iodoacetamide at 23 °C resulted in a decrease

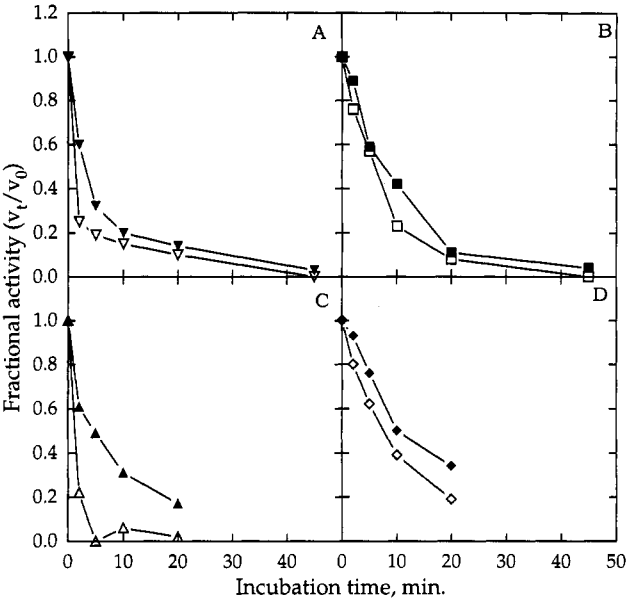

FIGURE 3: Inactivation of IMPDH by iodoacetamide in the presence and absence of IMP, NAD, and IMP + NADH. Open symbols represent results of assays with saturating amounts of IMP and NAD. Closed symbols represent results of assays with 100 μM 2-Cl-IMP. Panel A shows inactivation by 5 mM iodoacetamide only; panel B, inactivation by iodoacetamide in the presence of 200 μM IMP; panel C, inactivation by iodoacetamide in the presence of 400 μM NAD; panel D, inactivation by iodoacetamide in the presence of 200 μM IMP + 400 μM NADH.

in enzyme activity with incubation time. IMPDH incubated with iodoacetamide and 200 μM IMP lost activity at a slower rate. NAD in the incubation mixture had no effect on the inactivation rate; however, the combination of IMP and NADH reduced the inactivation rate by approximately the same as with IMP alone. Activity as measured by both the IMP/NAD and 2-Cl-IMP dehalogenation assay is shown in Figure 3. The inactivation rate with iodoacetamide in the absence of any substrates as measured by the IMP/NAD assay was approximately 7 times faster than that measured by the 2-Cl-IMP assay. It is possible that iodoacetamide is labeling the protein in a region that interferes with NAD binding, in addition to an IMP-site cysteine. Labeling of iodoacetamide at this other site would interfere with the IMP/NAD assay but not the 2-Cl-IMP assay. The implications of this are presented in the discussion below.

When the enzyme was incubated with IMP or IMP + NADH, the inactivation rates between the two assays were similar. In the presence of 400 μM NAD, the inactivation rate as measured by the IMP/NAD assay was approximately 6 times faster than that measured by the 2-Cl-IMP assay. The inactivation rates are summarized in Table 1.

Identification of Free Protein Sulfhydryls by Mass Spectrometry. The free sulfhydryls and intrachain disulfides contained in IMPDH were identified. IMPDH samples were alkylated with iodoacetamide in the absence of reducing agent (conditions which should preserve disulfide bonds) and digested with trypsin. The digests were analyzed by HPLC–MS using a C4 column in order to ensure that any large, hydrophobic peptides containing disulfide-linked cysteines would be eluted. As shown by the data in Table 2, all eight cysteines were observed as carboxymethanamido derivatives present in seven distinct peptides, corresponding to residues [1–57], [63–105], [137–149], [162–181], [243–259], [323–349], and [460–474]. Five of the seven cysteine-containing fragments have been sequenced by tandem-MS (Table 2). No fragments corre-

Table 2: Results of HPLC Analysis of Cysteine-Containing Peptides Obtained from Trypsin Hydrolysis of Reduced and Alkylated Human Type II IMPDH

time (min)[a]	identity[b]	mw_{obs}[c]	mw_{calc}[d]	sequence[e]
40.3	T52a*	1610.6	1610.8	460–474†
42.1	T30+31+32	2074.7‡	2075.3‡	242–259
43.4	T31+32	1946.7‡	1947.2‡	243–259†
45.4	T13	1429.7	1429.7	137–149†
53.8	T38	2853.6‡	2854.3‡	323–349†
61.5	T16+17	2541.1‡	2541.9‡	162–181†
76.4	T1	6696.5‡	6696.5‡	1–575¶
83.1	T4	4572.3‡	4573.2‡	63–105¶

[a] Peptides which account for 8 Cys residues (present as acetamido-Cys). [b] Peptides resulting from trypsin hydrolysis are numbered sequentially from N-terminus [* indicates cleavage by trypsin/chymotrypsin activites.] [c] Molecular weight, as monoisotopic ($C = 12.0000$) or average mass ($C = 12.0115$, indicated by ‡). [d] Sequences marked with ‡ were confirmed by tandem-MS experiments. [e] Includes leader sequence for construct (TMITPS). [¶ indicates that first five residues in peptide were confirmed by Edman microsequencing.]

sponding to peptides linked by disulfides were observed, and all of the major peaks which were eluted from the HPLC column corresponded to the fragments expected of type II IMPDH. Approximately 95% of the expected sequence of type II IMPDH has been identified by MS.

In a second set of experiments, IMPDH samples were first treated with iodoacetamide in the absence of reducing agent and then reduced by treatment with DTT and alkylated with a second reagent, 4-vinylpyridine. The peptide fragments resulting from trypsin or chymotrypsin digestion were analyzed by HPLC–MS. All peptides containing cysteine residues were found to be labeled by the first reagent, iodoacetamide. Thus all of the cysteine residues in IMPDH exist in the reduced state.

Identification of Covalently Labeled Cysteine Residues by Mass Spectrometry. The peptides obtained by digestion with trypsin from the 6-Cl-IMP-labeled and -unlabeled samples were analyzed by HPLC–MS. This dataset was searched for molecular weights that would correspond to all of the possible trypsin cleavage products of a protein that contained a PRMP-modified cysteine in place of a CMA-Cys at any of the eight possible positions. Peptides which contained a PRMP-modified cysteine were then sequenced by tandem-MS experiments and Edman microsequencing to verify the position of the modification.

When a 1:4 ratio of enzyme to 6-Cl-IMP was used, only one PRMP-modified cysteine residue, Cys331,[2] was found in the trypsin-digested sample of type II IMPDH. Figure 4, parts A and B, show ion chromatograms corresponding to the +2 charged molecular species for unlabeled T38 (m/z 1427.5) and PRMP-labeled T38 (m/z 1564.8). The reconstructed total ion chromatogram obtained from the LCMS analysis of this sample and the HPLC elution profile are shown in Figure 4, parts C and D. This peptide contains two cysteine residues (Cys-331 and Cys-336), and the position of the modified residue as Cys-331 was confirmed by tandem-MS and Edman microsequencing. The other seven cysteine residues were found to be unlabeled in this sample. Cys-331 was present in both labeled and unlabeled forms in a ratio of approximately 1:1.

When a sample of type II IMPDH was reacted with a 1000-fold excess of 6-Cl-IMP prior to reduction, alkylation, and digestion, LCMS analysis of the peptide fragments revealed

[2] All amino acid residue numbers cited are according to the sequence of Natsumeda *et al.* (1990).

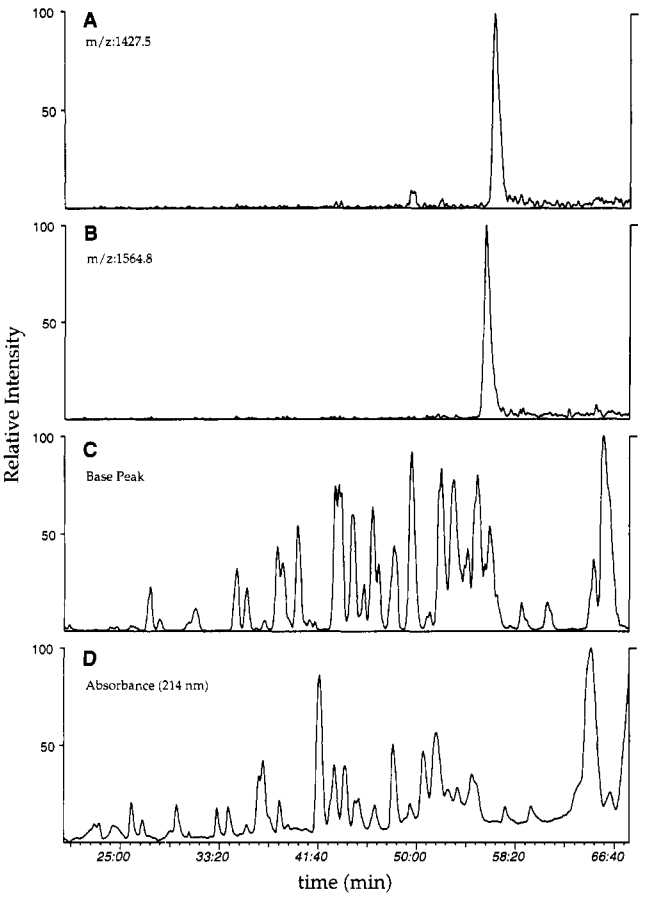

FIGURE 4: HPLC–MS elution profiles for trypsin hydrolysis of unlabeled and PRMP-labeled IMPDH: (A) reconstructed ion chromatogram for +2 ion of unlabeled T38 (residues [323–349]); (B) reconstructed ion chromatogram for +2 ion of PRMP-labeled T38 (residues [323–349]), where Cys-331 has undergone reaction with 6-Cl-IMP; (C) reconstructed total ionization chromatogram (most intense ion in each mass spectrum); (D) absorbance, 214 nm.

that a second cysteine, Cys-140, was also labeled but at a ratio of 1:2 unlabeled/labeled. Under these conditions, Cys-331 was found to be labeled at a ratio of 1:4 unlabeled/labeled.

DISCUSSION

Covalent modification reagents have been useful in identifying amino acid residues which are important for substrate binding and catalysis (Miles, 1977; Zhang *et al.*, 1992; Lundblad, 1991). It is particularly useful to have a reagent which is also a substrate analog, in this case, 6-Cl-IMP, since its primary reactive site would be the active site of the enzyme. In this study, 6-Cl-IMP was used to identify a cysteine residue located at the IMP binding site of IMPDH.

6-Cl-IMP was found previously to irreversibly inactivate IMPDH from *A. aerogenes* (Brox & Hampton, 1968). Gilbert and Drabble also implicated a cysteine residue in the *E. coli* enzyme as the target of 6-Cl-IMP (1980). In our experiments, inactivation of the human IMPDH by 6-Cl-IMP also showed that the enzyme reacts irreversibly to form a PRMP–IMPDH adduct. Tryptic digests of labeled protein and analysis by mass spectrometry identified a peptide fragment containing PRMP-labeled Cys-331 on the human enzyme. This result was confirmed by sequencing of the same peptide by Edman degradation. A second cysteine, Cys-140, was also found to be labeled by 6-Cl-IMP, but only when a large excess (1000× and at 100-fold Km of IMP) of reagent was used. All eight

cysteine residues are labeled by iodoacetamide, a nonspecific sulfhydryl reagent. Under the conditions used to label only Cys-331, IMP protects the enzyme from inactivation.

The structural similarity between 6-Cl-IMP and IMP and the protection of inactivation by IMP strongly suggest that the site at which the 6-Cl-IMP labels the protein is the IMP binding site. It is not known whether the labeled cysteine residue is directly involved in a catalytic mechanism via an enzyme–substrate intermediate or as a general base (for a discussion of mechanisms see Antonino and Wu, preceding paper in this issue). The electronic configuration of the purine ring of 6-Cl-IMP allows the formation of a stable enzyme–substrate covalent intermediate at the C6 position. The C2 carbon is the site at which the hydride transfer occurs. If there were a covalent intermediate formed via sulfhydryl attack at C2 of IMP in the catalytic mechanism, the cysteine residue would need to be properly positioned. Whether Cys-331 is in a position to react at C2 as well as C6 is not known, but we were not able to detect turnover of 6-Cl-IMP in the presence of NAD to form 6-Cl-XMP at high enzyme concentrations. It is possible that binding of 6-Cl-IMP may involve a conformational shift of the enzyme away from the one that binds to the substrate IMP. Such an altered conformation may place the nucleophilic cysteine residue, which is situated normally for reaction at C2, into a position where it can react rapidly with the C6 of 6-Cl-IMP.

Nucleotide binding sites of several enzymes have been compiled into a data base by Bork and Grunwald (1990). Using a property pattern approach, they identified one region of the *E. coli* IMPDH sequence as a possible nucleotide binding site. This region corresponds to residues 319–347 in the human IMPDH. The sequence is highly conserved among the known IMPDH sequences (Thomas & Drabble, 1985, *E. coli*; Tiedeman & Smith, 1985, *E. coli*; Collart & Huberman, 1988, human and Chinese hamster; Kanzaki & Miyagawa, 1990, *Bacillus subtilis*; Natsumeda *et al.*, 1990, human types I and II; Tiedeman & Smith, 1991, mouse; Wilson *et al.*, 1991, *Leishmania donovani*). For the dehydrogenases with known structures, the nucleotide binding sites are for NAD or NADP. For IMPDH, however, the PRMP-labeled Cys-331, which we have demonstrated to be the site of IMP binding, is located in this conserved region. If Cys-331 functions in catalysis and is positioned in proximity to the C2 or C6 carbon of IMP, it is reasonable to assume that it also must be near the NAD binding site where hydride transfer occurs. Thus, the amino acid sequence predicted to comprise the dinucleotide binding region may also contain amino acid residues which interact with IMP in or near the IMP binding site. Another possibility is that IMPDH is unique among the dehydrogenases in that the substrate binds at a site normally reserved for NAD binding in other dehydrogenases, and the NAD binding site is in a different region of this enzyme. IMPDH is unusual among the dehydrogenases for two reasons. First, both substrates, IMP and NAD, are nucleotides, and either substrate may be able to associate with this putative nucleotide binding pocket. Second, unlike other pyridine nucleotide-dependent dehydrogenases, IMPDH catalyzes ordered bi–bi kinetics in which the substrate, IMP, binds the enzyme prior to the cofactor, NAD.

The alternative substrate, 2-Cl-IMP, was employed to distinguish between modification reagents that affect the IMP site and those that affect the NAD site. 2-Cl-IMP does not react to form an irreversible covalent adduct with IMPDH, as does 6-Cl-IMP, but rather undergoes IMPDH-catalyzed dehalogenation (Antonino & Wu, 1994). The kinetics and inhibition of the dehalogenation reaction suggest that 2-Cl-IMP occupies the same enzyme site as IMP.

Studies with 6-Cl-IMP revealed no significant difference between the results of the IMP/NAD and 2-Cl-IMP assays, suggesting that this reagent does not react preferentially at the NAD binding site. However, there was a discrimination between the two assays when iodoacetamide was used to covalently label the enzyme. The dehalogenation reaction is inactivated more slowly than the dehydrogenation reaction (Figure 3). Because iodoacetamide is a relatively small molecule which reacts nonspecifically with free cysteine residues, it may react at sites inaccessible to a larger, more specific reagent such as 6-Cl-IMP. If iodoacetamide reacted with a cysteine in the NAD binding site, as well as with Cys-331 in the IMP binding site, inactivation as measured by the 2-Cl-IMP assay would be slower than that measured by the IMP/NAD assay, and incubation with NAD would be expected to reduce the inactivation rate for the IMP/NAD assay but not with the 2-Cl-IMP assay. We do observe a difference in the inactivation rates between the two assays, but do not observe any protection by NAD. The lack of protection by NAD may be the result of an obligatory ordered binding behavior, by which NAD alone may have a very low affinity for the enzyme. By themselves, however, these results suggest that the site or sites of inactivation are not explicitly in the NAD binding site but are near the NAD binding pocket. Alteration of one or more sulfhydryl groups near the active site may affect the conformation of the enzyme required for binding to NAD. Blockage of these secondary cysteines may impede NAD binding but does not interfere with IMP binding.

Sulfhydryl groups in proteins play vital functional roles, including metal binding, hydrogen-bond stabilization of substrates, acid–base catalysis, and nucleophilic attack at reactive sites to form covalent enzyme–substrate intermediates. Sulfhydryl groups also form disulfide bridges to stabilize protein tertiary and quaternary structure. We have shown that the eight cysteines of human type II IMPDH exist as reduced sulfhydryls on the active form of the enzyme and that the sulfhydryl group of Cys-331 is located at the IMP binding site. Cys-331 appears to be a very reactive sulfhydryl, but its role in catalysis is not known. It may provide noncovalent interactions such as hydrogen-bond stabilization to the carbonyl group at the C6 position of IMP or serve as a base to activate a water molecule for nucleophilic catalysis at the active site. It may also react at the C2 position to form a covalent adduct with IMP, which, after hydride transfer, becomes hydrolyzed by H_2O. Studies are in progress to distinguish between the covalent and noncovalent mechanisms postulated for this reaction.

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