Identification of the IMP Binding Site in the IMP Dehydrogenase from *Tritrichomonas foetus*†

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ABSTRACT: The IMP dehydrogenase from *Tritrichomonas foetus* has been identified as a potential target for antitritrichomonial chemotherapy. The gene encoding this enzyme was expressed in transformed *Escherichia coli*, and the recombinant protein was purified to homogeneity with an average yield of 3 mg of protein per liter of bacterial culture. Kinetic characterizations verified that the recombinant enzyme is in the authentic native state. 6-Cl-IMP, an irreversible inhibitor of the enzyme, was found to protect cysteine residue 319 of the enzyme against carboxymethylation by iodoacetamide. Radiolabeled IMP was covalently bound to the enzyme during the enzyme-catalyzed reaction via the formation of a specific adduct with cysteine residue 319. It is thus postulated that the conversion of IMP to XMP catalyzed by the IMP dehydrogenase from *T. foetus* is mediated by a nucleophilic attack of cysteine-319 in the enzyme protein to IMP at, most likely, its 2-position to facilitate a hydride transfer to NAD, resulting in the formation of a covalent intermediate between substrate and enzyme.

Inosine-5′-monophosphate dehydrogenase (IMPDH;[1] EC 1.1.1.205) catalyzes the NAD-dependent oxidation of IMP to xanthosine monophosphate (XMP), which constitutes the committed step in the biosynthesis of guanine nucleotides. It has been recognized as a potential target for immunosuppression (Eugui *et al.*, 1991), antiviral (Streeter *et al.*, 1973), antitumor (Weber, 1983), and antiparasitic chemotherapy (Wang *et al.*, 1984a). In *Tritrichomonas foetus*, a parasitic protozoan in the urogenital tract of cattle, inhibition of IMPDH leads to cessation of *in vitro* growth of the parasite, which can be selectively reversed by adding guanine or guanosine to the culture medium (Wang *et al.*, 1984b). This IMPDH has since been purified from *T. foetus* to apparent homogeneity and functionally characterized (Verham *et al.*, 1987; Hedstrom & Wang, 1990). It has a subunit molecular weight of 58 000, forms active tetramers at relatively high salt concentrations, and aggregates into hexamers at lower ionic strengths. Steady-state kinetic studies and product inhibition data suggest a sequential, ordered bi-bi kinetic mechanism for the enzyme-catalyzed reaction, in which IMP binds before NAD, and NADH is released before XMP. This unusual reaction mechanism, differing from those of all the other known NAD-dependent dehydrogenases, which either have the random kinetics or require NAD association prior to the other substrate, has been observed also in the human IMPDH-catalyzed reaction (Carr *et al.*, 1993). Recent studies by Antonino and Wu (1994) on human type II IMPDH indicated hydrolytic dehalogenation of 2-F-IMP and 2-Cl-IMP by the enzyme without the presence of NAD, thus supporting the model of nucleophilic attack at the C2-position of IMP by an enzymatic nucleophile or an OH^- from water at the beginning of the reaction. This nucleophile was subsequently identified to be cysteine-331 in the human type II enzyme by substrate protection experiments using the covalent modification reagents 6-chloroinosine 5′-monophosphate (6-Cl-IMP) and iodoacetamide (Antonino *et al.*, 1994). More recently, Wu *et al.* (unpublished) measured the formation of precipitable enzyme-bound radiolabeled IMP, and the identity of IMP-Cys331 was confirmed by HPLC−mass spectrometry analysis of the tryptic fragments of the enzyme−substrate complex.

The gene encoding *T. foetus* IMPDH was identified recently (Beck *et al.*, 1994). The amino acid sequence thus translated from the cloned gene shares only 30−35% sequence identity with IMPDHs from organisms ranging from bacteria, to protozoa, to human. A sequence alignment between the *T. foetus* enzyme and IMPDHs of other origins indicates that Cys319 in the *T. foetus* enzyme is the conserved cysteine residue among all IMPDHs and corresponds to Cys331 in the human IMPDH.

In the present investigation, we expressed the functional recombinant *T. foetus* IMPDH in transformed *Escherichia coli* in high yield and purified it to homogeneity. It was crystallized, and its three-dimensional structure is being resolved by X-ray diffraction in a separate study (Whitby *et al.*, 1995). This prompted us to try to identify the active site in the enzyme protein via biochemical means in a parallel investigation. The kinetics of inactivation of the enzyme by 6-Cl-IMP and inhibition by several other IMP analogs were monitored. 6-Cl-IMP protection against carboxymethylation of the enzyme by iodoacetamide and direct affinity labeling by radiolabeled IMP (Wu *et al.*, unpublished

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[1] Abbreviations: IMPDH, inosine-5′-monophosphate dehydrogenase; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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experiments), followed by trypsin digestion and HPLC fractionation, indicate that cysteine-319 is indeed the nucleophile in *T. foetus* IMPDH forming the covalent intermediate with IMP.

MATERIALS AND METHODS

Materials. 6-Cl-IMP, 6-thio-IMP, 2′-deoxy-IMP, 2′,3′-dialdehyde-IMP, mycophenolic acid, IMP, NAD, and NADH were purchased from Sigma Chemical Co. [8-^{14}C]IMP (50 mCi/mmol) was obtained from Movarek and [1-^{14}C]iodoacetamide (60 mCi/mmol) from Amersham. All other chemicals were of the highest purity commercially available.

Plasmid Construction and Transformation of E. coli. The original EMBL3 clone containing an *Eco*RI fragment of 1.6 kb encoding the entire *T. foetus* IMPDH (Beck *et al.*, 1994) was used as the template in polymerase chain reactions (PCRs). Two synthetic oligonucleotide primers were used in the reactions. Primer I (GGCA TATGGCAAAATACTACAACG) consists of an *Nde*I restriction site and the 5′-end of the sense strand DNA whereas primer II (AACCC GGGTTATTTTGGGTGATAGTCG) has the 5′-terminal of the antisense strand following a *Sma*I site. Five cycles of PCR with a melting temperature at 95 °C for 1 min, reannealing at 40 °C for 30 s, and elongation at 75 °C for 2 min were followed by another 25 cycles at an elevated reannealing temperature of 50 °C. The amplified DNA fragment was digested with *NdeI*/*Sma*I, purified in agarose gel electrophoresis, and cloned into the 3.26 kb *NdeI*/*Eco*RV linearized pBAce plasmid by the standard procedure (Craig *et al.*, 1991). This construct, referred to as pBimpdh, was transformed into *E. coli* strain H712 (Δ*guaB*), which has the IMPDH gene deleted (Nijkamp & De Haan, 1967). The plasmid DNA purified from the transformed and cloned *E. coli* cells was digested with *NdeI*/*Sma*I and examined in agarose gel electrophoresis, and the two ends of the DNA insert were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) for verification.

Expression and Purification of the Recombinant IMPDH. The transformed *E. coli* H712/pBimpdh cells were cultivated at 37 °C for 18 h to reach an optical density of 1.2 at 600 nm in low-phosphate culture medium supplemented with 100 μg/mL L-tryptophan and 100 μg/mL thiamin (Yuan *et al.*, 1990) for induction of the *phoA* promoter which controls the expression of the IMPDH gene in the plasmid pBimpdh. The cells were chilled on ice and harvested by centrifuging at 10000*g* in a refrigerated centrifuge for 15 min. The cell pellets were washed and resuspended in buffer A [0.1 M Tris-HCl, pH 8.0, + 1 mM dithiothreitol (DTT)] containing 1.0 mM phenylmethanesulfonyl fluoride (PMSF) and 10% glycerol, sonicated, and centrifuged at 14000*g* for 20 min to remove pelletable materials. The resulting crude cell-free extracts were fractionated with 45−75% ammonium sulfate, recovered in the pellet, and redissolved in a small aliquot of buffer B (20 mM Tris-HCl, pH 7.4, 4 mM DTT, and 10% glycerol), desalted through a prepacked Sephadex G-25M column (Pharmacia), applied to a Mono Q HR 10/10 column (Pharmacia) preequilibrated in buffer B, and eluted with a linear gradient of 0−0.5 M NaCl at a flow rate of 3.0 mL/min by HPLC. The fractions containing the IMPDH activity were pooled, precipitated with 75% ammonium sulfate, redissolved in buffer A, and stored frozen at −80 °C.

The IMPDH Assay. The enzyme activity was determined with a Beckman DU-7 spectrophotometer by monitoring the formation of NADH at 340 nm (Verham *et al.*, 1987). The standard reaction mixture contained 0.1 M Tris-HCl, pH 8.0, 0.1 M KCl, 1 mM DTT, 1 mM NAD, 3 mM EDTA, and 200 μM IMP. The reaction was started at 37 °C by adding the enzyme. One unit of enzyme activity was defined as the conversion of 1 nmol of NAD to NADH within 1 min.

For enzyme kinetic and inhibition studies, the pure IMPDH was present at 70 nM in the assays (Hedstrom & Wang, 1990). Potential irreversible inhibitors were assayed by preincubation with 7 μM of the pure enzyme in the assay mixture without substrates at 24 °C for varying lengths of time. The incubated mixture was then diluted 100-fold into the standard assay mixture containing both substrates at 37 °C to monitor the IMPDH activity (Antonino *et al.*, 1994). The fractional activity at various time intervals (v_i/v_o) was calculated, and the rate of inactivation, k_i, and the inactivation constant, K_i, were determined using iterative calculations with the program Sigma Plot by fitting to the equation:

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

where [I] is the inhibitor concentration, based on the 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 parts of the plot of v_i/v_o vs time were used.

Carboxymethylation of IMPDH with Radiolabeled Iodoacetamide. The purified recombinant IMPDH (300 μg) in 1.0 mL of 0.1 M Tris-HCl, pH 8.0, 0.1 M KCl, and 3 mM EDTA was preincubated with 200 μM 6-Cl-IMP at 37 °C for 2 h, precipitated with 10% trichloroacetic acid (TCA), washed with acetone, dried, and redissolved in 0.1 M NH_4HCO_3, pH 8.0, containing 8 M urea. After incubation at 37 °C for 30 min, the sample was treated with 8 mM [1-^{14}C]-iodoacetamide (3 mCi/mmol) at the same temperature for 30 min and diluted 4-fold with 0.1 M NH_4HCO_3, pH 8.0, to give a final urea concentration of 2 M. The protein sample was then digested in 10% trypsin (Sigma) at 37 °C for 20 h, and fractionated by HPLC using a Microsorb-MV C18 column (4.6 × 250 mm) preequilibrated in solvent A (0.115% trifluoroacetic acid and 5% acetonitrile). Approximately 3 nmol of the protein sample was injected into the column each time and eluted at 1 mL/min with a gradient of acetonitrile as follows: from 5% to 15.5% within 10 min, from 15.5 to 36.5% in 100 min, and a final elution for 10 min at 36.5%. The eluate, monitored at 214 nm, was collected in 1.0 mL fractions. One-tenth of each fraction was used for measuring the radioactivity in a Beckman LS-3801 scintillation spectrometer. The rest of the aliquot was used for peptide N-terminal sequence analysis at the Biomolecular Resource Center (BRC) of UCSF.

Labeling IMPDH with [8-^{14}C]IMP. The purified recombinant IMPDH (600 μg) was incubated at 24 °C with 0.4 mM [8-^{14}C]IMP (25 mCi/mmol) and 1.0 mM NAD in a 1.0 mL enzyme assay mixture for 10 s (Wu *et al.*, unpublished

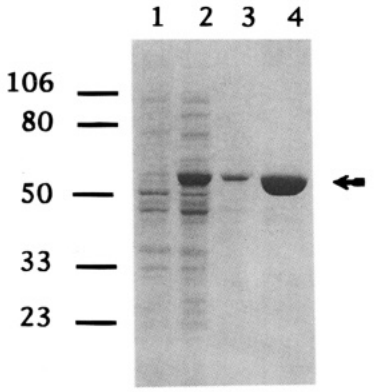

FIGURE 1: SDS−PAGE (12% polyacrylamide) stained with Coomassie blue. Lane 1, crude extracts of untransformed *E. coli* H712; lane 2, crude extracts of pBimpdh transformed *E. coli* H712 expressing *T. foetus* IMPDH; lane 3, the IMPDH sample from 45−75% ammonium sulfate fractionation; lane 4, the IMPDH sample from Mono Q column HPLC. Numbers on the left indicate the molecular mass (in kDa) of standard proteins.

Table 1: Purification of *T. foetus* IMPDH Expressed in *E. coli* H712[a]

fraction	vol (mL)	total act (units)	total protein (mg)	yield (%)	sp act (units/mg)	purification (n-fold)
crude extract	6.50	13295	63.3	100	210	1.0
45−75% ammonium sulfate	1.25	11756	34.0	88	346	1.6
Mono Q	4.50	8771	3.8	65	2308	11.0

[a] This batch of enzyme was derived from 1 L of mid-logarithmic phase cultures of transformed *E. coli*.

experiments). The enzyme protein was then precipitated with 10% TCA, denatured in 8 M urea, digested with trypsin, and analyzed by HPLC as previously described.

Protein Analysis. Protein concentration was determined with the Bio-Rad Bradford protein assay using IgG as a standard. The purity of the enzyme sample was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) in 12% gels as described by Laemmli (1970). Quantitation of the protein band stained in SDS−PAGE was by an LKB laser densitometer.

RESULTS

Expression and Purification of the Recombinant IMPDH. The crude cell-free extracts of the transformed *E. coli* H712/pBimpdh cells were analyzed in SDS−PAGE and compared with those from untransformed *E. coli* H712. The results presented in lanes 1 and 2 of Figure 1 demonstrate that the main difference between the two extracts lies in the presence of a major 58 kDa protein in the transformed cell extract. This protein, which constitutes about 10−20% of the total protein in *E. coli* H712/pBimpdh, is most likely the recombinant IMPDH from *T. foetus*. It remains mostly in the soluble fraction of the transformed bacteria, and can be purified to homogeneity in two simple steps of 45−75% ammonium sulfate fractionation and Mono Q HPLC. Data in Table 1 represent the typical outcome from one such purification effort. There was an 11-fold overall purification with a yield of 65%. The purified enzyme sample was examined in SDS−PAGE. Figure 1, lane 4, and a densitometric tracing analysis of the stained gel indicated 95% purity. This purified enzyme sample has a specific activity of 2308 units/mg, which agrees well with that of the purified authentic IMPDH from *T. foetus*, estimated at 2550 units/mg (Verham *et al.*, 1987). This current system of expression yields about 3 mg of pure IMPDH from a liter of *E. coli* culture. The specific activity of IMPDH in the crude extracts of transformed *E. coli* is about 100 times higher than that found in the crude extracts of *T. foetus*, which should facilitate considerably our future investigations on this enzyme protein.

Kinetic Studies on the Recombinant IMPDH. The steady-state kinetics of the *T. foetus* IMPDH-catalyzed reaction were reexamined using the purified recombinant enzyme. By the same experimental procedures as described previously with 70 nM pure recombinant enzyme (Verham *et al.*, 1987), the K_m values for IMP and NAD are calculated to be 14 and 500 μM, respectively, in the present studies, which agree quite well with the original values of 18 and 340 μM from the native enzyme. The k_{cat} value is found to be 0.8 s^{-1}, which is somewhat lower than the 1.2 s^{-1} value for the authentic *T. foetus* IMPDH as well as the values of 1.5 and 1.3 s^{-1} for the human IMPDH types I and II, respectively (Carr *et al.*, 1993).

Mycophenolic acid, a well-known inhibitor of IMPDH (Snyder *et al.*, 1972), was retested on the recombinant *T. foetus* enzyme by the standard steady-state treatment with an excessive concentration of enzyme protein (350 nM) in the assay. It again inhibited in an uncompetitive manner with respect to both IMP and NAD with an estimated K_i value of 9 μM versus both IMP and NAD. It agrees well with the 9.4 and the 8.9 μM apparent K_i values derived previously from the authentic enzyme (Verham *et al.*, 1987). The K_i values versus IMP on the human enzyme, 37 nM for type I and 9.5 nM for type II IMPDH, are considerably lower (Carr *et al.*, 1993). This major discrepancy in sensitivities toward mycophenolic acid between the parasite and human IMPDH has constituted one of the main bases for our continued interest in studying the parasite enzyme (see Discussion).

Substrate Specificities of T. foetus IMPDH. 6-Thio-IMP, a known substrate for the IMPDH from *Aerobacter aerogenes* (Hampton, 1963; Hampton & Nomura, 1967), was identified also as a substrate for the parasite enzyme with an estimated K_m value of 34 μM and a V_{max} of 1.95 nmol/min. It acted also as a competitive inhibitor versus IMP with a K_i value of 70 μM. 2-Deoxy-IMP is another substrate with a K_m value of 9 μM and a V_{max} value of 1.0 nmol/min. 2′,3′-Dialdehyde-IMP, however, is not a substrate for the enzyme up to a very high concentration of 1 mM. Although this compound acts as an affinity label and irreversible inhibitor of hypoxanthine−guanine phosphoribosyltransferases (Kanaaneh *et al.*, 1994), it did not inactivate *T. foetus* IMPDH after prolonged preincubation with the enzyme. Apparently, this compound cannot be used as an affinity label for IMPDH.

Identification of Substrate Binding Amino Acid in the Active Site of T. foetus IMPDH. 6-Cl-IMP has been reported to inactivate *A. aerogenes* IMPDH and form a covalent adduct with a cysteine residue in the IMP binding site of *E. coli* IMPDH (Hampton & Nomura, 1967; Gilbert & Drabble, 1980). It was found to compete with IMP and result in inactivating human type II IMPDH while not being converted to 6-Cl-XMP in the presence of NAD (Antonino *et al.*, 1994).

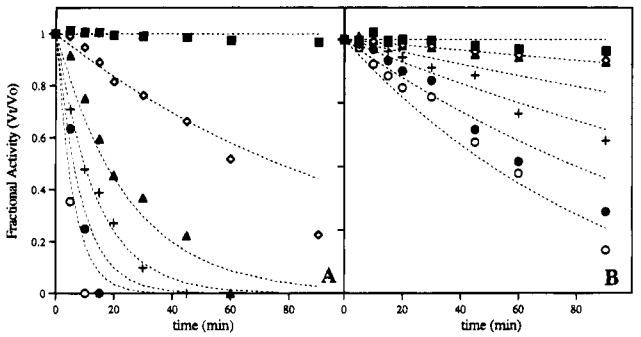

FIGURE 2: Inactivation of IMPDH by 6-Cl-IMP in the absence (panel A) and presence (panel B) of 100 μM IMP. Two micrograms of the recombinant enzyme was preincubated with varying concentrations of 6-Cl-IMP for different lengths of time and assayed for IMPDH activity. (■) No 6-Cl-IMP; (◊) 10 μM 6-Cl-IMP; (▲) 50 μM 6-Cl-IMP; (+) 100 μM 6-Cl-IMP; (●) 200 μM 6-Cl-IMP; (○) 400 μM 6-Cl-IMP. Data were fitted to eq 1 under Materials and Methods by nonlinear regression with the program Systat. Theoretical curves were calculated from the fitted values for K_i and k_i (see Results).

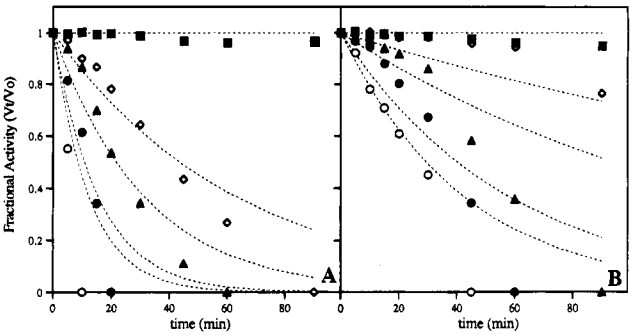

FIGURE 3: Inactivation of IMPDH by iodoacetamide in the absence (panel A) and presence (panel B) of 100 μM IMP. Reaction conditions were the same as in Figure 2, except that iodoacetamide was included in the preincubation instead of 6-Cl-IMP. (■) No iodoacetamide; (◊) 20 μM iodoacetamide; (▲) 50 μM iodoacetamide; (●) 200 μM iodoacetamide; (○) 500 μM iodoacetamide. Data were analyzed as described in Figure 2.

The adduct thus formed protected cysteine-331 from alkylation by iodoacetamide. In the current investigations, we preincubated *T. foetus* IMPDH (2 μg) with varying concentrations of 6-Cl-IMP ranging from 0 to 400 μM in a final volume of 20 μL for different lengths of time and assayed for IMPDH activities after an 100-fold dilution in the assay. The results, presented in Figure 2A, indicate that the enzyme is inactivated by 6-Cl-IMP in a time-dependent and drug concentration-dependent manner. 6-Cl-IMP is thus an irreversible inhibitor of *T. foetus* IMPDH. The dissociation constant K_i and the first-order rate constant of inactivation k_i, determined by fitting the data to eq 1 (see Materials and Methods), were 323 ± 100 μM and 0.303 ± 0.070 min^{-1}, respectively. When the two values are compared with the K_i of 78 ± 10 μM and the k_i of 0.21 ± 0.02 min^{-1} from human type II IMPDH (Antonino *et al.*, 1994), 6-Cl-IMP has apparently a lower affinity of binding to the parasite enzyme, but both enzymes appear to be inactivated by 6-Cl-IMP at similar rate constants. This irreversible inactivation can be, however, partially prevented by the presence of 100 μM IMP in the preincubation (Figure 2B). Thus, a total enzyme inactivation by 50 μM 6-Cl-IMP within 60 min can be completely prevented by the presence of 100 μM IMP (see Figure 2). Under these experimental conditions, 6-Cl-IMP has an estimated dissociation constant K_i of 532 ± 148 μM and an apparent rate constant of inactivation k_i of 0.023 ± 0.004 min^{-1}.

Similarly, when iodoacetamide was included in the preincubation instead of 6-Cl-IMP, at concentrations ranging from 0 to 500 μM, it also caused time-dependent and dose-dependent irreversible inactivation of the enzyme (Figure 3A). Iodoacetamide was estimated to have a K_i value of 103 ± 28 μM and a k_i value of 0.098 ± 0.018 min^{-1}. These results suggest that iodoacetamide has a greater binding affinity to IMPDH than 6-Cl-IMP, but inactivation proceeds at a slower rate. One possible explanation for it is that the crucial cysteine residue at the active site can be reached more readily by 6-Cl-IMP than iodoacetamide. The inactivation by iodoacetamide can also be partially blocked by 100 μM IMP (Figure 3B). The observed K_i and k_i values of iodoacetamide under such conditions are 162 ± 51 μM and 0.031 ± 0.005 min^{-1}, respectively. There is a similar

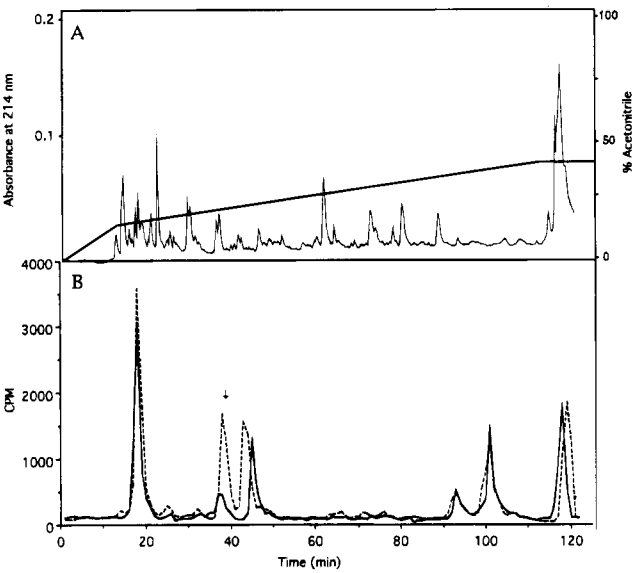

FIGURE 4: HPLC fractionation of the trypsin hydrolysate of IMPDH (300 μg) radiolabeled with 8 mM [1-^{14}C]iodoacetamide. Panel A: Profile of A_{214nm}. Panel B: Radioactivity profile of the trypsin hydrolysate of IMPDH with (solid line) and without (broken line) preincubation with 200 μM 6-Cl-IMP prior to treatment with radiolabeled iodoacetamide.

decrease of about 1.6-fold of binding affinities for 6-Cl-IMP and iodoacetamide when 100 μM IMP is present. This can be explained by assuming that each of the three substrates can bind to the same cysteine residue in the active site of IMPDH from *T. foetus*. This assumption is subsequently supported by the following experimental results.

In order to verify the assumption and identify the specific cysteine residue in the *T. foetus* enzyme involved in catalysis, 300 μg of the enzyme protein in 1.0 mL of solution was treated with 8 mM [1-^{14}C]iodoacetamide, denatured by 8 M urea, digested with trypsin, and analyzed in HPLC. The results shown in Figure 4B demonstrate that there are six radiolabeled peptide peaks in the protein digest. These results were compared with those from an enzyme sample preincubated with 200 μM 6-Cl-IMP at 37 °C for 2 h to ensure a total irreversible inactivation (see Figure 2A) prior to the [1-^{14}C]iodoacetamide treatment (Figure 4B). A single peptide peak migrating at about 48 min was identified in the HPLC analysis to be greatly diminished in its radiolabel when the sample had been pretreated with 6-Cl-IMP (Figure

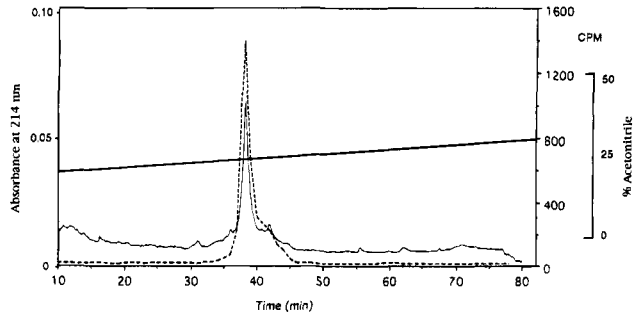

FIGURE 5: Second HPLC fractionation of the pooled fractions from Figure 4 (see the arrow in Figure 4B). Solid line, A_{214nm} profile; broken line, radioactivity profile.

4B). The fractions containing this peptide were collected from several identical HPLC runs of the sample without a 6-Cl-IMP pretreatment and purified by a second HPLC run (Figure 5). The N-terminal amino acid sequence of the purified peptide was analyzed by Edman degradation reactions. The results indicated an octapeptide sequence of IGIGGGSI(?)----- after 9 cycles of the reaction. The amino acid from cycle 9 was not identified. The yield for the first eight identified amino acids was 24, 10, 14, 10, 10, 8, 0.73, and 4.0 pmol, respectively. Among the individual fractions collected from the Edman reactions, only fraction 9 contained a substantial level of radioactivity, which also had the unidentified amino acid derivative. An analysis of the location of this particular peptide sequence in the entire protein of *T. foetus* IMPDH (Beck *et al.*, 1994) placed it at positions 311−318. Position 319 turns out to be a cysteine residue which was apparently carboxymethylated by the radiolabeled iodoacetamide in the present study. It is most likely the unidentified, radiolabeled amino acid derivative from cycle 9 of the Edman reactions. This cysteine residue was also protected by 6-Cl-IMP against carboxymethylation by iodoacetamide (see Figure 4B). By sequence alignment, Cys319 in *T. foetus* IMPDH is equivalent to the Cys331 in human IMPDH (Beck *et al.*, 1994), which was also found to be covalently modified by 6-Cl-IMP in previous studies (Antonino *et al.*, 1994).

In order to determine whether Cys319 in *T. foetus* IMPDH forms a covalent complex with IMP during the normal catalysis, the enzyme was incubated with [8-^{14}C]IMP and NAD in the assay mixture for 10 s. While the enzyme-catalyzed reaction was proceeding at a linear rate, the enzyme−substrate complex was precipitated with 10% TCA, denatured in 8 M urea, digested with trypsin, and analyzed in HPLC as previously described. The results in Figure 6A demonstrate that only a single peptide peak is radiolabeled. This radiolabeled peak was collected from several identical HPLC runs and subjected to a second HPLC elution as described above (Figure 6B). The purified peptide was sequenced in 10 cycles of Edman reaction with the following results: IGIGGGSI(X)I-----, with a yield for each individual amino acid of the following: 32, 28, 22, 28, 24, 15, 17, 8.7, (X), and 5.1 pmol, respectively.

A peak of radioactivity was recovered from fraction 9 containing the unidentified compound X. This unknown sample is thus most likely the adduct between [8-^{14}C]IMP and the Cys319 in *T. foetus* IMPDH, suggesting that the latter is modified by IMP as indicated for 6-Cl-IMP in the previous experiments. No [8-^{14}C]IMP labeling of the enzyme was detectable when NAD was omitted from the assay mixture.

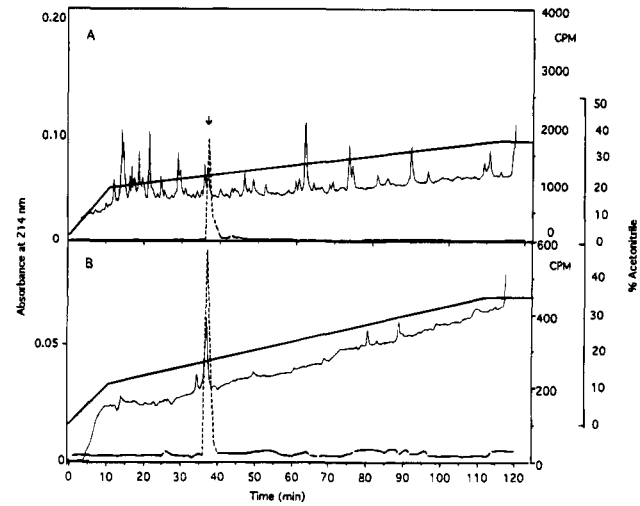

FIGURE 6: HPLC fractionation of the trypsin hydrolysate of IMPDH (600 μg) radiolabeled with 0.4 mM [8-^{14}C]IMP. Panel A: First round of fractionation. Panel B: Second round of fractionation of pooled radiolabeled fractions from panel A (see the arrow in panel A). Solid line, A_{214nm} profile; broken line, radioactivity profile.

Thus, we have obtained conclusive evidence of covalent bond formation between IMP and Cys319 during the enzyme-catalyzed reaction.

DISCUSSION

We have succeeded in our current studies to express the gene encoding *T. foetus* IMPDH in transformed *E. coli*, and the recombinant enzyme protein thus harvested and purified is apparently in the native form with a reasonably high yield. This success has already led us to crystallize the protein for X-ray analysis of its three-dimensional structure. Initial results from X-ray crystallography showed that the crystals belong to the cubic space group *P432* with a unit-cell edge of 157.25 Å (Whitby *et al.*, 1995). Complete native data sets have been collected to 2.3 Å resolution. Four putative heavy atom derivatives, apparently isomorphous with that of the native crystals, yielded complete data extending to 3.0 Å. It is anticipated that we should have the detailed three-dimensional structure of *T. foetus* IMPDH before long to compare with the current biochemical data.

In our previous investigations, we carried out thorough analysis on the steady-state kinetics of the reaction catalyzed by purified authentic *T. foetus* IMPDH (Verham *et al.*, 1987; Hedstrom & Wang, 1990). The results led to a proposal of possible mechanisms of the parasite enzyme-catalyzed reaction (Hedstrom & Wang, 1990). It postulates that a nucleophile must add to the 2-position of IMP before hydride transfer from the 2-position of IMP to NAD can occur. There are two likely candidates for this nucleophile: a cysteine thiol from within the active pocket of the enzyme or the OH^- from water. Subsequent investigations by Antonino *et al.* (1994) and Wu *et al.* (unpublished) indicating that both 6-Cl-IMP and IMP can specifically form a covalent adduct with Cys331 in the human type II IMPDH showed that Cys331 is the nucleophile in the human enzyme-catalyzed reaction. The present report provides another example of IMPDH which utilizes a cysteine thiol in the enzyme protein as the nucleophile to catalyze conversion of IMP to XMP. Although there is only a 34% sequence identity between the human and *T. foetus* IMPDH, the latter was also irreversibly

inhibited by 6-Cl-IMP, which in turn protected Cys319 of the enzyme from carboxymethylation by iodoacetamide. Furthermore, the NAD-dependent adduct formation between IMP and Cys319 has provided a direct demonstration that the latter is the nucleophile in the active site of *T. foetus* IMPDH. Although one cannot totally rule out the possibility of nonspecific IMP−enzyme complex formation during 10% TCA precipitation of the protein, it is unlikely in light of the fact that no such complex was formed in the absence of NAD. The relative ease in identifiying and isolating the IMP−enzyme covalent complex from the reaction mixture also suggests a slow rate of hydrolysis of the complex to produce XMP. This hydrolytic step must be partially rate-limiting in the IMPDH-catalyzed reaction. A closer examination of the protein sequences among the IMPDH's of various origins (Beck *et al.*, 1994) revealed the region surrounding this particular cysteine residue, GSICIT, to be remarkably well conserved. It is likely that this segment of the protein constitutes an important part of the active site with the cysteine residue in the peptide performing the crucial role of the nucleophile.

The present study has also confirmed one of our previous findings that mycophenolic acid is a much weaker inhibitor of *T. foetus* IMPDH when compared with human type II IMPDH (Verham *et al.*, 1987; Hedstrom & Wang, 1990; Carr *et al.*, 1993). Since mycophenolic acid is an uncompetitive inhibitor vs both IMP and NAD, mutually exclusive with NADH but capable of forming a ternary enzyme complex with ADP or adenosine diphosphate ribose (Hedstrom & Wang, 1990), it has been postulated that mycophenolic acid may bind to a transition state between E−IMP and E−XMP by partly occupying the dinucleotide binding site. Link and Straub (1995) have recently identified a human type II IMPDH-bound complex between IMP and the conserved Cys331 in the presence of mycophenolic acid, and thus provided experimental evidence in support of this hypothesis. With the availability of covalent complexes between 6-Cl-IMP and IMPDH from *T. foetus* or human, it should be possible to monitor the dissociation constants of mycophenolic acid binding to these two enzyme−inhibitor complexes and crystallize the ternary complexes between the enzyme−6-Cl-IMP complex and mycophenolic acid for X-ray diffraction analysis of the mycophenolic acid binding sites in *T. foetus* and human IMPDH (Whitby *et al.*, 1995). It is anticipated that there are significant discrepancies in this binding site between the two enzymes exploitable for designing specific inhibitors of *T. foetus* IMPDH.

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