Trapping of an IMP Dehydrogenase−Substrate Covalent Intermediate by Mycophenolic Acid

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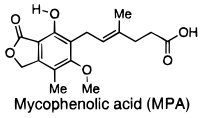
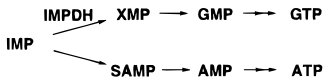
Mycophenolic acid (MPA), an antibiotic first isolated from a *Penicillium* culture in 1896, has been known as a potent inhibitor of inosine monophosphate dehydrogenase (IMPDH; EC 1.1.1.205) for over 25 years[1] (K_i < 10 nM, human IMPDH[2]). The branch-point enzyme IMPDH catalyzes the rate-limiting, committed, and penultimate step in *de novo* guanine nucleotide biosynthesis.[3] IMPDH catalysis proceeds through an ordered binding of substrates [inosine monophosphate (IMP) first, then nicotinamide adenine dinucleotide (NAD)] and an ordered release of products [NADH followed by xanthosine monophosphate (XMP)].[2,4] The fundamental role of IMPDH in cell proliferation has prompted widespread study of this enzyme and interest in modulation of its activity.[5] The antiproliferative and immunosuppressive action of MPA or derivatives has proven therapeutically valuable in the clinical treatment of psoriasis, rheumatoid arthritis, and allograft rejection.[6] Despite considerable study, conclusive evidence concerning the mechanism of IMPDH catalysis and the enzymatic species complexed by MPA has remained elusive.[4,7] The results we describe here provide evidence elucidating the mechanism of IMPDH catalysis and the mechanism of IMPDH inhibition by MPA.





Denaturation of IMPDH in the presence of saturating concentrations of NAD, ^{14}C8-IMP, and an inhibitory concentration of MPA, followed by trypsinolysis and analysis by high-performance liquid chromatography (HPLC), revealed a single tryptic fragment T38*(Figure 1A) not present in a control trypsinolysis of IMPDH alone. Electrospray mass spectral analysis showed that this modified peptide is derived from the enzyme−substrate (E−S) covalent adduct **3** (Figure 2A). Data for T38* is consistent with a structure bearing a covalent bond between the sulfur of ^{331}Cys and C2 of the substrate purine ring (T38*, Figure 1B).[8,9] Under these experimental conditions[10] the covalent intermediate is present as the major enzymatic species; the ratio of T38* to the peptide T38 (T38 is the corresponding peptide containing ^{331}Cys from the native enzyme) is 85:15 (Figure 1A). Control experiments without NAD or without IMP afforded no labeled peptide T38*. Thus MPA prevents the hydrolysis of an IMPDH−substrate covalent intermediate.

In a parallel experiment without MPA,[10] 3% of T38* was isolated relative to the quantity of T38* found in the presence

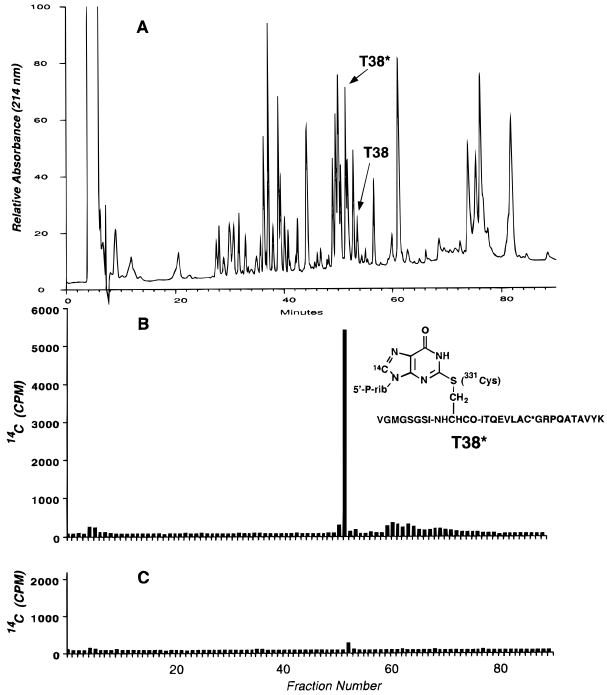


Figure 1. HPLC traces of tryptic digests containing covalent substrate−peptide T38*. (A) HPLC chromatogram of the tryptic digest derived from incubation of IMPDH, ^{14}C8-IMP, NAD, and MPA.[10] The ratio of modified peptide T38* to T38 (unmodified native peptide) is 85:15 (peak absorbance 214 nm). (B) Counts of 1 min samples from the HPLC effluent of trace A showed incorporation of radioactivity (derived from covalent linkage of ^{14}C8-IMP to ^{331}Cys) for the vial containing T38*. The structure determined for T38* is shown.[8] This modified peptide correlates to the E−S structure **3** in Figure 2A. The residue C* is a (aminocarbonyl)methyl cysteine. (C) Counts of samples from the HPLC effluent of a parallel experiment to that described in part A but without MPA.[10] This shows counts above background only for the vial containing T38* at 3% the magnitude of the counts in panel B. The presence of T38* was again confirmed by MS analysis. Due to turnover of the enzyme, IMP is not at saturating levels without the presence of inhibitor at this 15 min incubation.

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(7) Antonino, L. C.; Wu, J. C. *Biochemistry* **1994**, *33*, 1753−1759.
(8) The indicated peptide, T38*, had an observed MW of 3143.7 ± 0.7 Da (predicted 3144 Da) (in a separate experiment with ^{12}C8-IMP the mass was 3141.5 ± 0.7 Da (predicted 3142 Da)) and an N-terminal sequence of VGMGSGSI with a blank cycle at position 9 (catalytic ^{331}Cys) and greatly reduced signals for released PTH amino acids in subsequent cycles via Edman microsequencing. Tandem MS experiments confirmed that ^{331}Cys had been converted to a residue with a mass of 449 Da ± 0.1 Da (predicted 449 Da for Cys + IMP − 2H) and that ^{339}Cys was present as a (aminocarbonyl)methyl sulfide. MS techniques allowed the assignment of all major components observed in the tryptic digest of IMPDH and accounted for >96% of the predicted sequence of the enzyme. The absorption spectrum of T38* isolated by HPLC showed a λ_{max} of 248 nm, as is typical for C2-thioalkyl inosines; see: Wong, C. G.; Meyer, R. B., Jr. *J. Med. Chem.* **1984**, *27*, 429−432. The mass measurements, UV spectrum, and requirement of NAD for the formation of T38* are consistent with the structure of T38* presented in Figure 1B corresponding to covalent intermediate **3** in Figure 2A, and are not consistent with the isolation of the tetrahedral intermediate **2** from Figure 2A.
(9) A nucleophilic cysteine (^{331}Cys, human type II IMPDH) has been identified that reacts with the affinity labeling agent 6-chloro-IMP; see: (a) Brox, L. W.; Hampton, A. *Biochemistry* **1968**, *7*, 2589−2596. (b) Antonino, L. C.; Straub, K.; Wu, J. C. *Biochemistry* **1994**, *33*, 1760−1765.
(10) IMPDH II[2](10 μM), ^{14}C8-IMP (200 μM, 56mCi/mmol, Moravek Biochemicals Inc.), NAD (400 μM), and MPA (10 μM) were incubated in aqueous Tris (50 μM), KCl (50 μM), EDTA (1.5 mM), and 1% DMSO (enzyme added last, 200 μL final volume) for 15 min at 23 °C. The protein was precipitated with trichloroacetic acid, centrifuged, washed with acetone, dissolved (8 M urea, pH 8, NH_4HCO_3), treated with excess dithiothreitol and iodoacetamide, and digested with trypsin (7.5 μL, 0.4 mg/mL, 20 h, 37 °C). To demonstrate covalent intermediate formation in the uninhibited pathway, an experiment without MPA but otherwise identical to that above was performed. On-line HPLC-MS and tandem MS were carried out as in ref 9b.

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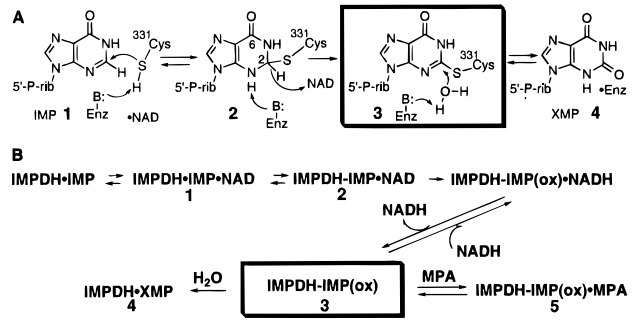

Figure 2. (A) The proposed steps leading to formation of E−S covalent intermediate **3** and its hydrolysis in the catalytic mechanism of IMPDH. E−S covalent intermediate **3** is isolable and is the target of the inhibitor MPA. MPA complexes thiopurine intermediate **3**, preventing its hydrolysis to form the product XMP. (B) The ordered binding scheme of IMPDH catalysis and inhibition by MPA. The numbered species correlate to the numbered structures in part A. This scheme is consistent with the ordered kinetics measured for IMPDH and the uncompetitive inhibition pattern of MPA. After release of NADH,[13] MPA binds to covalent intermediate **3**, forming E−S covalent intermediate·MPA complex **5**, and prevents XMP formation. Uncompetitive inhibition by MPA is a consequence of ordered binding.

of MPA (Figure 1C). Covalent intermediate **3** is not uniquely formed due to the presence of MPA, since this same intermediate is formed in the uninhibited turnover of IMPDH. This result suggests that MPA does not divert IMPDH along an unnatural mechanistic pathway, but instead prevents consummation of the normal catalytic cycle. We propose that IMPDH follows the covalent catalytic pathway detailed in Figure 2A. Isolation and characterization of the covalent E−S intermediate in the IMPDH catalytic pathway derived from the enzyme undergoing turnover with its natural substrates IMP and NAD constitute strong evidence to support the mechanism proposed in Figure 2A and avoid the ambiguities associated with the use of unnatural substrates in enzyme mechanism studies.[11]

We have measured near single turnover of NAD to NADH in the trapping of the covalent E−S intermediate by MPA, and we have identified the enzymatic intermediate species to which MPA binds; NADH is not complexed to the MPA-trapped E−S covalent intermediate. Ultrafiltration isolation of the MPA-trapped E−S covalent intermediate, and HPLC analysis[12] of the filtrate revealed a quantity of the product NADH that was 1.8 times the molar quantity of IMPDH used for complex formation. This measurement indicates that MPA interrupts catalysis mostly within the first catalytic cycle of IMPDH. Denaturation of this isolated protein complex followed by ultrafiltration and HPLC analysis showed no presence of NADH in the filtrate; denaturation of the inhibited complex does not release NADH.[13] This demonstrates that the MPA-inhibited enzymatic species is not bound to NADH [*i.e.*, IMPDH−IMP(ox)·NADH·MPA], but is the binary complex IMPDH−IMP(ox)·MPA. These results are consistent with the ordered kinetic scheme in Figure 2B where MPA prevents E−S hydrolysis by binding to the covalent intermediate **3** after decomplexation of NADH. The specific binding of MPA to this idiosyncratic species **3** in the IMPDH catalytic pathway may play an important role in the selectivity of inhibition of IMPDH relative to the inhibition of other dehydrogenases by MPA *in vivo* and could be a critical factor contributing to the clinical efficacy of MPA or its derivatives.

Inhibition by MPA is uncompetitive relative to both IMP and NAD; *i.e.*, MPA inhibition is not overcome by an increase in concentration of either substrate.[2,4] Our results reveal the mechanistic event that is the basis for this uncompetitive inhibition; MPA binds to the IMPDH−IMP covalent intermediate **3** and thereby does not compete with substrate binding.

The molecular details concerning how MPA prevents the hydrolysis of the E−S covalent intermediate are still not understood. The vague structural similarity of MPA to the nucleotides IMP or NAD has prompted speculation concerning the location of the MPA binding site in IMPDH.[4,5a,14] Since NADH is not bound to the E−S·MPA complex **5** (Figure 2B), it is attractive to imagine that MPA binds in the nicotinamide mononucleotide portion of the NAD/NADH site and interferes with the hydrolysis of the nearby thiopurine. The uncompetitive inhibition pattern of MPA and multiple inhibitor studies[4] are consistent with this binding mode. Yet the present experimental information does not refute the possibility that MPA binds at a site distinct from the NAD binding region of IMPDH when disrupting the mechanism of covalent intermediate hydrolysis.

The trapping of the IMPDH E−S covalent intermediate by MPA exemplifies an intriguing mode of small molecule/protein interaction. Such inhibition of catalysis by the noncovalent binding of a small molecule to prevent the subsequent chemical reaction of a covalent E−S intermediate in an ordered kinetic scheme is precedented. Some choline analogs display uncompetitive inhibition kinetics and prevent hydrolysis of the acetylcholine esterase acyl-enzyme covalent intermediate.[15] Indeed, this mode of action for uncompetitive inhibitors of enzymes with covalent mechanisms may be widespread; the pursuit or even design of uncompetitive inhibitors that selectively bind to covalent E−S intermediates may prove to be a fruitful branch of drug development for enzyme targets of known or suspected covalent mechanism.[16]

Supporting Information Available: Electrospray mass spectrum, tandem mass spectral data, and absorbance mass spectrum (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(11) After our isolation and characterization of the IMPDH−IMP covalent intermediate **3** from human IMPDH, we communicated our results to Dr. John C. Wu. He subsequently collaborated with Wang *et al.* in repeating experiments analogous to ours but using *Tritrichomonas foetus* IMPDH and similarly observed covalent catalysis in the uninhibited turnover of this protozoan enzyme; see: Huete-Perez, J. A.; Wu, J. C.; Whitby, F. G.; Wang, C. C. *Biochemistry* **1995**, *34*, 13889−13894.

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(13) A solution of the inhibited complex (final volume 800 μL) was formed as described in footnote 10 but using non-radiolabeled IMP and a 10 min incubation at 23 °C. Ultrafiltration (Centricon 10 kDa filter, 3750 rpm, 20 min) and assay of the filtrate by HPLC[12] showed a concentration of 18 μM NADH (1.8 × enzyme concentration). Addition of 1% trichloroacetic acid (400 μL, 23 °C) to the protein complex remaining on the filter, centrifugation (3750 rpm, 30 min), and HPLC analysis showed the absence of NADH in the filtrate. Addition of 8 M urea (300 μL, pH 8, NH_4HCO_3) to the protein afforded two 150 μL homogeneous samples. Ultrafiltration of one sample and HPLC analysis again showed no NADH in the filtrate. Trypsinolysis and HPLC analysis of the remaining sample of denatured protein afforded a tryptic map with 85% T38* relative to 15% T38, demonstrating the expected presence of the E−S covalent intermediate.

(14) Prior proposals that MPA inhibition is through binding to IMPDH·XMP are contradicted by the demonstrated binding of MPA predominantly to the E−S covalent intermediate; see: Hupe, D. J.; Azzolina, B. A.; Behrens, N. D. *J. Biol. Chem.* **1986**, *261*, 8363−8369.

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