Conformational Changes and Stabilization of Inosine 5'-Monophosphate Dehydrogenase Associated with Ligand Binding and Inhibition by Mycophenolic Acid*

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The effects of substrate, product, and inhibitor (mycophenolic acid) binding on the conformation and stability of hamster type II inosine 5'-monophosphate dehydrogenase (IMPDH) have been examined. The protein in various states of ligand occupancy was compared by analyzing susceptibility to in vitro proteolysis, the degree of binding of a hydrophobic fluorescent dye, secondary structure content as determined by far-UV circular dichroism spectra, and urea-induced denaturation curves. These analysis methods revealed consistent evidence that IMPDH undergoes a local reorganization when IMP or XMP bind. NAD⁺ produced no such effect. In fact, no evidence was found for NAD⁺ binding independently of IMP. It is proposed that IMPDH adopts an open conformation around its nucleotide binding sites in the absence of substrates and that binding of IMP stabilizes a closed conformation that has a higher affinity for NAD⁺. The data also suggest the enzyme remains in the closed configuration throughout the catalytic steps and then reverts to the open conformation with XMP release, thereby consummating the enzyme cycle. Mycophenolic acid inhibition appeared to impart even greater stability. We propose that localized conformational changes occur during the normal and mycophenolic acid-inhibited reaction sequences of IMPDH.

Inosine 5'-monophosphate dehydrogenase (IMPDH; EC 1.1.1.205) catalyzes the conversion of IMP to XMP, the committed step in the de novo biosynthesis of guanine nucleotides (1-4). Now partially characterized from many organisms, IMPDH is well conserved with 35% or more sequence identity from bacterial and protozoan to human proteins (5, 6). Since the enzyme appears to be necessary for cellular replication, some view it as an attractive target for anti-microbial chemotherapy; however, others have recognized further therapeutic possibilities for IMPDH inhibition.

Human and hamster possess two closely related isoenzymes (designated types I and II). In both species the isoforms share 84% (of 514 residues) sequence identity (5). The existence of similar isoenzymes in mammals suggests a subtle distinction of roles. Many studies have shown that IMPDH activity is elevated in tumors and other actively proliferating tissues (3, 4, 7-15). In some cases the elevated activity seems to stem from induction of the type II isoenzyme (9, 11, 12), although similar up-regulation of both isoforms in activated T-lymphocytes has also been reported (14). These observations suggest that inhibition of IMPDH, or perhaps selective inhibition of individual isoforms, has potential for immunosuppressive or anti-neoplastic therapies. Indeed, inhibition of IMPDH in cultured cells results in decreased guanine nucleotide levels. Ultimately, this leads to the induction of differentiation in neoplastic cells (8) and the suppression of lymphocyte activation (16).

Mycophenolic acid (MPA), an uncompetitive inhibitor of IMPDH that was originally identified in certain Penicilium cultures (17), is a potent immunosuppressant, both in vitro and in vivo (for reviews, see Refs. 18-20). Mycophenolate mofetil, a morpholinoethylester pro-drug of MPA, has been developed as a therapy for organ transplantation. The drug has been approved in the United States and Europe for the prevention of acute rejection in kidney transplant recipients, but broader clinical use may be complicated by drug toxicity (for reports and reviews of clinical findings, see Refs. 21-24). Therefore, with the goal of increasing drug efficacy and decreasing toxic side effects, there is great interest in characterizing the inhibition of IMPDH by MPA and other compound classes.

Many species of IMPDH have been isolated, both from natural and recombinant sources. All possess a similar homotetrameric quaternary arrangement of about 55-57-kDa subunits, and the numerous enzyme kinetic characterizations (reviewed recently by Wu (19) and (25)) suggest a common mechanism that is unusual among dehydrogenases. In most other dehydrogenases substrate binding is either random or the hydride acceptor binds first (26). This binding often induces a large conformational change that prepares the enzyme for the catalytic events (27-30). In the case of IMPDH, catalysis proceeds via an ordered bi-bi kinetic mechanism wherein IMP (the hydride donor) binds first and NAD⁺ binds second. Thus, the substrate binding order is inverted relative to many other dehydrogenases. Following substrate binding and hydride transfer, IMPDH first releases NADH and, finally, XMP (31-36). Recent independent studies, in our laboratory (37, 38) and by others (39), have identified the probable mechanism of IMPDH inhibition by MPA. The inhibitor interrupts the normal enzyme turnover cycle, after both substrates have bound, after hydride transfer and after NADH release, by trapping the enzyme with a precursor of XMP still covalently bound to the enzyme active site cysteine. Our colleagues (38) have recently solved a high-resolution x-ray crystal structure of hamster type II IMPDH with MPA bound. This structure confirms that MPA traps a covalent adduct of IMPDH and a precursor of XMP. It also establishes a structural framework necessary to better understand the wealth of kinetic analyses already published.
However, no studies have yet investigated the effects of ligand binding on the conformation and/or stability of IMPDH. A better understanding of the dynamic behavior of IMPDH upon ligand binding should prove valuable for inhibitor design, especially in light of the inverted order of substrate binding.

We report the effects of substrate, product, and inhibitor binding on the conformation and stability of hamster type II IMPDH. The protein was examined in various states of ligand occupancy, using in vitro proteolysis, fluorescence quenching techniques, and circular dichroism (CD) spectroscopy. The urea-denatured denaturation of such samples was also examined using CD spectroscopy. These complementary analysis methods revealed consistent evidence that IMPDH is locally reorganized and stabilized by the binding of IMP or XMP, but not by NAD+. MPA inhibition appears to impart even greater stability. We propose that local conformational changes occur during the normal and MPA-inhibited reaction sequences of IMPDH.

EXPERIMENTAL PROCEDURES

Materials—Highest grade available IMP (free acid), NAD+, NADH (disodium salt), XMP (sodium salt), 6-chloro-IMP (sodium salt), and ANS (8-anilino-1-naphthalenesulfonic acid) were purchased from Sigma. MPA was obtained from Calbiochem. Proteinase K (from Tritirachium album, EC 3.4.21.14), α-chymotrypsin (bovine pancreatic, EC 3.4.21.7), and Glu-C (endoproteinase Glu-C, from porcine pancreas, EC 3.4.21.19) were obtained from Boehringer Mannheim. Elastase (porcine pancreatic, EC 3.4.21.36) was obtained from Worthington.

IMPDH Purification and Enzyme Parameters—Expression in Escherichia coli, purification, and enzymatic characterization of hamster type II IMPDH has already been described (37). The resultant protein appeared >99% pure as judged by SDS-PAGE (40) and N-terminal sequence analyses. Analytical size-exclusion chromatography and light-scattering measurements showed the protein was virtually monodisperse with a weight-average (Mw), number-average (Mn), and Z-average molecular weights of 228,300 and 227,800, respectively (Mw/Mn = 1.002). The enzyme contained undetectable levels of other oxidoreductase activities. The observed kcat for IMP and NAD+ were 22 and 37 μM, respectively. The observed Km for MPA (with respect to NAD+) was about 11 μM (37).

For the present study, the enzyme was stored at −70 °C at a concentration of 10 mg/ml in Buffer A (300 mM KCl, 50 mM Tris-HCl, 2 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol, pH 8.0, at 4 °C) prior to use. Enzyme activity was determined spectrophotometrically at 22 °C, by measuring the conversion of NAD+ to NADH via the increase in absorbance at 340 nm. Typical assays contained substrate concentrations of 300 and 250 μM for IMP and NAD+, respectively, and 100 μM IMPDH in Buffer A. Protein concentrations were determined by UV spectroscopy, using a specific molar extinction coefficient (A280) of 23,800 M−1 cm−1 (determined according to Ref. 41), using a molar extinction coefficient of 23,800 M−1 cm−1 for Tyr of 1400 M−1 cm−1. The final concentration of each protein was determined by the Bradford method (42) or by automated protein sequencer.

RESULTS

**IMPDH Sensitivity to In Vitro Proteolysis**—Fig. 1A presents a comparison, by SDS-PAGE, of the sensitivity of hamster IMPDH II to proteolysis by proteinase K, elastase, α-chymotrypsin, and Glu-C. For each enzyme, two proteolytic fragments were formed (apparent M, about 45,000 and 12,000) more rapidly, and at lower protease concentrations, than any products generated by other cleavage events (see below). These four proteases were chosen because they cleave at the C-terminal side of quite different recognition sequences (viz. proteinase K, N-substituted, hydrophobic aliphatic and aromatic residues; elastase, residues bearing uncharged nonaromatic side chains; α-chymotrypsin, Tyr, Phe, Trp, Leu, Met, Ala, Asp, and Glu residues; Glu-C, specifically Glu and Asp residues). This made it unlikely that the pattern would be dominated by protease sequence preferences. Instead, it suggests that IMPDH contains a well-exposed region about 12 kDa from either terminus. This is supported by the studies of Gilbert et al. (46), who showed rapid generation of two fragments (apparent M, about 42,000 and 14,000) upon treatment of E. coli IMPDH with trypsin or Pronase. The protease-sensitive region of hamster IMPDH II was identified by N-terminal sequencing. Consistent with the gel pattern, each protease yielded similar fragments. The larger fragment from elastase, α-chymotrypsin, and Glu-C proteolysis began at the authentic N terminus of the native IMPDH sub-
unit (with the N-terminal Met, resulting from E. coli expression, not present). Proteinase K digestion generated a large fragment beginning either at residue Gly-8 or Tyr-11 of the unproteolyzed subunit. To generate the small fragment (∼12 kDa) the proteases cleaved between Gln-427 and Asn-428 for proteinase K (lane 5), as described under “Experimental Procedures.” After incubation, the samples were compared by SDS-PAGE. B, ligands were added to IMPDH at a final concentration of 1 mM, as indicated in the figure. The samples were preincubated for 10 min prior to the addition of elastase and then proteolyzed for 60 min. The IMP + NAD’ + MPA sample (lane 7) originally contained 1 mM each of IMP, NAD’, and MPA. The IMP + NAD’ sample (lane 5) represents the uninhibited control, wherein IMP and NAD’ were originally added at concentrations designed such that unproteolyzed IMPDH would convert the substrates to XMP and NADH throughout the course of the protease incubation period. Standard molecular markers were run in lanes M, and the respective molecular masses (in kDa) are shown.

Time Course of Proteolysis of IMPDH—Fig. 2 presents the time dependence of elastase proteolysis of IMPDH, unprotected (Fig. 2A) and protected by IMP (Fig. 2B). Aliquots withdrawn at the indicated times were examined by SDS-PAGE. Standard molecular markers were run in lane M, and the respective molecular masses (in kDa) are shown.

Fig. 1. Protease sensitivity of IMPDH. A, IMPDH was incubated for 1 h without protease addition (lane 1), for 1 h with elastase (lane 2), for 1 h with Glu-C (lane 3), for 18 h with α-chymotrypsin (lane 4), and for 10 min with proteinase K (lane 5), as described under “Experimental Procedures.” After incubation, the samples were compared by SDS-PAGE. B, ligands were added to IMPDH at a final concentration of 1 mM, as indicated in the figure. The samples were preincubated for 10 min prior to the addition of elastase and then proteolyzed for 60 min. The IMP + NAD’ + MPA sample (lane 7) originally contained 1 mM each of IMP, NAD’, and MPA. The IMP + NAD’ sample (lane 5) represents the uninhibited control, wherein IMP and NAD’ were originally added at concentrations designed such that unproteolyzed IMPDH would convert the substrates to XMP and NADH throughout the course of the protease incubation period. Standard molecular markers were run in lanes M, and the respective molecular masses (in kDa) are shown.

Fig. 2. Proteolysis time course of IMPDH. IMPDH was incubated with elastase without added ligands (A) or in the presence of 1 mM IMP (B). Aliquots withdrawn at the indicated times were examined by SDS-PAGE. Standard molecular markers were run in lane M, and the respective molecular masses (in kDa) are shown.

Ligand-induced Conformational Changes in IMPDH for residues deemed less likely to be directly involved in ligand binding. As shown in Fig. 1B, IMPDH was much less sensitive to proteolysis by elastase in the presence of IMP (lane 3) or XMP (lane 8) than in the absence of added ligand (lane 2). By contrast, the enzyme was not protected by NAD’ (lane 4), and it was only marginally protected by NADH (lane 9). In equilibrium binding studies, Xiang et al. (25) saw no evidence for NAD’ binding to IMPDH and observed weak binding of NADH at 4 °C and no binding at 37 °C. When IMPDH was incubated with both IMP and NAD’, and the conditions were designed to allow the enzyme to convert substrates to products throughout the elastase incubation period (data not shown), IMPDH was again protected from proteolysis (lane 5). MPA was added in addition to IMP and NAD’ before proteolysis was started. As demonstrated previously (37–39), under these conditions the substrates are partially turned over, leaving free NADH and only IMPDH, MPA, and covalently bound XMP in the inhibited complex, as well as excess IMP and NAD’. The MPA-inhibited complex of IMPDH was considerably more resistant to elastase proteolysis than any other form of the enzyme, showing no evidence for cleavage (lane 7) under conditions that produced essentially quantitative cleavage of the unproteolyzed protein (lane 2). MPA alone did not lead to stabilization of IMPDH against proteolysis (not shown). IMPDH that had been completely inactivated by 6-chloro-IMP (data not shown), which forms a covalent adduct with the active site cysteine (31, 47), was not protected (lane 6).

The MPA-bound complex of IMPDH as a substrate for the protease.
that the proteolysis rate was much lower in the presence of IMP. The unprotected protein was almost completely proteolyzed in 45 min, whereas in the presence of IMP some full-length protein was detectable even after 10 h (see Fig. 4A, below). Fig. 3 shows the results of analyses of the IMPDH enzymatic activity of the same protected and unprotected samples undergoing proteolysis. These data demonstrate that this conspicuous internal cleavage event resulted in inactive enzyme. Densitometric scanning of stained gels, monitoring the disappearance of the full-length subunit, produced virtually identical decay curves (data not shown). It was also demonstrated, by combined size-exclusion chromatography and SDS-PAGE analysis, and light scattering experiments, that the two major fragments of proteolysis remained associated (results not shown). Therefore, inactivation resulted from the localized effects of the proteolytic cleavage and not from dissociation of motifs that together constitute the catalytic machinery.

Fig. 4 extends the proteolytic incubation period to show the greater stability of the MPA-inhibited sample. After 10 h about 20% of the full-length subunit was detectable in the IMP-protected sample (Fig. 4A), whereas about 70% remained uncleaved in the MPA complex (Fig. 4B). Over this time scale some band broadening of the larger fragment was seen for the unprotected protein (data not shown). Sequence analyses showed this was probably due to variable degradation of the C terminus. No further proteolysis was observed for the unprotected enzyme. By contrast, in the presence of IMP, and less prominently in the MPA-inhibited sample, were susceptible at another site (before residues Ala-223 or Arg-224 with elastase). Susceptibility to the second pathway only became evident with the ligand-induced attenuation of the first. Also, when unprotected IMPDH was rapidly proteolyzed by the first pathway, little evidence of the second pathway was later seen. Therefore, the two pathways seem mutually exclusive. The appearance of the second pathway may reflect direct conformational effects of IMP or MPA binding. However, it is also possible that cleavage via the first pathway made IMPDH unrecognizable by elastase via the second pathway. In either scenario, the IMPDH-IMP sample would be susceptible to both pathways (as observed) because of the equilibrium between free and IMP-bound protein.

**ANS Fluorescence in the Presence of Different Nucleotides—** IMPDH contains 17 tyrosine residues (per subunit) and no tryptophan residues, so ligand binding effects and conformational changes are not easily investigated by measuring intrinsic protein fluorescence. Hager et al. (48) analyzed the fluorescence changes in IMPDH upon binding of the competitive inhibitor mizoribine monophosphate, but the use of their approach did not result in significant fluorescence changes when MPA and the enzyme substrates and products were added (results not shown). Therefore, we analyzed the binding of ANS to IMPDH, in the presence of different ligands. The binding of ANS, a fluorescent hydrophobic dye, is often used as a measure of the amount of hydrophobic surface on proteins (49). As shown in Fig. 5, IMPDH had the same ANS fluorescence in the presence and absence of NAD+, whereas in the presence of IMP or XMP the intensity decreased by about 40%. These findings are consistent with the protection of IMPDH by IMP and XMP.
in the proteolysis experiments and the lack of protection by NAD$^+$. Interestingly, IMPDH that had been inactivated by 6-chloro-IMP had an ANS fluorescence intermediate between unliganded IMPDH and the IMP-bound form. The ANS fluorescence of the MPA-inhibited complex could not be interpreted because control samples containing NADH exhibited very high fluorescence, and NADH is generated (by partial substrate turnover) in the course of MPA inhibition.

Circular Dichroism Analysis of IMPDH—Fig. 6 presents the far-UV CD spectra of unliganded IMPDH, IMPDH with IMP, and MPA-inhibited enzyme (incubated with IMP, NAD$^+$, and MPA). These are the first CD analyses reported for IMPDH. All of the spectra were very similar. Addition of ligands did result in some minor perturbation of ellipticities, especially below 200 nm. The XMP spectrum is not shown because of unusual behavior below 186 nm (data not shown). We are investigating the cause of this, but we are presently unable to obtain reliable secondary structure data for that complex. The results of secondary structure analyses derived from the spectra are shown in Table I. The secondary structure content for the unliganded and IMP-bound forms of IMPDH is quite similar (about 30% $\alpha$-helix and 24% $\beta$-sheet/strand), whereas the MPA-inhibited complex has a slightly lower $\alpha$-helix (27%) and higher $\beta$-sheet/strand (30%) content. The proportions of the different secondary structure components, as calculated from the CD spectra, are quite consistent with the numbers calculated from the x-ray structure of the MPA complex (38). However, the x-ray structure has identified only about 83% of the residues in the protein.

Urea Denaturation of IMPDH—Urea-induced unfolding of IMPDH was analyzed by monitoring the magnitude of the ellipticity minimum at 225 nm, at different urea concentrations. This was done for IMPDH with and without the ligand combinations described already. After preincubating the IMPDH/ligand mixtures for 10 min at 22°C, the samples were made up to the selected urea concentrations and then incubated for a further 15 h (at 22°C) to allow complete equilibration. As shown in Fig. 7, all samples except the MPA-inhibited complex showed symmetrical, sigmoidal decreases in ellipticity as the urea concentration was increased. This behavior is usually interpreted in terms of a simple two-state denaturation process (50). Since IMPDH is a homotetramer, these curves probably describe the combination of denaturation and dissociation processes (other data not shown), so a detailed interpretation according to a two-state model may be inappropriate. Nevertheless, the urea concentration at the midway point of the transitional portion of such curves is often used as an indicator of relative protein stability (50). The midway point for native IMPDH occurred at 5 M urea. The shape and position of the curve was unaffected by the presence of NAD$^+$. In the presence of IMP or XMP the curve shapes still appeared the same, but the midway points occurred at 5.7 M urea. This suggests the protein is stabilized by the latter ligands. The MPA-inhibited complex appeared to be much more stable toward urea unfolding. Even at the highest urea concentration

![Graph 1](image1)

**Graph 1**. ANS fluorescence. ANS fluorescence spectra of IMPDH (●), IMPDH with 50 $\mu$M NAD$^+$ (○), IMPDH with 50 $\mu$M IMP (□), IMPDH with 50 $\mu$M XMP (▲), and IMPDH inactivated with 50 $\mu$M 6-chloro-IMP (▲) were recorded as described under “Experimental Procedures.”

![Graph 2](image2)

**Graph 2**. CD spectra of IMPDH. Spectra of IMPDH (●), IMPDH with 500 $\mu$M IMP (□), and IMPDH inactivated with 200 $\mu$M MPA (▲). See “Experimental Procedures” for details.

| Protein                  | $\alpha$-Helix | Anti-parallel $\beta$-sheet | Parallel $\beta$-sheet | $\beta$-Turns | Other | Total |
|--------------------------|----------------|----------------------------|-----------------------|---------------|-------|-------|
| No added ligand          | 30 ± 1         | 15 ± 1                     | 9 ± 1                 | 18 ± 1        | 28 ± 0 | 100   |
| +IMP                     | 31 ± 1         | 15 ± 2                     | 9 ± 1                 | 17 ± 1        | 28 ± 1 | 100   |
| MPA-inhibited complex    | 27 ± 2         | 19 ± 3                     | 11 ± 1                | 14 ± 3        | 29 ± 2 | 100   |
| X-ray structure of MPA-inhibited complex* | 32             | 24                         |                       |               |       | 30    |

* Percentages of the secondary structural elements present in the determined x-ray crystal structure of the MPA-IMPDH complex, consisting of 425 out of 514 residues (38).
Ligand-induced Conformational Changes in IMPDH

Conformational Changes during the IMPDH Substrate Binding Sequence—Controlled in vitro proteolysis is often used to probe protein conformation and stability, especially when direct structural information is lacking. This approach has been used in studies to map surface loops, domain boundaries, and protein-protein or protein-ligand interaction sites (e.g. bovine brain calcineurin (51)) that were later confirmed by high resolution structural studies (52).

Our studies identify the region around residues 412–441 of the IMPDH subunits as being particularly vulnerable to proteolytic cleavage. Proteolysis in this region proceeded far more rapidly than any other internal cleavage, but binding of IMP or XMP dramatically reduced the rate of proteolysis. Without a high resolution structure of the protein, the simplest interpretation for this would be that this region forms an open "loop" or "flap" on the surface of the enzyme when no ligands are bound and that the binding of IMP or XMP induces a localized conformational change. The ligand-bound configuration, in effect, closes this putative "loop" or "flap" region by causing it to adopt a more compact arrangement as it collapses around the nucleotide. No detailed structural information is available for IMPDH without ligands bound, but the x-ray crystal structure of IMPDH complexed with MPA and XMP, recently solved by our colleagues (38), supports the simple model presented above. The structure shows XMP is partially buried, with the hypoxanthine ring making multiple hydrogen bonds to a portion of the protein described as a "flap motif." This flap, as delineated by the x-ray structure (38), spans residues 400–450 and has a pair of β-strands at its borders (residues 406–413 and residues 443–448). It is striking that the portion of this flap that connects the β-strands (residues 414–442) almost exactly coincides with the region (residues 412–441) we have independently identified as the part of IMPDH most vulnerable to proteolysis in the absence of ligands and that binding of IMP or XMP greatly reduces its vulnerability.

In most dehydrogenases, binding of the hydride acceptor or cofactor either occurs first or the binding can proceed in random order (26). In such cases conformational changes induced by binding of the hydride acceptor are well documented (27–30). In a striking example of this, the structure of glycolate oxidase verges on a molten globule-like state in the absence of the co-factor, FMN (30). Binding of the co-factor induces widespread structural reorganization and stabilization, whereupon the enzyme active site residues are oriented optimally for catalysis. Our findings suggest that the hydride donor, IMP, induces local changes in a binding site flap of IMPDH. This may reflect a mechanism analogous to that in other dehydrogenases with an inverted order of substrate binding. NAD⁺-bound enzyme is not an intermediate in the IMPDH reaction mechanism, and our present studies show NAD⁺ did not stabilize IMPDH against proteolysis or urea denaturation in the absence of IMP. Indeed, we found no evidence, using any of the dissimilar analytical methods described herein, for NAD⁺ binding to the protein independently of IMP. Recently, Xiang et al. (25) have analyzed equilibrium binding of ligands to IMPDH and saw no evidence for binding of NAD⁺. Also, detailed sequence comparisons (37, 38) suggest that the IMP binding site of IMPDH, and the NAD⁺ binding site of dehydrogenases that bind NAD⁺ first, probably evolved from a common ancestral motif. Both substrates are nucleotides, so the structural inference seems plausible. All of this considered, we propose that the binding of NAD⁺ to IMPDH is facilitated by local conformational changes induced by IMP binding. Given that IMP and XMP binding invariably resulted in identical effects, we propose that the normal enzyme cycle is consummated by a reciprocal rearrangement of the enzyme upon release of XMP.

The results of ANS fluorescence and CD analyses are consistent with these contentions. The fluorescence experiments suggest a decrease in hydrophobic surface of IMPDH results when IMP or XMP bind. This could be due to steric effects where the ligands bury hydrophobic residues lining their common binding pocket, or a conformational change in the protein, or a mixture of both effects. 6-chloro-IMP irreversibly inactivates IMPDH by binding to the IMP site and forming a stable covalent bond from the 6-position of its purine ring to the enzyme active site cysteine (47). In the present study, IMPDH inactivated by 6-chloro-IMP showed fluorescence behavior intermediate between that of the unliganded protein and the IMP- or XMP-bound forms, yet 6-chloro-IMP gave no proteolytic protection (see below). These findings suggest that a conformational change in the protein contributed to the fluorescence decrease observed when IMP or XMP bind. The CD spectra show the ligands did not alter the overall characteristics of the protein conformation. Since the proposed conformational change could occur without a change in secondary structure content, our model is consistent with the results of the CD analysis that rule out a global rearrangement of the protein upon ligand binding.

Inactivation by 6-Chloro-IMP and Inhibition by MPA—We have proposed that IMP induces a closed conformation in IMPDH which persists throughout the reaction sequence until XMP release. In the context of this model, we surmise that the binding of 6-chloro-IMP leaves the enzyme in slightly different state than the enzymatically productive IMP-bound conformation. Unlike IMP and 2-chloro-IMP, which became linked via the 2-position of the purine ring to the active site cysteine (47), 6-chloro-IMP forms a covalent link from the 6-position. Perhaps this orients the substrate analog differently, making it unable to induce the same local changes that occur when IMP binds and assists NAD⁺ binding.

FIG. 7. Urea denaturation curves of IMPDH. The ellipticity at 225 nm was taken from corrected spectra of IMPDH samples incubated in varying urea concentrations, as a measure of the secondary structure integrity. The ellipticity values are plotted versus the urea concentration. Symbols: native IMPDH (+), IMPDH in 100 mM IMP (●), IMPDH in 100 mM IMPDH (□), IMPDH in 100 mM NAD⁺ (○), IMPDH in 100 mM XMP (■), and IMPDH inhibited by MPA (▲). The samples were preincubated with their respective ligands for 10 min at 22 °C prior to addition of urea. The MPA-inhibited sample was preincubated in 100 mM IMP, 100 mM NAD⁺, and 100 mM MPA. See “Experimental Procedures” for details.

used (8 M) the buffer-corrected ellipticity had not plateaued at a minimum value, indicating the protein was still not fully denatured and/or dissociated. To rule out the possibility of very slow unfolding and demonstrate the attainment of equilibrium conditions, the samples were re-analyzed after a further 24 h. These curves (data not shown) were virtually identical to those shown in Fig. 7.

DISCUSSION
Previous studies have shown MPA inhibits IMPDH by trapping a transient intermediate of the normal enzyme cycle, after hydride transfer and NADH release, but before a covalent bond with XMP is severed (37–39). The extreme proteolytic stability of MPA-inhibited IMPDH can be explained by the tight binding of MPA and further stabilization of the closed IMPDH-XMP complex. Whereas a somewhat modest but similar stabilization against urea denaturation was observed for both IMP and XMP, the MPA-inhibited complex was extremely stable. Only partial denaturation was achieved after 40 h incubation at 22 °C in 8 M urea. From these data we cannot determine a free energy of stabilization by binding of MPA or the other ligands, because more complete unfolding curves would be needed (50).

We are currently examining the effects of stronger chaotropic denaturants. Secondary structure analyses of CD spectra of IMPDH without bound ligands and of the MPA complex predicted the latter had slightly higher β-sheet/strand contents and slightly lower α-helix content than native IMPDH. The proportions of different secondary structure components, as calculated from the CD spectra, were quite consistent with the numbers calculated from the x-ray structure of the MPA complex (38) (see Table I). However, the x-ray structure only identifies about 83% of the residues in the protein. Because of this, and because a high resolution structure of unliganded IMPDH without bound ligands and of the MPA complex (38) (see Table I). However, the x-ray structure only identified the latter had slightly higher α-helix propensity and which lose α-helix propensity upon MPA-binding.

In summary, we show evidence that IMPDH adopts an open conformation around its nucleotide binding sites in the absence of substrates and that binding of IMP induces a conformation around its nucleotide bindingsite in the absence of substrate and that binding of IMP induces a conformation around its nucleotide bindingsite in the absence of substrate and that binding of IMP induces a transientintermediate of thenormal enzymecycle, afterhydride transfer and NADH release, but before a covalent bond with XMP is severed (37–39). The extreme proteolytic stability of MPA-inhibited IMPDH can be explained by the tight binding of MPA and further stabilization of the closed IMPDH-XMP complex. Whereas a somewhat modest but similar stabilization against urea denaturation was observed for both IMP and XMP, the MPA-inhibited complex was extremely stable. Only partial denaturation was achieved after 40 h incubation at 22 °C in 8 M urea. From these data we cannot determine a free energy of stabilization by binding of MPA or the other ligands, because more complete unfolding curves would be needed (50).

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