The Conformation of Inosine 5'-Monophosphate (IMP) Bound to IMP Dehydrogenase Determined by Transferred Nuclear Overhauser Effect Spectroscopy*

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IMP dehydrogenase (IMPDH) catalyzes the NAD-dependent synthesis of xanthosine 5'-monophosphate which is the rate-limiting step in guanine nucleotide biosynthesis. Although IMPDH is the target of numerous chemotherapeutic agents, nothing has been known about the conformation of the enzyme-bound substrates. The conformation of IMP bound to human type II IMP dehydrogenase has been determined by two-dimensional transferred nuclear Overhauser effect NMR spectroscopy at 600 MHz. NOE buildup rates were determined by recording NOESY spectra at numerous mixing times. The cross-relaxation rates determined from the initial NOE build-up rates were used to calculate inter-proton distances of bound IMP. The conformation of the enzyme-bound IMP was obtained by molecular modeling with energy minimization using the experimentally determined inter-proton distance constraints. The glycosidic torsion angle of the bound nucleotide is anti and the sugar is in the C2'-endo-conformation. This conformation places H2 of IMP, which is transferred to NAD in the reaction, in a position clear of the rest of the molecule in order to facilitate the reaction.

IMP dehydrogenase (IMPDH)† is a key enzyme in the biosynthesis of guanine nucleotides (1, 2) and is a target of numerous anticancer and antiviral drugs (3, 4). The steady state kinetic mechanism of the enzyme from various sources has been shown to be steady state ordered sequential BiBi in which IMP binds before NAD, and XMP is released before NADH (3, 5–7). Our previous study of the human tumor form of IMP dehydrogenase (human type II IMPDH or IMPDH-h2) extended this mechanism by analysis of the activation of the enzyme by various monovalent cations and proposed a steady state mechanism including the monovalent cation activator (8). In this mechanism, the enzyme binds K+ first, IMP second, and then NAD; the product NADH is released before XMP. Due to the central metabolic role of IMPDH, much effort continues to be devoted to studies of inhibitors such as mycophenolic acid and their biological effects (4, 9–14).

A preliminary crystallographic analysis of IMPDH from Tritrichomonas foetus was reported recently (15). Our previous work showed that there were four IMP-binding sites per 223-kDa IMPDH-h2 tetramer (8). In other studies, the IMP-binding site was shown to be near Cys-331 (16). Additionally, a covalent adduct of IMPDH-h2 with IMP was found in protein which had been incubated with substrates and the inhibitor mycophenolic acid and then denatured (17, 18). The conformations of the substrates (IMP and NAD) and products (NADH and XMP) bound to IMPDH have yet to be determined. Knowledge of these conformations is central to understanding substrate and inhibitor recognition and enzyme function.

One-dimensional transferred nuclear Overhauser effect (TRNOE) and two-dimensional transferred nuclear Overhauser spectroscopy (TRNOESY) are unique techniques used to determine the conformation of a small ligand molecule bound to a macromolecule or molecular assembly in solution (19–23). TRNOE and TRNOESY have been used to study structures of substrates, inhibitors, and coenzymes bound to numerous enzymes (24–32), peptides bound to membranes (33–35), peptides bound to proteins (36), and an antigen bound to antibody (37). TRNOE and TRNOESY methods have been shown to be very powerful in determining conformations of nucleotides bound to enzymes (25–29). The NOE cross-peaks for each proton pair of a nucleotide can be easily identified and inter-proton distances of the enzyme-bound nucleotide can thus be determined by their relative NOE intensities. A molecular model of the enzyme-bound nucleotide can then be constructed using an energy minimization molecular modeling program with the NOE determined inter-proton distance constraints. This report presents the determination of the conformation of IMP (Fig. 1) bound to IMPDH-h2 by the TRNOESY method and molecular modeling, showing that IMP adopts an anti conformation for the glycosidic bond and 1E pucker for the sugar ring.

EXPERIMENTAL PROCEDURES

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1 The abbreviations used are: IMPDH, inosine 5'-monophosphate dehydrogenase; IMPDH-h2, the human type II inosine 5'-monophosphate dehydrogenase isozyme; IMP, inosine 5'-monophosphate; XMP, xanthosine 5'-monophosphate; NOE, nuclear Overhauser effect; TRNOE, transferred nuclear Overhauser effect; TRNOESY, transferred nuclear Overhauser effect spectroscopy; HDO, hydrogen deuterium oxide.

D2O (99.9%), DCl (99% D), NAD, and the free acid of IMP were purchased from Sigma. Perdeuterated Tris-d11 (99%), dithiothreitol-d10 (98%), and glycero-d8 (99%) were purchased from CDN Isotopes Inc.

IMPDH-h2 Preparation—The construction of the IMPDH-h2 expression system was described in our previous report (8). The standard assay for the IMPDH-h2 activity was carried out in 100 mM Tris-HCl (pH 8.1) buffer at 37 °C with 0.2 mM IMP, 0.2 mM NAD, and 10 mM K+ as described before (8). The reaction rate was determined by the increase in absorbance at 340 nm.

The enzyme purification protocol was modified to use larger chromatographic columns. The crude cell lysate from a 2-liter culture (the supernatant collected after breaking the cells in a French press followed by centrifugation to remove cell debris) was loaded onto a Blue Sepha...
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rose CL-6B (Pharmacia Biotech Inc.) column (5 × 30 cm). IMPDH-h2 was eluted by a linear gradient of 20–500 mM KCl in 2000 ml of buffer A (20 mM Tris, 1 mM dithiothreitol, 20 mM KCl, pH 8.1, with HCl). Fractions with high IMPDH activity were collected and desalted by dialysis against buffer A (two changes of 1000 ml each). This solution was loaded onto a Q-Sepharose (Pharmacia) anion exchange column (5 × 30 cm) and eluted by a linear gradient of 100–700 mM KCl in 2000 ml of buffer A. The fractions with high IMPDH activity were collected and dialyzed against buffer B (20 mM Tris, 1 mM EDTA-Na4, 1 mM dithiothreitol, 20 mM KCl, pH 8.1, with HCl) with two changes of 1000 ml each. The purity of the final IMPDH-h2 solution was verified by SDS-gel electrophoresis and estimated to be at least 95%.

Sample Preparation for TRNOESY Measurements—For NMR studies, the buffer was exchanged to replace the buffer B of the IMPDH-h2 solution with perdeuterated NMR buffer (99.9% D2O containing 20 mM Tris-d11, 1 mM dithiothreitol-d10, 20 mM KCl, pH 8.1, with DCl); the exchange was carried out using a Centriprep-10 concentrator (purchased from Amicon Inc.) at 8 °C. An IMPDH-h2 solution was first concentrated to 0.5 ml. It was diluted with the NMR buffer to 5 ml and then concentrated to 0.5 ml. The dilution-concentration cycle was repeated three times. The IMPDH-h2 concentration of the final solution was determined by comparing its activity to the activity of newly purified enzyme. We have determined that each IMPDH-h2 tetramer has four active sites (8). The final active site or IMP-binding site concentration in the NMR sample was 0.11 mM.

The free acid of IMP was dissolved in water to 4 mM and was titrated with KOH to pH 8.1. H2O was removed from 5 ml of this IMPK+ solution under reduced pressure using a SpeedVac device (Savant Inc.). This dried IMPK+ was then redissolved in 0.5 ml of the IMPDH-h2 solution in NMR buffer. The [IMP]/[IMP-binding site] ratio was 36. Additional measurements were made at 4 mM IMP, 0.4 mM IMPDH-h2, and 2 mM IMP, 0.2 mM-IMPDH-h2 with analogous results to those described below.

NMR Measurements and Data Processing—A Bruker DMX600 NMR spectrometer and standard pulse programs were used. The solution of IMPK and IMPDH-h2 was placed in a 5-mm diameter NMR tube for NMR measurements. The sample temperature was maintained at 10 °C. NOESY data were collected in TSP340 mode with 256 t1 increments and 2,000 t2 points. Thirty-two scans were accumulated for each t2 value. The longitudinal relaxation times of the protons of IMP (assigned in their initial NOE build-up rate (Rij) relative to that of H1 and H2. The digital resolution is the same as that used in the TRNOESEY experiments.

The NOE build-up curves for each cross-peak were constructed and simulated with a polynomial equation using the SCIENTIST program (developed by MicroMath Scientific Software) to determine the initial NOE build-up rate, Rij, as described under "Results." The inter-proton distance, rij, is defined as the distance between H1 and H2. The uncertainty in distances was allowed without energy penalty. The torsion angles of the energy minimization model were determined from the lowest energy structure.

RESULTS

Fig. 2 shows the 600 MHz one-dimensional 1H NMR spectrum of a IMP and IMPDH-h2 mixture at the resolution used for TRNOESY measurements. The peaks at 8.44, 8.04, 5.91, 4.55, 4.24, and 4.09 ppm are assigned to H1, H3, H4, H5, H6, and H7, respectively (25). The peak at 3.72 ppm belongs to the residual HDO signal. The peaks are slightly broadened due to the slow tumbling of the 223-kDa protein.

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Fig. 3 shows the NOESY spectrum obtained at 200 ms mixing time. The diagonal peaks correspond to those seen in the spectrum in Fig. 2. Clear NOE cross-peaks can be seen for every proton pair except those between H4 and other protons. The NOESY spectra at shorter mixing times show the same characteristics but weaker NOE cross-peaks. The NOESY spectrum at 200 ms mixing time of a control sample without the protein showed no NOE cross-peaks. Spectra obtained at twice
the IMP concentration showed cross-peaks similar to those in Fig. 3. The volumes of the diagonal peak for H1 (5.91 ppm) at different mixing times were measured, and the nearly linear increase of the peak volume with decreasing mixing time was extrapolated to zero mixing time to obtain the NMR peak volume at zero mixing time. The volume of each NOE cross-peak at different mixing times was measured and the percentage NOE intensities ($I_{noe}$) were calculated by dividing NOE peak volumes by the NMR peak volume of H1 at zero mixing time. The NOE build-up curves for each cross-peak were constructed and simulated. The relation between NOE intensity ($I_{noe}$) and mixing time ($t_m$) can be described by Equation 2 (25):

$$I_{noe} = 1 - \exp(-R t_m)$$

(Eq. 2)

which can also be written as:

$$I_{noe} = R_{ij} t_m - \left(\frac{1}{2} R^2 t^2_m + \frac{1}{6} R^3 t^3_m + \ldots\right)$$

(Eq. 3)

where $R$ is the relaxation matrix which has elements, $R_{ij}$, that describe the initial build-up rate of the NOE peak between H$_i$ and H$_j$ (25).

In this study, the NOE peaks which showed a clear linear increase in their intensities with mixing time were simulated by Equation 4.

$$I_{noe} = R_{ij} t_m$$

(Eq. 4)

Those NOE peaks which showed deviation from the linear build-up at longer mixing times were simulated by Equation 5:

$$I_{noe} = R_{ij} t_m - a_{ij} t^2_m$$

(Eq. 5)

where $a_{ij}$ is a constant. The NOE peak between H$_i$ and H$_j$ partially overlapped with diagonal peaks of H$_i$ and H$_j$ (Fig. 3) and thus was simulated by Equation 6 to include the contribution from diagonal peaks as a constant $c_{ij}$.

$$I_{noe} = c_{ij} + R_{ij} t_m - a_{ij} t^2_m$$

(Eq. 6)

The build-up curves for the NOE peaks are shown in Fig. 4. The simulation parameters $R_{ij}$, $a_{ij}$, and $c_{ij}$ are listed in Table I. The distance of 2.90 Å between H$_{19}$ and H$_{29}$ was used as the internal reference since this distance is approximately independent of the ribose conformation. Other inter-proton distances ($r_{noe}$) were calculated using Equation 1 and the initial build-up rate $R_{ij}$. These distances are shown in Table I.

Molecular modeling with constrained energy minimization was used to construct the minimum energy model of IMP bound to IMPDH-h2. All the NOESY determined inter-proton distances, $r_{noe}$ in Table I, were used as distance constraints with approximately 10% variation allowed. The inter-proton distances ($r_{noe}$) of this model are also shown in Table I. The torsion angles are shown in Table II (39, 40). The inosine base adopts anti-orientation about the glycosyl C$_1$-N$_9$ bond relative to the sugar moiety. The sugar ring assumes nonplanar C$_2$-endo-conformation ($^\circ$E). The pseudorotation phase angle, $P$, is 166° calculated from Equation 7 (40).

$$P = \tan^{-1}[(n_1 + n_4 - n_5 - n_8)(2 n_2 \sin36^\circ + \sin72^\circ)]$$

(Eq. 7)
The angles $\phi$, $\psi$, and $\chi$ of IMP bound to IMPDH-h2 (166°) are derived from the energy minimization modeling only since there were no appropriate experimental constraints.

### DISCUSSION

The good fits of the simulated NOE build-up curves to the experimental data for NOE intensities at different mixing times in Fig. 4 and the small standard deviations of the parameters $R_{ij}$ indicate the high reliability of the NOE determined inter-proton distances $r_{\text{nue}}$. The inter-proton distances, $r_{\text{em}}$, of the energy-minimized model are generally very close to the experimental values $r_{\text{nue}}$. The difference between $r_{\text{em}}$ and $r_{\text{nue}}$ is within 20% for all the proton pairs of the sugar ring. In constructing the energy-minimized model, the magnetic interactions between IMP and the protein and the effects of the solvent were not included. This simplification may contribute to the deviations of some $r_{\text{em}}$ values from $r_{\text{nue}}$ (Table I).

No NOE cross-peaks were observed for a control sample of GMP without IMPDH-h2, or a sample with an equal amount of thermally-inactivated enzyme, which confirms that the observed NOE cross-peaks in the NOESY spectra (Fig. 3) of the IMP and IMPDH-h2 mixture are due to the binding of IMP to the enzyme. The NOE cross-peaks and the broadening of the NMR peaks indicate the existence of IMP-IMPDH-h2 complexes which are in fast exchange with free IMP and IMPDH-h2 molecules. Two mechanisms have been proposed for the IMPDH-catalyzed reaction (41, 42). One is a non-covalent mechanism which does not involve a bond formation between the enzyme and IMP. Another involves covalent catalysis with transient bond formation between the sulfur of Cys-331 and C2 of IMP (17); if this reaction occurs in the absence of NAD it must be rapidly reversible.

The sugar puckers of a nucleotide is defined by its pseudorotation phase angle (40, 45). In different enzyme-nucleotide complexes, the pseudorotation phase angle varies and the nucleotides take different sugar pucker (25–28, 43, 44). The pseudorotation phase angle of IMP bound to IMPDH-h2 (166°) is comparable to that of 2° puckering (162°) and the torsion angle $\theta_4$ ($-2°$) approximates 0° (40). Therefore, the sugar puck of the bound IMP is very close to envelope C2-endo (2E).

The strong NOE cross-peaks between H8 and protons in the sugar ring in the NOESY spectrum (Fig. 3) are direct evidence for an anti-conformation for the inosine base versus the sugar ring. The energy-minimized model of the bound IMP confirmed the anti-conformation. Similar anti-conformations are taken by enzyme-bound ATP and ADP in many other systems with many having torsional angles of $-50°$ comparable to that found here (25–28, 43, 44). For IMPDH the anti-conformation of the inosine base keeps H8 away from the bulk of the sugar moiety and opens it for binding and reaction and thus is consistent with the structural constraints required for substitution at C2.

As this article was being completed, a crystal structure of hamster IMPDH in a complex with the inhibitor mycophenolic acid and a covalent thioimidate adduct between the active site cysteine and C2 of oxidized IMP was published; this structure supports the anti-conformation of the glycosidic bond but suggests a C2-endo-conformation of the sugar (46). Whether the differences in sugar conformation are due to the differences in the complexes studied or to differences in experimental conditions remains to be ascertained.

Another enzyme that catalyzes a reaction at the C2 position of a nucleotide is the subsequent enzyme in the pathway of GMP synthesis, GMP synthetase. In this reaction, the oxygen at position 2 of XMP is replaced by a NH2 moiety. Unfortunately in the recently reported crystal structure of GMP synthetase, XMP was not found although it was essential for crystal formation (47). Thus the conformational similarities for substrate binding utilized by these enzymes remain unclear at present. It will be revealing to learn the similarities of substrate recognition for enzymes that catalyze these sequential reactions.

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