Inosine 5'-monophosphate dehydrogenase binds nucleic acids *in vitro* and *in vivo*

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Inosine 5'-monophosphate dehydrogenase (IMPDH) is the rate-limiting enzyme in the *de novo* biosynthesis of guanine nucleotides. In addition to the catalytic domain, IMPDH contains a subdomain of unknown function composed of two cystathione β -synthase domains. Our results, using three different assays, show that IMPDHs from *Tritrichomonas foetus*, *Escherichia coli*, and both human isoforms bind single-stranded nucleic acids with nanomolar affinity via the subdomain. Approx. 100 nucleotides are bound per IMPDH tetramer. Deletion of the subdomain decreases affinity 10-fold and decreases site size to 60 nucleotides, whereas substitution of conserved Arg/Lys residues in the

subdomain with Glu decreases affinity by 20-fold. IMPDH is found in the nucleus of human cells, as might be expected for a nucleic-acid-binding protein. Lastly, immunoprecipitation experiments show that IMPDH binds both RNA and DNA *in vivo*. These experiments indicate that IMPDH has a previously unappreciated role in replication, transcription or translation that is mediated by the subdomain.

Key words: cystathione β -synthase (CBS) domain, DNA-binding protein, inosine 5'-monophosphate (IMP) dehydrogenase, nucleic acid, RNA-binding protein.

INTRODUCTION

Inosine 5'-monophosphate dehydrogenase (IMPDH) catalyses the rate-limiting and first committed step of guanine nucleotide metabolism, the oxidation of IMP (inosine 5'-monophosphate) to XMP (xanthosine 5'-monophosphate) with the reduction of NAD. IMPDH controls the size of the guanine nucleotide pool, which in turn controls many physiological processes, including replication, transcription, signalling and glycosylation [1]. Inhibitors of IMPDH are antiproliferative, which makes the enzyme an important drug target for immunosuppressive, antiviral and cancer chemotherapy [2–5].

Intriguingly, IMPDH may have a more complex role in cellular regulation than generally appreciated. Virtually all IMPDHs contain a subdomain of unknown function in addition to the catalytic domain [6]. The catalytic domain is an α - β barrel of approx. 400 residues. The approx. 100 residue subdomain consists of two cystathione β -synthase (CBS) domains and protrudes from the opposite end of the barrel from the active site. The subdomain can be deleted with no effect on enzymic activity [7,8]. The presence of the subdomain indicates that IMPDH has another, as yet undefined, role in metabolism in addition to the production of XMP. Autosomal dominant retinitis pigmentosa has recently been attributed to mutations in the subdomain of human IMPDH type I [9,10], although the mechanism of this pathology is not understood.

We have serendipitously discovered that IMPDH binds to single-stranded nucleic acid with nanomolar affinity. Moreover, this nucleic-acid-binding activity involves the subdomain, and is the

first and only function assigned to this region. Further, we show that IMPDH is located in the nucleus of human cells and binds both RNA and DNA *in vivo*. While this work was in progress, another laboratory found that *Saccharomyces cerevisiae* IMPDH binds to telomeric sequences [11]. These observations suggest that IMPDH has a previously unappreciated role in RNA and/or DNA metabolism.

EXPERIMENTAL

Materials

All oligonucleotides were obtained from Qiagen Operon (Alameda, CA, U.S.A.). Heparin, poly(dT), poly(dU), poly(dA), poly(dC) and protein A–Sepharose were obtained from Sigma. [γ -³²P]ATP was obtained from PerkinElmer Life Sciences (Boston, MA, U.S.A.). Alkaline phosphatase was purchased from Roche (Indianapolis, IN, U.S.A.). Polynucleotide kinase, RNase One ribonuclease and RQ1 DNase were purchased from Promega (Madison, WI, U.S.A.). The cDNA for human IMPDH type I was a gift from Dr Beverly Mitchell (University of North Carolina, Chapel Hill, NC, U.S.A.).

Construction of *Tritrichomonas foetus* R212E (i.e. Arg²¹² → Glu²¹²)/R217E and K230E/R231E IMPDH double mutants

Site-directed mutagenesis was performed on the plasmid *pTfI* [12] using the QuikChange mutagenesis kit from Stratagene. The following primers and their exact complements were used

Abbreviations used: DTT, dithiothreitol; CBS, cystathione β -synthase; ChIP, chromatin immunoprecipitation; dT₆₀ etc., 60-mer (etc.) thymidine homo-oligonucleotide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IMP, inosine 5'-monophosphate; IMPDH, inosine 5'-monophosphate dehydrogenase; K_d , dissociation constant; NLS, nuclear localization signal; R212E etc., substitution of Arg²¹² with Glu²¹² etc.; SSB, single-stranded DNA-binding protein; ssDNA, single-stranded DNA; *Tf* Δ SD IMPDH, *Tritrichomonas foetus* IMPDH with the subdomain deleted; XMP, xanthosine 5'-monophosphate.

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to create the R212E/R217E and K230E/R231E double mutants respectively: 5'-CGTTATATCGTTTTCGAAAAAGATTATGACGAATCGCAAGTCTGTC-3' and 5'-CGAACTCGTCGATT-CGCAAGAAGAATATTTGGTCGGAGCAGG-3'. The nicked vector products were transformed into *Escherichia coli* ultra-competent XL-1 Blue cells. To ensure that no undesired mutations were introduced, the resulting IMPDH cDNAs were sequenced using the ABI PRISM 377 DNA Sequencer.

Protein purification

Human type II, *T. foetus* and *E. coli* IMPDHs, and *T. foetus* IMPDH with the subdomain removed (*Tf*ΔSD IMPDH), were purified as described previously [8,12–14]. The human IMPDH type I was expressed in *E. coli* using the pKK223-3 vector (Pharmacia, Piscataway, NJ, U.S.A.). The protein was purified using Cibacron Blue–Sepharose and IMP–Sepharose as described previously for human IMPDH type II [13]. The two double mutants of *T. foetus* IMPDH (R212E/R217E and K230E/R231E) were also expressed in *E. coli* and purified according to the same protocol as *T. foetus* IMPDH [12]. A His-tagged version of the subdomain of human type I IMPDH was expressed in *E. coli*, independently of the catalytic domain. The sequence (single-letter symbols) of this expressed construct is MRGSHHHHHG-MASMTGGQQMGRDPEPEFQANEVRKVKNFEQGFITDPV-VLSSHTVGDVLEAKMRHGFSGIPITETGTMGSKLVGIVTS-RDIDFLAEKDHTTLLSEVMTPRIELVVPAGVTLKEANEILQRSKKGLPIVNDCEVAIIARTDLKKNRNSKLDPAAN-KARKEAELAAATAQQ. This protein was purified using an immobilized-metal affinity chromatography column with an imidazole elution followed by an 80 % (w/v) ammonium sulphate precipitation. All protein preparations were > 95 % homogeneous as judged by SDS/PAGE.

ssDNA (single-stranded DNA) filter-binding studies

All experiments were performed in 50 mM Tris/HCl, pH 8.0, 1 mM DTT (dithiothreitol) and 50 mM KCl, unless otherwise indicated. Pools of ssDNA were assayed for binding to IMPDH from various sources using dot-blot filter binding. The sequence of ssDNA in the pool was GGGAAATGGATCCACATCTACGAATTC-N₃₀-TTCAGTGCAGACTTGACGAAGCTT, where N₃₀ denotes a random sequence 30 bases long. 5'-³²P-labelled ssDNA (2 nM) and protein were mixed in 10 mM Tris/HCl, pH 7.5, 50 mM KCl and 1 mM DTT for 10 min at 25 °C. The mixture was filtered on a vacuum manifold (Schleicher and Schuell, Keene, NH, U.S.A.) equipped with a piece of pure nitrocellulose membrane (Schleicher and Schuell) over a piece of Hybond (Amersham Biosciences, Piscataway, NJ, U.S.A.). The nitrocellulose membrane traps protein complexes and the Hybond traps free nucleic acids. The radioactivity bound to each filter was quantified to determine the ratio between free and protein-bound DNA. The fraction of nucleic acid bound to the nitrocellulose was fitted to the following equation using Sigmaplot (ISI Researchsoft):

$$f = \frac{R[\text{IMPDH}]}{K + [\text{IMPDH}]} + B \quad (1)$$

where f is fraction of nucleic acid bound, R is the maximum specific bound fraction, K is the binding constant, and B is the fraction bound to the membrane in the absence of protein. Dot-blot analysis of the *T. foetus* IMPDH double mutants was performed in a similar manner but using 100-mer oligonucleotide (dT₁₀₀).

Binding monitored by intrinsic protein fluorescence

T. foetus IMPDH (30 nM) and *Tf*ΔSD IMPDH (25 nM) were titrated with oligonucleotides or double-stranded DNA. Fluorescence was observed with a Hitachi Instruments F-2000 fluorimeter (San Jose, CA, U.S.A.). The excitation and emission wavelengths were 295 nm and 330 nm respectively. This excitation wavelength was chosen to avoid inner filter effects from the oligonucleotides. The fluorescence quenching fitted a simple binding function as described in Equation 1. A similar experiment was performed with high concentrations of *T. foetus* IMPDH (0.40 μM) and *Tf*ΔSD IMPDH (0.9 μM) to determine the binding site size.

UV cross-linking binding assay

The cross-linking assay was modified for use with IMPDH [15]. IMPDH (200 nM) from various sources was mixed with 5'-³²P-radiolabelled dT₁₀₀. This mixture was UV-irradiated for 1 h with a hand-held UV lamp (model UVGL-25 mineral lamp), then separated by SDS/PAGE. The gels were exposed on Molecular Dynamics phosphor screens (Amersham Biosciences) that were scanned with the Molecular Dynamics Storm 840 reader.

Enzymic activity

T. foetus IMPDH was incubated in the presence and absence of saturating dT₁₀₀ (300 μM). This enzyme was added to assay solution containing 10 mM Tris/HCl, pH 8.0, 1 mM DTT, 5 mM EDTA, 50 mM KCl and sub-saturating amounts of substrate (10 μM IMP and 200 μM NAD⁺). The production of NADH was followed at 340 nm using a Hitachi Instruments U-2000 spectrophotometer.

Cellular localization

Confluent human cells (K562, HeLa, PC3) were fixed with 3.7 % paraformaldehyde on to slides and permeabilized with 0.1 % Triton X-100. The primary antibody, anti-human IMPDH polyclonal rabbit antiserum, was incubated with the cells at 37 °C for 1 h. Anti-rabbit antibody conjugated to rhodamine was then incubated with the cells for 30 min at 37 °C. The cells were co-stained with Hoechst stain and viewed with an Olympus IX70 inverted fluorescence microscope (Melville, NY, U.S.A.).

Immunoprecipitation and digestion of nucleic acid

Nucleic acid bound to IMPDH was immunoprecipitated from K562 human cells with anti-human IMPDH antisera. Confluent human cells were cross-linked with 0.8 % formaldehyde for 20 min, quenched by 0.14 M glycine at 22 °C for 5 min, lysed at 4 °C for 30 min in RIPA buffer (10 mM Tris/HCl, pH 8, 140 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 1 % deoxycholic acid, 1 mM PMSF) and sonicated. The supernatants were pre-cleared with Protein A–Sepharose and incubated with pre-immune serum, anti-IMPDH antiserum or 3.4 μg of purified recombinant human type I IMPDH followed by anti-IMPDH antiserum and precipitated with Protein A–Sepharose. The immunoprecipitate was washed extensively, eluted at 65 °C with 1 % SDS, and the cross-links were reversed by incubating at 65 °C overnight. The resulting samples were treated with alkaline phosphatase to remove 5'-phosphates and followed by polynucleotide kinase/[γ-³²P]ATP to label the nucleic acid. Each sample was split into three: a control, treated with RNase One, or treated with RQ1

DNase. The nucleic acid was isolated by filter binding on a Hybond membrane and exposed as described above. Radioactivity (expressed as intensity) was compared relative to the mean of each anti-IMPDH antiserum (at least three replicate experiments were performed). The data from three independent experiments were combined and the standard errors of the mean were calculated. The significance of the difference between two groups was analysed using a two-tailed *t* test analysis assuming unequal variance.

ChIP (chromatin immunoprecipitation) experiments

A modified ChIP experiment was performed to demonstrate that human IMPDH binds DNA *in vivo*. Human cells were labelled at approx. 70 % confluency for 6 h with 1 μ Ci/ml 2 Ci/mmol [*methyl*-³H]thymidine. The cells were cross-linked with 0.8 % formaldehyde for 15 min, then were lysed at 4 °C for 1 h using 1 % Triton X-100, 0.1 % SDS and 1 % deoxycholic acid. The DNA was sheared using a micro-sonicator with 9×10 s pulses at 80 % power. The extracts were pre-cleared with Protein A-Sepharose for 1 h at 4 °C. The extracts were treated with anti-human IMPDH polyclonal antiserum for 3 h, then precipitated with Protein A-Sepharose. The immunoprecipitate was eluted with 1 % SDS and radioactivity was counted with a scintillation counter. Alternatively, the immunoprecipitate was incubated at 65 °C overnight to remove the cross-links and treated with phenol/chloroform to remove protein. The putative nucleic-acid fraction was treated with alkaline phosphatase to remove 5'-phosphates. Alkaline phosphatase was removed by phenol/chloroform extraction, and the nucleic acid was precipitated with ethanol and NaCl. The nucleic acid was labelled with [γ -³²P]ATP and polynucleotide kinase, and the protein was removed by phenol/chloroform extraction and ethanol precipitation. The resulting nucleic acid was treated with RNase or DNase and analysed on an 8 % denaturing acrylamide gel.

RESULTS

IMPDH binds ssDNA in a filter-binding assay

We observed that IMPDH bound to nucleic acids with nanomolar affinity in the course of aptamer selection. Typical filter-binding assays are shown in Figure 1. IMPDHs from *E. coli* and *T. foetus* and both human isoenzymes (type I and type II) bind a random pool of 80-mer oligonucleotides, suggesting that nucleic-acid binding is a general property of IMPDH. While the *E. coli* and *T. foetus* enzymes bind approx. 75 % of the random pool, the two human isoenzymes bind only 10–20 % (approx. 10^{11} sequences). This observation indicates that the human isoenzymes are more specific than the microbial enzymes. For comparison, thrombin, a protease that does not have a role in nucleic-acid metabolism but does have two positively charged exosites, binds only 0.01 % of a random oligonucleotide pool in similar aptamer selection experiments [16].

The data are described by a simple binding function (Equation 1). The values of K_d (dissociation constant) for nucleic-acid binding to the various IMPDHs are: *T. foetus*, 2.3 ± 0.5 nM; *E. coli*, 8 ± 2 nM; human type I, 8 ± 7 nM; and human type II, 20 ± 4 nM (note that these concentrations refer to tetramers of IMPDH). *T. foetus* IMPDH also binds a 100-mer of poly(dT) (dT₁₀₀) with high affinity ($K_d = 2$ nM, Figure 1). These values are lower than or comparable with those reported generally for other single-stranded nucleic-acid-binding proteins, which suggests that the nucleic-acid-binding activity of IMPDH is physiologically relevant (2–200 nM; [17–19]).

Figure 1 IMPDH binds ssDNA in a filter-binding assay

5'-³²P-labelled ssDNA was incubated with varying concentrations of IMPDH from different organisms (IMPDH concentration is given in tetramers). The fraction bound was measured as described in the Experimental section. The lines represent the best fits of the data to a simple binding model (Equation 1). (A) A random pool of ssDNA (0.2 nM, sequence: 5'-GGGAATGGATCCACATCTACGAATTC-N₃₀-TTCACTGCAGACTTGACGAAGCTT-3'); human type I IMPDH (●); human type II IMPDH (○); *T. foetus* IMPDH (□); and *E. coli* IMPDH (■). (B) A random pool of ssDNA as above (2 nM): wild-type *T. foetus* IMPDH (□); *Tf*ΔSD IMPDH (●); and human type I IMPDH subdomain (○). (C) dT₁₀₀ (4 nM): wild-type *T. foetus* IMPDH (□); R212E/R217E (●); and K230E/R231E (○).

Nucleic acids can be UV cross-linked to IMPDH

In order to further demonstrate that IMPDH binds nucleic acid, IMPDH was incubated with 5'-³²P-labelled dT₁₀₀ and irradiated with UV light for 1 h. This treatment cross-links *T. foetus* IMPDH to the dT₁₀₀ as can be seen by comparing lanes 1 and 2 of Figure 2 (left-hand panel). A high-molecular-mass band appears (> 200 kDa), which is consistent with the molecular mass of a tetramer of IMPDH plus oligonucleotide. The high-molecular-mass band decreases in the presence of increasing concentrations of unlabelled dT₁₀₀ (Figure 2, right-hand panel). The simplest explanation for this observation is that a single oligonucleotide

Figure 2 UV cross-linking of oligo(dT) to IMPDH from various sources

$5'$ - 32 P-labelled dT₁₀₀ is UV cross-linked to IMPDH as described in the Experimental section. Left-hand panel: lane 1, dT₁₀₀ alone; lane 2, dT₁₀₀ + *T. foetus* IMPDH; lane 3, dT₁₀₀ + *Tf*ΔSD; lane 4, dT₁₀₀ + human type II IMPDH; lane 5, dT₁₀₀ + human type I IMPDH; lane 6, dT₁₀₀ + human type I His-tagged subdomain; and lane 7, dT₁₀₀ + *E. coli* IMPDH. Middle and right-hand panels respectively: *T. foetus* IMPDH (WT) and *Tf*ΔSD (Δsubdomain) were incubated with $5'$ -[32 P]-dT₁₀₀ and 0, 3, 6, 12 and 50 μ M unlabelled dT₁₀₀ in 50 mM Tris/HCl, pH 8.0, 1 mM DTT and 50 mM KCl at 0 °C.

cross-links all four subunits. This conclusion is substantiated in the site size experiments described below. No cross-linking was observed to the two human IMPDH isoenzymes, although it would be incorrect to conclude that these enzymes do not bind dT₁₀₀ (Figure 2, lanes 4 and 5). Some cross-linking is observed in the *E. coli* IMPDH reaction, but the band is smeared and more consistent with a monomer (Figure 2, lane 7). These variations in cross-linking patterns probably reflect differences in the structures of the nucleic-acid-binding sites among the different IMPDHs. These experiments provide further evidence that IMPDHs bind nucleic acids.

Nucleic acids do not inhibit IMPDH activity

We monitored the effects of nucleic acids on the IMPDH reaction to determine if nucleic acids bind at the active site. *T. foetus*

IMPDH was incubated with sub-saturating concentrations of substrates (10 μ M IMP compared with $K_m = 2$ μ M and 200 μ M NAD⁺ compared with $K_m = 150$ μ M) in the presence and absence of 3.5 μ M dT₁₀₀ ($1000 \times K_d$, as determined below). If dT₁₀₀ binds in the dinucleotide site, as observed for many dehydrogenases, then activity should be reduced to less than 1% under these conditions. However, no inhibition was observed; the initial rates of NADH production were identical (within experimental error) in the presence and absence of dT₁₀₀ (66 and 70 nM s⁻¹ respectively). Thus, nucleic acids do not bind in the active site.

The IMPDH subdomain binds nucleic acids

The above observations suggest that nucleic acids bind to the 'back-end' of the α - β barrel away from the active site and in the vicinity of the subdomain. Interestingly, the junction between the catalytic domain and the subdomain contains many positively charged residues (Figure 3). Therefore we determined if deletion of the subdomain impaired nucleic-acid binding. A variant of *T. foetus* IMPDH lacking the subdomain (*Tf*ΔSD) has enzymic properties identical with the wild-type enzyme [8]. *Tf*ΔSD binds to the random pool of oligonucleotides with lower affinity than wild-type ($K_d = 12 \pm 2$ nM versus 2 nM for wild-type) (Figure 1B). UV cross-linking experiments with *Tf*ΔSD also demonstrate reduced binding to dT₁₀₀ (Figure 2, right-hand panel).

To further demonstrate that nucleic acids bind to the subdomain, we substituted conserved positively charged residues in the junction between the catalytic domain and subdomain with glutamic acid, constructing R212E/R217E and K230E/R231E in *T. foetus* IMPDH (Figure 3). These mutations do not affect enzymic activity (Table 1), but impair nucleic-acid binding (Figure 1C). Both mutants bind dT₁₀₀ with an approx. 20-fold higher value of K_d than wild-type IMPDH (Figure 1C: the values of K_d are 7 ± 2 nM, 70 ± 10 nM, and 120 ± 30 nM for wild-type, R212E/R217E and K230E/R231E tetramers respectively). These experiments confirm that nucleic acids bind to the back-end of the protein near the subdomain.

Figure 3 Structure of IMPDH

The tetramer of hamster IMPDH (Protein Database 1jr1 [6]) is shown because the subdomain is disordered in all of the structures of *T. foetus* IMPDH. Red denotes Asp and Glu residues, blue denotes Arg and Lys residues, orange denotes the substrate and inhibitor mycophenolic acid. Green residues are analogous to Arg²¹², Arg²¹⁷, Lys²³⁰ and Arg²³¹ in *T. foetus* IMPDH. Residues 412–420 have been omitted so that the active site could be readily visualized. Some residues in the N-terminus (1–10), subdomain (121–124, 129–130, 135–149, 156–177 and 206–208) and active-site flap (421–436) are disordered in this structure. The left-hand and right-hand panels are related by a rotation of 180 degrees about the y-axis. Note the high density of Arg and Lys residues at the junction between the catalytic domain and subdomain.

Table 1 Steady-state kinetic parameters for mutant *T. foetus* IMPDHs

Results are means $\pm$ S.E.M. under conditions of 50 mM Tris/HCl, pH 8.0, 100 mM KCl, 1 mM DTT, 1 mM EDTA, 25 °C.

	Wild-type*	R212E/R217E	K230E/R231E
k_{cat} (s^{-1})	1.9 ± 0.2	1.2 ± 0.1	1.2 ± 0.1
K_m (IMP) (μM)	1.7 ± 0.4	5 ± 2	5 ± 2
K_m (NAD) (μM)	150 ± 30	120 ± 20	90 ± 20
K_{ii} (NAD) (μM)	7 ± 2	5 ± 2	5 ± 2

* Data from [12].

We expressed the subdomain of human IMPDH type I in order to determine if the barrel domain is required for nucleic-acid binding. The subdomain alone binds to the random pool of oligonucleotides with an affinity of 8 ± 2 nM (Figure 1B). Interestingly, approx. 75 % of the pool binds to the subdomain alone, whereas only approx. 10 % bound to human IMPDH type I. Unfortunately the subdomain does not UV cross-link to dT₁₀₀ (Figure 2, lane 6).

The properties of nucleic-acid binding

We chose to characterize the nucleic-acid-binding properties of *T. foetus* IMPDH in more detail because this enzyme has a strong intrinsic protein fluorescence signal that is quenched in the presence of oligonucleotides (Figure 4, Table 2). No quenching is observed when a variety of double-stranded complexes of oligonucleotides are added to solutions of *T. foetus* IMPDH (results not shown). This observation indicates that IMPDH binds single-stranded, but not double-stranded, nucleic acids. No quenching is observed in the presence of heparin (a mixture of anionic polysaccharides). The failure to bind either double-stranded DNA or heparin suggests that IMPDH binds specifically to single-stranded nucleic acids, not just to polyanions. Note that the concentration units are 'per nucleotide'. Table 2 shows that longer oligonucleotides bind with higher affinity, suggesting that the binding-site size is > 60 nucleotides. IMPDH binds to RNA as well as ssDNA (Table 2). Poly(dT), poly(U) and poly(A) bind with similar affinity, suggesting that IMPDH binds to unstructured nucleic acids. The other homo-oligonucleotides dA, dG and dC do not bind appreciably to IMPDH. In addition, no binding is observed with 20–55-mers of various sequences selected from laboratory stocks, further confirming that the binding site is > 60 bases. We also failed to observe binding to cognate RNA, which suggests that IMPDH does not regulate its own translation.

As observed in the filter-binding assay, deletion of the subdomain decreases the affinity of dT₁₀₀ and dT₆₀ relative to wild-type, indicating that the subdomain is involved in nucleic-acid binding. Interestingly, dT₁₀₀ and dT₆₀ bind to *Tf*ΔSD with similar affinity. This observation suggests that deletion of the subdomain has also decreased the size of the nucleic-acid-binding site.

IMPDH binds approx. 100 bases of nucleic acid

Following the experiments of Lohman and co-workers with *E. coli* SSB (single-stranded DNA-binding protein) [20], we further characterized poly(dT) binding because this homopolymer has little structure. To determine the size of the nucleic-acid-binding site, we monitored the quenching of IMPDH fluorescence by dT₁₀₀ under tight binding conditions (Figure 4). Extrapolation of the linear portions of the graph to their intersection indicates that the site size is approx. 96 nucleotides/tetramer of IMPDH. The alternative stoichiometry of 24 nucleotides/monomer is

Figure 4 ssDNA quenches the fluorescence of *T. foetus* IMPDH

(A) *T. foetus* IMPDH (30 nM; ●) and *Tf*ΔSD (25 nM; ○) were titrated with dT₁₀₀ in 50 mM Tris/HCl, pH 8.0, 1 mM DTT and 50 mM KCl at 25 °C. The excitation and emission wavelengths were 295 nm and 330 nm respectively. The lines represent fits to a simple binding function. (B) *T. foetus* IMPDH (400 nM) was titrated with dT₁₀₀ under the same conditions. This enzyme concentration is greater than the K_d and tight-binding conditions apply. The linear portions of the graph are extrapolated to their intersection point, and the x co-ordinate of the intersection equals the number of bases that bind to an IMPDH monomer [49].

Table 2 The nucleic acid binding specificity of *T. foetus* IMPDH

Conditions: 50 mM Tris/HCl, pH 8.0, 1 mM DTT and 50 mM KCl at 25 °C. Changes in protein fluorescence were used to monitor nucleic-acid binding. The value of K_d per site is based on the site size of approx. 100 nucleotides (nt) for wild-type and approx. 60 nucleotides for *Tf*ΔSD, and is only calculated for oligomers ≥ 100 bases. n.a., not applicable.

Ligand	Length (nt)	K_d (μM) per base	K_d (μM) per site
Wild-type <i>T. foetus</i> IMPDH			
dT	100	0.35	0.004
dT	60	1.4	n.a.
dT	30	15	n.a.
dT	14–18	$\gg 4$	n.a.
Poly(dT)	Undetermined	4	0.040
dA	60	> 46	n.a.
dC	60	50	n.a.
dG	60	> 96	n.a.
Poly(U)	Undetermined	0.45	0.005
Poly(A)	Undetermined	1.9	0.019
Poly(G)	Undetermined	> 250	n.a.
Heparin	n.a.	> 25	n.a.
<i>Tf</i> ΔSD			
dT	100	3.6	0.06
dT	60	2	0.03

Figure 5 IMPDH is localized in the nucleus of human cells

Human K562 and PC-3 cells were grown to near confluence. (A and B) K562 cells; (C and D) PC-3 cells. The panels show the fluorescence of the central slices of these cells observed using a fluorescence microscope. The first picture in each panel shows the antibody staining. Cells in panels (A) and (C) were treated with polyclonal rabbit antibody raised against human type II IMPDH, followed by rhodamine-conjugated anti-rabbit IgG antibody (red). Cells in panels (B) and (D) were treated with rhodamine-conjugated anti-rabbit IgG antibody alone. The second picture in each panel shows cells fixed and stained with Hoechst stain (blue) to identify the nucleus. The third panel shows the overlays of the antibody and Hoechst staining. Scale bars represent 20 μm .

unlikely, because 30-mers have low affinity. A similar site size (110 nucleotides/tetramer) is obtained when IMPDH is titrated with poly(dT) of length > 150 bases (results not shown). The simplest model for this binding site is that the nucleic acid interacts with all four subunits, as suggested by the cross-linking experiments. This site size indicates that the true K_d for dT₁₀₀ binding to *T. foetus* IMPDH is 4 nM (dividing the apparent K_d per nucleotide by the site size gives the true K_d). This value is comparable with that determined in the filter-binding experiments. As expected, deletion of the subdomain reduces the site size to 60 nucleotides/tetramer (Table 2).

Human IMPDH is located in the nucleus

We performed a series of immunofluorescence experiments to examine the localization of IMPDH. Polyclonal antibodies raised against recombinant human type II IMPDH were used as the primary antibody. These antibodies are specific for IMPDH in human cell extracts, but cannot distinguish between the two isoforms of human IMPDH (S. E. Mortimer and L. Hedstrom, unpublished results). In K562 leukaemia cells, IMPDH co-localizes with the Hoechst staining (Figures 5A and 5B). In PC-3 prostate cancer cells, IMPDH is found in both the cytoplasm and nucleus (Figures 5C and 5D; note that IMPDH does not stain the nucleolus). IMPDH was also observed in both the cytoplasm and nucleus of HeLa cells (results not shown).

IMPDH is associated with nucleic acids *in vivo*

Our binding experiments clearly show that IMPDH binds single-stranded nucleic acid *in vitro*. To determine if IMPDH binds nucleic acids *in vivo*, K562 cells were cross-linked with formaldehyde and sonicated to fragment the DNA, as described in the Experimental section. IMPDH was immunoprecipitated with anti-human IMPDH antisera. The presence of nucleic acid was detected by treating with alkaline phosphatase followed by poly-

Figure 6 Immunoprecipitation of IMPDH-bound nucleic acid

Human K562 cells cross-linked and immunoprecipitated with pre-immune serum (A), anti-IMPDH antiserum (B–D) or recombinant human type I IMPDH and anti-IMPDH antiserum (E) as described in the Experimental section. Co-immunoprecipitated nucleic acid was labelled with [γ -³²P]ATP and split into three equal aliquots: control (B), RNase-treated (C), or DNase-treated (D). Data are shown relative to average sample B values for each of three independent experiments and error bars are S.E.M. including all experiments. Statistical analysis was performed using a two-tailed *t* test assuming unequal variance. Statistical significance is defined as $P < 0.05$. The asterisks denote samples that are significantly different from sample B ($P = 0.02$, $P = 1 \times 10^{-5}$, and $P = 0.01$ for samples A, C and E respectively). Samples B and D are statistically similar, as are samples A and E ($P = 0.3$ and $P = 0.11$ respectively).

nucleotide kinase/[γ -³²P]ATP and the labelled nucleic acids were isolated by filter binding (Figure 6). Approx. 1.5-fold more radioactivity was recovered with anti-IMPDH antiserum than with the pre-immune-serum control. The addition of recombinant human IMPDH before immunoprecipitation decreased the radioactivity to the level of the pre-immune serum. This experiment demonstrates that the excess radioactivity is associated with IMPDH. Together these results show that IMPDH binds nucleic acids *in vivo*.

Table 3 Immunoprecipitation of DNA with anti-human IMPDH antisera

K562 cells were labelled with [^3H]thymidine and cross-linked with formaldehyde as described in the Experimental section. IMPDH was precipitated with polyclonal anti-human IMPDH rabbit antiserum.

Experimental conditions	Counts per min
Experiment 1	
No antiserum, no cross-linking	60
Antiserum, no cross-linking	70
No antiserum, plus cross-linking	160
Antiserum, plus cross-linking	330
Experiment 2	
No antiserum plus cross-linking	120
Antiserum plus cross-linking	880
Above plus recombinant human type II IMPDH	120

Since RNA is the predominant single-stranded nucleic acid *in vivo*, we determined if IMPDH-bound nucleic acid is RNA by treating the above samples with RNase. As expected, the radioactivity was degraded, indicating that most of the nucleic acid is RNA. This experiment demonstrates that IMPDH binds RNA *in vivo*.

A small decrease in radioactivity was also apparent when the samples were treated with DNase. Although this decrease is not statistically significant, since single-stranded DNA is much less abundant than RNA, even a small decrease could indicate that IMPDH also binds DNA *in vivo*. Therefore, we performed a second experiment to determine if IMPDH associates with DNA. K562 cells were treated with [^3H] thymidine to label DNA, and immunoprecipitated as above. Only a background level of radioactivity is observed in the immunoprecipitate in the absence of cross-linking (Table 3, Experiment 1). Upon cross-linking, significantly more radioactivity was recovered in the presence of anti-human IMPDH antiserum. This experiment indicates that IMPDH is associated with DNA *in vivo*. A second experiment was performed to demonstrate further that the radioactivity was specifically associated with cellular IMPDH. As before, more radioactivity was observed in the presence of anti-human IMPDH than in the absence (Table 3, Experiment 2; the larger amount of precipitated radioactivity in this experiment can be attributed to more efficient labelling of DNA). This radioactivity was reduced to background levels in the presence of excess recombinant human IMPDH type II, demonstrating that the radioactivity must be specifically associated with cellular IMPDH and does not fortuitously precipitate. The simplest explanation for these results is that IMPDH is also associated with DNA *in vivo*.

DISCUSSION

We have used three different assays to demonstrate that IMPDH binds single-stranded nucleic acids. Most importantly, the affinity of this interaction is in the low nanomolar range, comparable with the affinities observed for nucleic-acid interactions with known nucleic-acid-binding proteins [17–19]. This high affinity argues strongly against a fortuitous non-specific interaction, as does the failure to bind double-stranded DNA or heparin. The site size is 100 nucleotides, consistent with the low affinity of small oligonucleotides. This site size is larger than that of *E. coli* SSB (35 or 65 nucleotides per tetramer), but can be easily accommodated by the size of the IMPDH tetramer (approx. 55 kDa/monomer versus approx. 19 kDa/monomer for SSB). Lastly, IMPDH is found in the nucleus, and immunoprecipitation experiments demonstrate that IMPDH binds RNA and DNA *in vivo*.

The human IMPDHs do not possess nuclear localization signals (NLS) that can be recognized by either PredictNLS [21] or PSORT II [22]. However, the prediction of protein localization is uncertain at best. Many nuclear proteins lack identifiable NLSs, and the recognition sequences have yet to be determined for approx. 50 % of the known nuclear import factors [23], so the absence of an NLS in IMPDH is not unusual.

Several other dehydrogenases are also nucleic-acid-binding proteins, most notably glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH has been independently isolated as a tRNA-, RNA- and DNA-binding protein [17,24–27]. Nucleic acids bind in the NAD site of GAPDH, which has a Rossmann fold common to many other dehydrogenases [18,28,29]. GAPDH binds transcription regulatory elements [24], has a uracil glycohydrolase activity [30], is involved in nuclear/cytoplasmic transport of tRNA and oligonucleotides [17,18], activates ribozymes [29,31] and cleaves RNA [32]. These activities appear to be important in transcription, DNA repair, apoptosis and viral replication [33–36] and may be mediated by different GAPDH isoforms [37].

Unlike other dehydrogenases, the NAD site of IMPDH is not involved in nucleic-acid binding, as demonstrated by the failure of nucleic acids to inhibit the IMPDH reaction. Instead, nucleic acids interact with the 'back-end' of the enzyme and the subdomain. Nucleic-acid binding is the first function associated with the subdomain, which consists of two structural motifs similar to the regulatory domain of CBS [38]. CBS domains are also found in chloride channels, ABC transporters and protein kinases where their function is unknown [38]. CBS domain mutations have been implicated in a wide array of inherited diseases, including myotonia (chloride channel 1 [39]) and familial hypertrophic cardiomyopathy (protein kinase $\text{A}\gamma 2$ [40]). Perhaps nucleic-acid binding is a general property of CBS domains, and alterations in nucleic-acid-binding activity are a common mechanism underlying these pathologies. Mutations in the subdomain of the type I isoenzyme are implicated in retinitis pigmentosa [9,10], as are mutations in several splicing proteins and transcription factors [41]. It seems likely that the pathological effects of IMPDH mutations result from the disruption of nucleic-acid binding, and delineation of the function of IMPDH in nucleic-acid metabolism will reveal a novel regulatory mechanism that must be crucial for photoreceptor function.

Our experiments indicate that IMPDH has a previously unsuspected role in nucleic-acid metabolism. Such additional functions can have profound therapeutic consequences, as illustrated by recent work on thymidylate synthase, which is a translational repressor in addition to its well-known role in pyrimidine biosynthesis [42,43]. The selective toxicity of a thymidylate synthase inhibitor for *Plasmodium falciparum* arises from differences in translational repression rather than differences in inhibition of enzymic activity. Likewise, the nucleic-acid binding of IMPDH could have an unappreciated role in the efficacy of IMPDH-targeted chemotherapy.

Assigning physiological roles to proteins or subdomains is a difficult task, and as yet we can only speculate on the role of IMPDH in nucleic-acid metabolism. IMPDH protein concentrations increase dramatically when the guanine nucleotide pool is low, and IMPDH inhibitors cause cells to arrest at the G1/S interface [44]. In yeast, the transcription of IMPDH is linked to growth state, and decreases in post-diauxic and stationary cultures [45]. It is possible that IMPDH has an active role in these processes, maybe stabilizing ssDNA until replication can resume, or perhaps blocking transcription until guanine nucleotide levels are restored. Alternatively, since the demand for guanine nucleotides is high in translation, IMPDH could bind to mRNA and block translation

until guanine nucleotide concentrations are restored. Nucleic-acid binding may simply increase the local concentration of IMPDH where the cellular demand for guanine nucleotides is high. IMPDH associates with proteins involved in transcription, translation, nuclear transport and DNA repair in genome-wide protein interaction screen, and, like GAPDH, could be involved in all of these processes [46–48]. Perhaps, like GAPDH, IMPDH has multiple isoforms to satisfy diverse functions. Indeed, the most striking differences between the two human IMPDH isoenzymes are found in the subdomain. The experiments reported here are a necessary prelude to uncovering the role of the IMPDH subdomain *in vivo*.

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