**A nucleotide-dependent conformational switch
controls human IMP dehydrogenase polymerization
to modulate their catalytic activity**

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Abstract

IMP dehydrogenase (IMPDH) catalyzes the rate-limiting step in the *de novo* GTP biosynthetic pathway and plays essential roles in cell proliferation. As a clinical target, IMPDH has been studied for decades but it has only been within the last years that we are starting to understand the complexity of the mechanisms of its physiological regulation. Here, we report structural and functional insights into how adenine and guanine nucleotides control a conformational switch that modulates the assembly of the two human IMPDHs into cytoophidia and allosterically regulates their catalytic activity. *In vitro* reconstituted micron-length cytoophidia show catalytic activity comparable to unassembled IMPDH but, in turn, are more resistant to GTP/GDP allosteric inhibition. Therefore, IMPDH cytoophidia formation facilitates the accumulation of high levels of guanine nucleotides when the cell requires it. Finally, we demonstrate that most of the IMPDH retinopathy-associated mutations abrogate GTP/GDP-induced allosteric inhibition and alter cytoophidia dynamics.

Keywords

IMP dehydrogenase, cytoophidia, conformational switch, allosteric regulation, X-ray crystallography and scattering

Introduction

Inosine 5'-monophosphate dehydrogenase (IMPDH) is the enzyme that catalyzes the rate-limiting step in the *de novo* guanine nucleotide biosynthetic pathway, which plays a crucial role in the regulation of the cellular pools of purine nucleotides. Therefore, IMPDH is a key enzyme involved in the control of cell viability, division and proliferation. Indeed, IMPDH is the target of a diverse number of drugs presently used in clinical chemotherapy such as immunosuppressors, antivirals, antitumorals or antibiotics [1].

IMPDH is composed of an archetypal TIM barrel catalytic domain and a Bateman regulatory domain, which is inserted within a loop of the catalytic module. In solution, the basic units of most IMPDHs are homotetramers that can dimerize in different ways to form octamers or higher order oligomers [2-7].

Despite the fact that IMPDH is a therapeutic target with important clinical relevance that has been widely studied during the last decades [1], the available information on its physiological regulation is rather limited. More specifically, the molecular details on how the regulatory and catalytic domains communicate to modulate the catalytic activity remain mostly unclear. Indeed, it has been only since the last few years that we are beginning to understand the mechanistic and structural details of IMPDH allosteric regulation [2, 3, 5-7].

Using the IMPDH enzyme from the industrial fungus *Ashbya gossypii* as a model, we have recently reported a molecular mechanism by which purine nucleotides allosterically modulate the catalytic activity. The competition of adenine or guanine nucleotides for their binding sites within the regulatory Bateman domain controls a conformational switch that modulates the catalytic activity by shifting from extended to compacted IMPDH octamers. Extended, ATP-induced octamers are fully active, while the catalytic activity of GTP/GDP-induced compacted octamers is significantly reduced [6, 7]. The relevance of this allosteric mechanism of regulation is stressed by the observation that most of the described missense mutations in human IMPDH associated to severe retinopathies, such as Retinitis Pigmentosa and Leber Congenital Amaurosis [8], map into the nucleotide binding sites of the Bateman regulatory domain. Based on these results, it was proposed that the retinopathy-associated

1 mutations might interfere with nucleotide binding and disrupt the allosteric
2 regulation of human IMPDH, generating constitutively active IMPDH mutants,
3 which is in agreement with the dominant hereditary character of these mutations
4 [7].
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8 Adding more complexity to the mechanisms of IMPDH regulation, the
9 human enzyme has been reported to polymerize into mesoscale intracellular
10 assemblies such as purinosomes [9] and micrometric structures denoted as *rod*
11 *and rings* or *cytoophidia* [4, 10, 11]. Thereby, human IMPDH, as well as CTP
12 synthase (CTPS) and some other metabolic enzymes, possess the particular
13 ability to self-assemble into higher order structures. This macromolecular
14 organization has been suggested to modulate enzyme activity and play
15 important roles in regulating cell metabolism by controlling intracellular
16 nucleotide homeostasis [12, 13]. Human IMPDH, in response to intracellular
17 guanine-nucleotide depletion, assembles into micron-length cytoophidia that
18 disappear once the guanine-nucleotide levels are restored. Remarkably, the
19 physiological functions of IMPDH cytoophidia remain unclear, although it has
20 been reported that polymerization correlates with rapid cell proliferation [11, 14,
21 15]. Nonetheless, the *in vitro* reconstituted polymers show identical activity for
22 polymerized and non-assembled human IMPDH [5]. To reconcile these results,
23 it has been recently speculated that filamentous IMPDH might be more resistant
24 to GTP/GDP inhibition, facilitating the intracellular accumulation of GTP when
25 required [15], but no experimental evidence supporting this hypothesis has been
26 obtained. Moreover, there is an evident lack of information regarding the
27 structural details and the molecular mechanisms governing the dynamics of
28 IMPDH cytoophidia assembly *in vitro*. Indeed, to our best knowledge, the *in vitro*
29 reconstituted IMPDH filaments studied so far consist of a few particles
30 organized into chains polymerized by a back-to-back assembly mechanism [2,
31 4, 5] that do not resemble the micrometer-long fibrillar IMPDH cytoophidia that
32 are observed in cells [10, 11, 16-21]
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54 In this study, we report the *in vitro* reconstitution of human IMPDH
55 cytoophidia and their structural and functional characterization. We show that
56 micron-length human IMPDH cytoophidia spontaneously assemble *in vitro*
57 under macromolecular crowding conditions in the absence of nucleotides but
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1 disassemble when either GTP or ATP are present in the solution, resembling
2 what is observed within cells. The *in vitro* reconstituted IMPDH cytoophidia are
3 catalytically functional, with similar activity to non-assembled IMPDH, but they
4 are significantly more resistant to GTP/GDP allosteric inhibition. Our results
5 show that the cytoophidia dynamics and the allosteric regulation of the catalytic
6 activity are tightly linked to the purine nucleotide-controlled conformational
7 switch that we have previously reported for the *Ashbya gossypii* fungal IMPDH
8 [6, 7]. They further provide a plausible structural mechanism for the nucleotide-
9 dependent dynamics of cytoophidia assembly. Finally, we present the high-
10 resolution crystal structures of HsIMPDH2 complexed to GTP and GDP and
11 demonstrate that most of the retinopathy-associated missense mutations map
12 into the allosteric nucleotide binding sites of human IMPDH, altering nucleotide
13 binding and subsequently abrogating GTP/GDP-induced allosteric inhibition.
14 Additionally, these mutations also alter cytoophidia dynamics, suggesting a
15 potential pathological role for these assemblies that deserves to be further
16 investigated.

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18 Altogether, the results presented here yield valuable information to
19 understand the complex mechanisms of physiological regulation of IMPDH in
20 humans, as well as the roles that these mechanisms might have in the
21 development of certain retinopathies.

22 **Results**

23 *A nucleotide-controlled conformational switch operates in both human IMPDH*
24 *isoforms*. By using the IMPDH enzyme from the industrial fungus *Ashbya*
25 *gossypii*, we have recently reported a conformational switch that modulates the
26 catalytic activity in response to the binding of purine nucleotides within the
27 Bateman regulatory domain: adenine nucleotides (ATP, ADP and AMP) induce
28 active IMPDH octamers in an extended (~115 Å-long) conformation, while
29 guanine nucleotides (GTP and GDP) induce inhibited octamers in a compact
30 (~95 Å-long) conformation [6]. Experimental evidence of the existence of this
31 conformational switch in the isoform 2 of human IMPDH (HsIMPDH2) was
32 recently reported by using electron microscopy on ATP and GTP-induced

1 helical protofilaments [5]. Here, we have further investigated the nucleotide-
2 conformational switch in both human IMPDH isoforms (HsIMPDH1 and
3 HsIMPDH2) *in vitro* by using Small Angle X-ray solution Scattering (SAXS).
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6 HsIMPDH1 and HsIMPDH2 wild type proteins at the concentration used
7 for the SAXS experiments (2.5 mg/mL) are mostly octamers in solution but the
8 addition of nucleotides induces stochastic oligomerization (see below) that
9 results in poly-disperse solutions [2, 4, 5], which are not appropriate for SAXS
10 analysis. Thereby, we made use of the missense mutation Y12A that has been
11 previously reported to abrogate polymerization of HsIMPDH2 *in vitro* and in
12 cultured cells [5]. Indeed, in the absence of nucleotides, HsIMPDH1-Y12A and
13 HsIMPDH2-Y12A mutant enzymes remained as monodisperse tetramers in
14 solution, as demonstrated by size-exclusion chromatography (Supplemental
15 Figure 1A). This observation indicates that the octamers that exist in solution in
16 the absence of nucleotides are most probably formed by the back-to-back
17 interaction of the catalytic domains of two tetramers with a crucial role for
18 residue Y12, as suggested in previous reports [2, 5].
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22 The addition of ATP or GDP to HsIMPDH1/2-Y12A induces the
23 association of two tetramers to form octamers (Figures 1A and 1B).
24 Remarkably, the SAXS profiles of both human IMPDH isoforms resemble those
25 described for the *A. gossypii* ATP or GDP-induced octamers (Supplemental
26 Figures 1B-D), demonstrating that the conformational switch previously
27 proposed for the fungal *Ashbya gossypii* AgIMPDH [6] is also present in both
28 isoforms of the human IMPDH enzyme.
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31 As shown in Supplemental Figures 1E-F, ATP induces the formation of
32 octamers more potently than GDP for both HsIMPDH1 and HsIMPDH2.
33 Interestingly, significant differences are observed between both isoforms. In
34 HsIMPDH1, the GDP-induced inhibition of the catalytic activity correlates with
35 octamer compaction (Figure 1C). In contrast, GDP-induced compact octamers
36 of HsIMPDH2 are formed before inhibition occurs, both in the absence or
37 presence of ATP in the solution (Figure 1D), highlighting mechanistic
38 differences between both human isoforms that might be relevant *in vivo*.
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1 In summary, our data show that the nucleotide-controlled conformational
2 switch initially described for the *A. gossypii* enzyme also operates in both
3 human IMPDH isoforms, where the binding of ATP or GDP induces octamers,
4 formed by the interaction of the Bateman domains of two tetramers. The
5 presence of adenine nucleotides in the solution induce octamers with an
6 extended conformation that are catalytically active, while guanine nucleotides
7 induce the compaction of these octamers, significantly inhibiting their enzymatic
8 activity.
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10 *Retinopathy-associated mutations abrogate the allosteric inhibition of human*
11 *IMPDH.* To gain insights into the binding modes of purine nucleotides to the
12 Bateman domain of human IMPDH, we attempted to obtain high resolution
13 structural information by X-ray crystallography. This issue is especially relevant
14 since, based on the *A. gossypii* fungal IMPDH structure, we have previously
15 proposed that most of the retinopathy-associated missense mutations in
16 HsIMPDH1 alter GTP/GDP-mediated allosteric inhibition [7]. Thereby, we
17 solved the high-resolution crystal structures of HsIMPDH2 in complex with GTP
18 and GDP, which are essentially identical (Supplemental Figure 2A). A detailed
19 description of these structures can be found in the Supplemental Information.
20 Both structures unequivocally demonstrate that most of the retinopathy-
21 associated mutations either: i) placed at the interface of two interacting
22 Bateman domains: R224P, L227P and R231P (Supplemental Figure 2C) or ii)
23 are directly involved in nucleotide binding at the allosteric sites of the Bateman
24 domain: D226N for GTP/GDP2 (Supplemental Figure 3B) and N198K and
25 K238E for GTP3/GDP3 (Supplemental Figure 3C).
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45 We then introduced most of the missense mutations described in the
46 literature [8] into HsIMPDH1 and purified the mutant enzymes for their analyses
47 *in vitro*. Our results showed that the mutants had catalytic activity *in vitro* that
48 fell within the same range (between 1.1 and 0.6-fold the activity of the WT;
49 Figure 2) as the WT enzyme. ATP showed no significant effect on the catalytic
50 activity of both the WT and the mutant enzymes (between 1.0 and 0.6-fold the
51 activity of the enzyme without nucleotide; Figure 2). On the other hand, those
52 mutations that map within the Bateman domain nucleotide binding sites
53 (N198K, D226N and K238E; Supplemental Figure 3) or at the interphase
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1 between the Bateman domains (R224P, L227P and R231P; Supplemental
2 Figure 2C) cannot be allosterically inhibited *in vitro* neither by GTP/GDP (Figure
3 2), which thereby results in constitutively activated mutant enzymes.
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6 *In vitro reconstitution of micron-length human IMPDH cytoophidia assembly.* We
7 then investigated *in vitro* the ability of IMPDH to reversibly assemble into
8 mesoscale structures that have been described to form within cells in response
9 to the depletion of the intracellular guanine nucleotide pool [10, 19, 21]. It is
10 important to stress here that several reports have shown that ATP and GTP
11 induce the formation of IMPDH polymers *in vitro* [3-5], but these polymers
12 consist of a few IMPDH octamers stacked back-to-back and organized into
13 single filaments. Thereby, these polymers do not represent the IMPDH micron-
14 length filamentous bundles that have been reported to form in cells [10, 18, 19,
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25 These data suggest that an important factor is missing *in vitro*, but what
26 could be this factor? An initial hint came from the published purification protocol
27 of recombinant human IMPDH enzymes from *E. coli* [21]. This protocol uses 1.5
28 M urea to keep the protein “soluble” in the supernatant after the initial
29 centrifugation of the bacterial cell extracts. However, 1.5 M urea concentration
30 is much lower than the midpoint of the urea-induced denaturation curve
31 (approximately 5M [22]), which indicates that there is no need to denature the
32 IMPDH to “solubilize” it. We then hypothesized that IMPDH properly folds in the
33 cytoplasm of *E. coli*, however the high background concentration of
34 macromolecules in the initial cell extracts favored the formation of large IMPDH
35 assemblies that readily pelleted during a soft centrifugation.
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45 We therefore tested the influence of macromolecular crowding on IMPDH
46 filament assembly *in vitro* under different experimental conditions by monitoring
47 protein assembly with turbidimetry. Our results showed that at high Ficoll-70
48 concentrations both human IMPDH isoforms (HsIMPDH1 and HsIMPDH2)
49 spontaneously assemble into large polymers, as indicated by the large increase
50 in the turbidity of the solution (Figure 3A and Supplemental Figure 4A), and
51 further corroborated by electron microscopy (EM; upper panels of Figure 3B
52 and Supplemental Figure 4B). At Ficoll-70 concentrations ≥ 150 mg/mL, most of
53 the IMPDH is included within these polymers. These results led us to conclude
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1 that the formation of micron-length cytoophidia is due to the macromolecular
2 crowding conditions, since mesoscale polymerization can be detected neither at
3 low crowding agent concentrations nor in its absence.
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6 As a negative control, we used the Y12A missense mutation that has
7 been previously reported to abrogate polymerization of HsIMPDH2 in cultured
8 cells [5]. In accordance to this report, we were not able to detect significant
9 assembly of the HsIMPDH2-Y12A mutant *in vitro* in dilute solutions or in
10 crowding conditions (Figure 3C). In contrast, we observed significant
11 polymerization of the HsIMPDH1-Y12A mutant under macromolecular crowding
12 conditions, although to a lower extent than the wild-type protein (Figure 3C).
13 These results agree with previous reports that describe a higher tendency to
14 polymerize of HsIMPDH1 than HsIMPDH2 [23]. Additionally, we tested whether
15 the IMPDH enzymes from different organisms, other than humans, can also
16 form cytoophidia *in vitro*. Within Figure 3C, it can be observed in that, in
17 contrast to the two human isoforms, the eukaryotic *Ashbya gossypii*
18 (AgIMPDH), the bacterial *E. coli* (EcIMPDH) and *B. subtilis* (BsIMPDH)
19 enzymes are unable to form cytoophidia in our experimental conditions.
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23 To further corroborate that eukaryotic AgIMPDH does not form
24 cytoophidia, we ectopically overexpressed this gene in HeLa and HEK293T
25 cells. As it is shown in Supplemental Figure 4C, in contrast to HsIMPDH2 that
26 showed a large amount of cytoophidia in essentially all the transfected cells, no
27 detectable polymer formation was observed for AgIMPDH, despite the large
28 amount of protein observed into the cytoplasm. Thereby, our results
29 demonstrate that the ability of human IMPDH to form cytoophidia is not
30 universal, since not all –eukaryotic or prokaryotic- IMPDH enzymes are able to
31 assemble into large aggregates. In agreement with our results, other authors
32 could neither find IMPDH polymers in the model yeast *S. cerevisiae* [24].
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36 The huge polymers of human IMPDH that formed *in vitro* under
37 macromolecular crowding conditions are composed of octamers that associate
38 into bundles of twisted and curved filaments that intricate into three dimensions
39 without apparent regular or defined structure (red rectangle in the upper right
40 panel of Figure 3B and upper right panel of Supplementary Figure 4B).
41 Nonetheless, ordered bundles of parallel protofilaments were eventually
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observed within the huge polymers in some of the HsIMPDH2 micrographs (blue rectangle in the upper right panel in Figure 3B). Direct distance measurements and power spectra analysis by fast-fourier transform (FFT) of the protofilaments within these bundles showed a repetitive longitudinal spacing of $58.2 \pm 0.4 \text{ \AA}$. This longitudinal spacing is compatible with the expanded ATP-induced $\sim 115 \text{ \AA}$ -long octamers previously observed by X-ray crystallography for AgIMPDH [6] and by SAXS for human IMPDH.

Remarkably, when ATP or GDP were present, the turbidity of the solution was significantly lower than in their absence (Figure 3A and Supplemental Figure 4A), indicating that these nucleotides inhibited IMPDH cytoophidia assembly. The EM analysis of the polymers formed in the presence of purine nucleotides revealed either isolated octamers or helical oligomers, extended for ATP and compacted for GDP (Figure 3B and Supplementary Figure 4B). A detailed analysis of the polymers formed in the presence of purine nucleotides can be found in the Supplemental Information, including the 3D reconstructions of ATP and GDP-induced protofilaments formed under macromolecular crowding conditions (Supplementary Figure 6).

Moreover, once the cytoophidia were spontaneously formed *in vitro*, the addition of either ATP or GDP depolymerized them, but the extent of this depolymerization strongly depended on the incubation time before nucleotide addition, *i.e.* the cytoophidia maturation time [18]. The more mature the cytoophidia are, the longer it takes to depolymerize them by GDP or ATP (Supplemental Figure 5).

In summary, both human IMPDH isoforms spontaneously assemble *in vitro* into micron-length cytoophidia under macromolecular crowding conditions that resemble those found in the cytoplasm. Under these conditions, the presence of purine nucleotides in the solution strongly reduce IMPDH cytoophidia formation, by both inhibiting assembly and inducing disassembly. Thus, our experimental setup reconstitutes *in vitro* the reversible nucleotide-controlled cytoophidia assembly phenomenon observed in cells.

Retinopathy-associated missense mutations in IMPDH1 alter the nucleotide-dependent cytoophidia dynamics in vitro. We next studied the nucleotide-

1 dependent dynamics of cytoophidia assembly *in vitro* for the IMPDH1 enzymes
2 containing the retinopathy-associated mutations. All the mutants have the ability
3 to spontaneously assemble into cytoophidia to a similar extent than the wild
4 type enzyme (Figure 4). Nonetheless, those mutants where the GTP/GDP
5 allosteric inhibition is abrogated have also altered nucleotide-controlled
6 regulation of cytoophidia assembly (Figure 4) and disassembly (Supplemental
7 Figure 7A). The only exception is the mutant N198K that cannot be allosterically
8 inhibited by GTP/GDP but possesses cytoophidia dynamics comparable to the
9 wild-type enzyme (Figure 4 and Supplemental Figure 7A). These data might
10 indicate that the non-canonical guanine nucleotide binding site is needed for
11 allosteric inhibition mediated by GTP/GDP, but is not implied in the regulation of
12 cytoophidia assembly by nucleotides.
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22 Remarkably, our data on cytoophidia assembled *in vitro* perfectly agree
23 with a recent report that showed that HsIMPDH1 mutant enzymes R224P,
24 D226N and R231P formed irreversible cytoophidia in transfected HEK293T
25 cells when treated with guanosine [14].
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30 *IMPDH cytoophidia are catalytically active in vitro but more resistant to*
31 *GTP/GDP inhibition*. We next studied the effects of the polymerization into
32 micron-length cytoophidia on the catalytic activity *in vitro*. To perform these
33 experiments, we used as control the Y12A mutants that have compromised
34 polymerization ability (Figure 3C). Remarkably, we observed no significant
35 differences between the catalytic activities of the Y12A mutants and the wild-
36 type enzymes, neither in crowded nor in non-crowded solutions (Supplemental
37 Table 2, Figures 5A and 5B). The slight increase in the K_M values between
38 crowded and non-crowded conditions are most probably due to excluded
39 volume and substrate limited diffusion effects, produced by the high
40 concentrations of Ficoll-70. Indeed, similar differences in the K_M and $K_{1/2}$ values
41 have been reported for a number of enzymes using similar crowding agents
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54 As an additional control, just before the measurements, we centrifuged
55 the samples (5.000 x g for 10 min), so that the cytoophidia were pelleted and
56 the tetramers, octamers and small oligomers remained in the supernatant. The
57 catalytic activity in the supernatant of the wild-type enzymes under crowding
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conditions was significantly reduced (Supplemental Figure 7B), demonstrating that most of the catalytic activity comes from the IMPDH cytoophidia. Additionally, most of the Y12A mutant enzyme, that have compromised cytoophidia formation (Supplemental Figure 7C), cannot be pelleted using these centrifugation conditions, therefore leaving the activity in the supernatant comparable to the activity prior to centrifugation (Supplemental Figure 7B). From these experiments, we can conclude that IMPDH polymerization into micron-length cytoophidia did not significantly affect the catalytic activity in our *in vitro* experimental conditions.

Since GTP and GDP promote both cytoophidia disassembly and catalytic activity inhibition, it can be hypothesized that IMPDH polymerization might serve to fine-tune the allosteric regulation of the enzyme. To test this hypothesis, we studied how GDP affects the catalytic activity of the cytoophidia assembled in our *in vitro* system. As observed in Figure 5C, non-mature cytoophidia (without incubation before GDP addition) demonstrated and inhibition of the catalytic activity while being depolymerized (red data) that showed no significant differences to the inhibition of the unassembled protein (green data). In contrast, the cytoophidia that had been pre-incubated for 2 hours before GDP addition, were more resistant to GDP-induced depolymerization (see also Supplemental Figure 5) and to a certain extent were resistant to GDP allosteric inhibition (blue data). As expected, this behavior can also be observed for HsIMPDH2 but to a lesser extent (Figure 5D), given that HsIMPDH2 is more sensitive to GDP-induced depolymerization than HsIMPDH1 (Supplemental Figure 5). Moreover, HsIMPDH2 was depolymerized at lower GDP concentrations than those required to inhibit its catalytic activity (Figure 5D). This observation agrees with the data presented above showing that GDP induces octamer compaction at lower concentrations than those required for catalytic activity inhibition (Figure 1D).

Discussion

Despite there is wide knowledge on the enzymatic reaction mechanisms of IMPDH and its inhibition by a variety of molecules, there is an evident lack of

1 information on its physiological mechanisms of regulation. Indeed, it has only
2 been since the last few years that we have obtained the first experimental
3 evidences on how the signal triggered by the binding of nucleotides to the
4 regulatory Bateman domain is transmitted to the catalytic domain to modulate
5 the enzymatic activity of IMPDH. To this respect, it has been recently described
6 a conformational switch -controlled by purine nucleotides- that modulates the
7 catalytic activity of eukaryotic IMPDH enzymes. The switch was first proposed
8 by crystallography and X-ray scattering using the enzyme AgIMPDH, from the
9 industrial fungus *Ashbya gossypii* [6] and was recently observed by electron
10 microscopy with HsIMPDH2, the second isoform of human IMPDH [5]. We
11 further demonstrate here that this conformational switch operates in both
12 human IMPDH isoforms, suggesting that it might be conserved within all
13 eukaryotic IMPDH enzymes. Moreover, a similar conformational switch,
14 modulated by adenine nucleotides, has also been reported for some prokaryotic
15 IMPDHs [2], despite the fact that these enzymes are not regulated by guanine
16 nucleotides [7]. This observation suggests that the conformational switch is
17 inherent to the acquisition of the Bateman regulatory domain, before the
18 divergence of prokaryotic and eukaryotic enzymes. Nevertheless, the latter
19 evolved separately to generate an additional non-canonical nucleotide binding
20 site that allows the inhibition of the enzyme by GTP and GDP, the end products
21 of the IMPDH's metabolic pathway.

22 Remarkably, we have found functional differences between the two
23 human isoforms of IMPDH, where GTP/GDP-mediated allosteric inhibition of
24 the catalytic activity is nicely correlated with octamer compaction in HsIMPDH1.
25 In contrast, HsIMPDH2 octamer compaction occurs at lower GTP/GDP
26 concentrations than those required for catalytic inhibition. These data, observed
27 both in HsIMPDH2- Y12A (Figure 1D) and HsIMPDH-WT (Figure 5D), indicate
28 that additional features, other than octamer compaction itself, are required to
29 inhibit HsIMPDH2. Possibly, the catalytic inhibition of IMPDH requires the
30 binding of GTP/GDP to the three allosteric sites within the Bateman domain,
31 while octamer compaction can happen with only a partial occupancy of these
32 sites. Thereby, distinct affinities of GTP/GDP among the three nucleotide-
33 binding sites of HsIMPDH1 and HsIMPDH2 might be the cause of the observed

1 differences. Additionally, our data show that HsIMPDH1 has a significantly
2 higher tendency to filament than HsIMPDH2, in accordance to previous reports
3 [23]. Future experimental studies will elucidate whether the different sensitivities
4 to GTP/GDP-mediated allosteric inhibition and/or the different propensities to
5 polymerize of the human IMPDH isoforms might have physiological
6 consequences.
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11 Presently, it is clearly accepted that the filamentation of enzymes to form
12 cytoophidia serves as a general mechanism for the regulation of metabolism by
13 fine-tuning protein functional and structural properties [12, 13]. Indeed, enzyme
14 filamentation seems to be rather extended with more than 20 metabolic
15 enzymes reported to filament in *S. cerevisiae* [24]. Perhaps the most
16 representative examples of cytoophidia-forming enzymes are those that
17 catalyze the limiting steps in the *de novo* biosynthetic pathways of pyrimidine
18 and purine nucleotides, CTP synthase (CTPS) and IMPDH, respectively.
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27 Human CTPS filaments lock the enzyme into an active conformation,
28 providing a mechanism for increasing the catalytic activity in response to
29 metabolic state [26]. Similarly, human IMPDH cytoophidia formation correlates
30 with rapid cell proliferation [11, 14, 15], however the *in vitro* reconstituted
31 polymers show identical activity for both polymerized and non-assembled
32 IMPDH [5]. In any case, the reported structural and functional data for IMPDH
33 cytoophidia *in vitro* are based on polymers, induced by ATP or GTP, that are
34 formed by the repeated back-to-back stacking of tetramers and octamers into
35 single filaments [4, 5], which do not represent the micrometer long and thick
36 bundles observed in cells [10, 19]. The results presented here demonstrate that
37 these polymers do not represent cytoophidia but rather the remnants of their
38 depolymerization. Our experimental conditions yield IMPDH polymer bundles *in*
39 *vitro* with similar dimensions to the micron-length cytoophidia found in cells.
40 Moreover, the dynamics of these polymers is modulated by purine nucleotides
41 *in vitro* in the same way as it has been observed in cells, as it will be discussed
42 below. Thereby, our conditions allow a much better biochemical and structural
43 characterization of this biological process.
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59 The nucleotide-mediated steric self-compatibility of the Bateman
60 interface and the highly symmetric structure of the octamers promote the
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assembly of IMPDH into the relatively thin and extended filaments that have been previously reported to form *in vitro* [4, 5]. Nonetheless, once these filaments are formed, they are not able to significantly associate side by side to form the bundles that constitute cellular cytoophidia. The higher order assembly into bundles of filaments proceeds only in the absence of nucleotides and seems to be a cooperative process, much less favorable than the nucleotide-induced single filament assembly is. Consistent with these observations, IMPDH assembly into micron-length cytoophidia depends on macromolecular crowding conditions, indicating that this assembly reaction is not only driven by enthalpy due to specific interface interactions, but rather is also driven by entropy as a consequence of excluded volume effects [27]. Indeed, in a crowded *milieu*, oligomerization is greatly favored to minimize the overall crowding by enhancing protein association, which can thereby reduce the excluded volume [28]. The cytoophidia assembled under these conditions are constituted by IMPDH octamers associated without an apparent order, although highly ordered specimens are also observed in the HsIMPDH2 micrographs. This observation indicates that the association of IMPDH octamers under crowding conditions confers high flexibility and dynamicity to the cytoophidia, which is in further agreement with the observed variety of shapes that easily inter-convert in cells.

Our *in vitro* results reconstitute what it is observed in cells: IMPDH assemble into micron-length cytoophidia upon guanine nucleotide depletion but cytoophidia disassemble after guanine levels recover. This observation might also apply for the adenine nucleotides, because it is assumed that the levels of the adenine and guanine nucleotide pools rise or decrease in parallel, at least to a certain extent. Several mechanisms explain the parallel changes in adenine and guanine nucleotide levels. Firstly, lowering the GTP levels might provoke a decrease in the adenine nucleotide levels through a dual action by: i) a reduced synthesis of AMP through adenylosuccinate synthetase, for which GTP serves as cofactor, and ii) a greater degradation of AMP by AMP deaminase, which is inhibited by GTP. Secondly, an interplay exists between the syntheses of AMP/ATP and GMP/GTP from IMP, where GTP is required to synthesize AMP

at the level of adenylosuccinate synthase, and ATP is required for the synthesis of GMP at the level of GMP synthase.

How do nucleotides modulate cytoophidia formation? A plausible explanation is that adenine and guanine nucleotides induce IMPDH octamer conformations that allow a few octamers to stack back-to-back and form short lineal filaments [4, 5], but these filaments do not significantly associate laterally to form the large bundles that constitute the cytoophidia. In contrast, in the absence of purine nucleotides, human IMPDH adopts a conformation that under crowding conditions favors both filament formation and filament bundling. A schematic representation of this hypothesis is shown in Figure 6. In support of this hypothesis, three different conformations for eukaryotic IMPDHs have been reported (Figure 6): i) ATP-induced extended active octamers (**5MCP**, [6]), ii) GTP/GDP-induced compacted inhibited octamers (**6I0O**, **6I0M** in this work and **4Z87** [7]), and iii) extended octamers with no nucleotide bound at the Bateman domain (**1NF7** and **1NFB**). The structures of the apo-enzyme and the ATP-induced IMPDH octamers have similar dimensions, but they significantly differ in the bending of the Bateman module that increases upon ATP binding (Figure 6). Therefore, it is then tempting to speculate that the less bent Bateman module of the apo-enzyme octamers favors the lateral packing of the single filaments that result in large micrometric bundles. On the contrary, the increased bending of the Bateman module, induced by the binding of either ATP or GTP/GDP to the allosteric sites, avoids the lateral association of octamers and single protofilaments that consequently avoids cytoophidia formation (Figure 6). Indeed, the crystal packing of **1NF7** (Supplemental Figure 8A) would be compatible with the repetition pattern observed in the ordered bundles of HsIMPDH2 found in some of the micrographs of the *in vitro* reconstituted cytoophidia (Figure 3B) and in a previous report [19], suggesting that this crystal might be mimicking the packing of IMPDH within the cytoophidia.

Our results demonstrate that assembly into cytoophidia does not significantly change the catalytic activity of purified IMPDH *in vitro*. These results agree with a previous report that could neither find differences in catalytic activity between a mutant unable to polymerize (HsIMPDH2-Y12A) and

1 the wild type enzyme, both *in vitro* and in cultured cells [5]. In turn, IMPDH
2 assembly modulates the sensitivity of the enzyme to allosteric inhibition.
3 Remarkably, the more mature the cytoophidia (longer incubation times) are, the
4 more resistant to GTP/GDP-induced octamer compaction they become and
5 thereby more resistant to depolymerization and subsequent inhibition of the
6 catalytic activity. In other words, the more mature a cytoophidia is, the longer it
7 takes to depolymerize by GTP/GDP. At this point, it remains unknown how
8 maturation time correlates to resistance to GDP-induced octamer compaction *in*
9 *vitro*, but it is clear that it might have important physiological implications. The
10 longer a cell remains in a state of guanine nucleotide deprivation, the longer it
11 will take to recover, i.e. the longer IMPDH should work actively in the absence
12 of negative feedback inhibition. Therefore, IMPDH cytoophidia formation and
13 maturation might constitute a mechanism to temporal and spatially regulate the
14 ability of IMPDH polymers to maintain especially high intracellular levels of
15 guanine nucleotides when required, e.g. for active cell proliferation or after a
16 pharmacological depletion of the guanine nucleotide pools. At this point, it is
17 important to stress that this study was performed *in vitro*, from purified
18 components, but it is envisaged that *in vivo* post-translational modifications and
19 interaction partners will play an important role in the regulation of the dynamics
20 of IMPDH cytoophidia assembly. Moreover, experiments with CTPS are
21 presently being performed in our laboratory, since a coordinated response to
22 simultaneously increase purine and pyrimidine nucleotide biosynthesis *de novo*
23 is expected as the result of CTPS and IMPDH filamentation.

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42 Finally, we demonstrate that the mutations in HsIMPDH1 associated to
43 human retinopathies map into the allosteric nucleotide binding sites, which
44 therefore alter the nucleotide-controlled conformational switch. Subsequently,
45 they also disrupt allosteric inhibition mediated by GTP/GDP and modify the
46 cytoophidia dynamics *in vitro*. Thereby, our data indicate that the HsIMPDH1
47 mutations within the first instance, will affect the intracellular levels of the purine
48 nucleotide pools in retinal cells that, in a medium/long term, will lead to visual
49 receptor cell death and subsequent retinal degeneration. Future studies are
50 needed to elucidate the molecular details on how the alterations in the
51 nucleotide pools develop into retinopathies. Moreover, it will also be very
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1 interesting to explore the potential physiological roles that cytoophidia play in
2 retinal development. Indeed, in preliminary experiments, we were able to detect
3 IMPDH cytoophidia in the retina of adult mice as 1-2 μ m long structures, mostly
4 localized in the periphery of the retina, although they were detected throughout
5 this organ (Supplemental Figure 8B). Altogether, these data indicate that
6 cytoophidia form in the retina *in vivo* under normal developmental conditions
7 and suggest a potential physiological role of cytoophidium that might be altered
8 by the retinopathy-associated missense mutations of human IMPDH1.
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10 In summary, our results provide novel and valuable information for
11 understanding the physiological regulation of IMPDH as well as deciphering the
12 putative roles of IMPDH cytoophidia *in vivo*, both under physiological and
13 pathological conditions. Moreover, given that IMPDH is a widely validated drug
14 target in several types of diseases, the reported results might eventually support
15 future studies to determine the therapeutic potential of the cytoophidium as a
16 useful target and/or biomarker.
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18 **Materials and Methods**

19 Proteins and nucleotides. Expression and purification of IMPDH proteins was
20 performed as previously described [7, 21]. Nucleotides were purchased from
21 Sigma-Aldrich and Jena Bioscience. Missense mutations were introduced into
22 the bacterial expression vectors by using QuickChange II Site-directed
23 mutagenesis protocol (Agilent Technologies).
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25 Protein polymerization analysis *in vitro*. Protein polymerization was monitored
26 by turbidimetry at 340 nm on a spectrophotometer and/or light scattering at 410
27 nm on a spectrofluorimeter. Control experiments were also performed by
28 pelleting the polymers by centrifugation and determining the protein
29 concentration in pellets and supernatants. Samples of protein polymers were
30 taken from each experiment for further analysis by electron microscopy (see
31 below).
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33 Enzyme kinetics assay. IMPDH activity was assayed, as previously reported [6],
34 at 32°C using 96 or 384-well microtiter plates by monitoring the reduction of
35 NAD⁺ and the subsequent increase in absorbance at 340 nm. Human IMPDH
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1 enzymes at 100 µg/mL in buffer 100 mM Tris-HCl, 100 mM KCl, 2 mM DTT,
2 1mM MgCl₂ (final free Mg⁺² concentration), pH 8.0 were assayed using as
3 substrates 0.5 mM NAD⁺ and from 0.019 to 5 mM IMP. The resulting
4 experimental data were fitted by non-linear regression analysis to the Michaelis-
5 Menten and -allosteric sigmoidal- equations using GraphPad Prism (GraphPad
6 Prism Software, Inc.).
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11 To monitor enzyme kinetics under macromolecular crowding conditions,
12 where turbidity might interfere with the measurement at λ=340 nm, the catalytic
13 reaction was stopped by incubating the samples with 4M urea (final
14 concentration) to disaggregate protein assemblies and avoid the turbidity of the
15 solution. These experiments demonstrated that the constant background at
16 λ=340 nm due to the presence of turbidity in the solution could be easily
17 subtracted from the raw measurements, without affecting the precise estimation
18 of the kinetic parameters.
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26 For control experiments, HsIMPDH1/2-WT and Y12A mutant enzymes
27 under macromolecular crowding conditions were centrifuged at 5.000 g during
28 10 minutes at 25°C, to pellet cytoophidia, and the catalytic activity of the
29 supernatants was measured as described above.
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34 Small Angle X-ray Scattering (SAXS). SAXS measurements were performed at
35 the B21 beamline at the Diamond Light Source. Protein samples at 2.5 mg/mL
36 in buffer 20 mM TrisHCl, 300 mM KCl, 1 mM MgCl₂ (final free Mg⁺²
37 concentration), 3 mM DTT, pH 8.0, were measured in the presence of
38 increasing amounts of nucleotides (total concentration of nucleotides ranging
39 from 0.02 to 3 mM). Experimental data were reduced following standard
40 methodologies by using the program PRIMUS. The fractions of tetramers,
41 expanded and compacted octamers were calculated with the program
42 OLIGOMER and the theoretical solution scattering profiles with CRY SOL. All
43 these programs are found within the ATSAS software suite [29].
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54 Crystallization and structure determination. Crystals of the complexes
55 HsIMPDH2-GTP and HsIMPDH2-GDP were grown at 22°C using the vapor
56 diffusion method by mixing a protein solution at 7.5 mg/ml in 10 mM Tris-HCl,
57 150 mM KCl, 1 mM DTT, 7.5 mM GTP (or GDP), 5 mM total MgCl₂, pH 8.0,
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1 with an equal volume of mother liquor consisting of 25% PEG-1500, 0.1 M
2 buffer MIB (malonic acid, imidazole and boric acid), pH 9.0, for HsIMPDH-GTP
3 and 0.1 M sodium citrate, pH 5.5, 0.2 M lithium sulfate and 15% ethanol, for
4 HsIMPDH2-GDP. Protein crystals were flashed-cooled in liquid nitrogen and
5 data were collected at 100 K, using monochromatic X-rays of ≈ 1 Å wavelength,
6 at the MX beamlines of the Diamond Light Source synchrotron (UK) or the
7 XALOC beamline in the ALBA synchrotron (Spain). Crystals of HsIMPDH2-
8 GDP were cryo-protected by immersion in NVH oil before being flashed-cooled
9 in liquid nitrogen. Diffraction intensities were indexed and integrated by using
10 the software autoPROC [30].
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19 The experimental data were phased by molecular replacement using the
20 program PHASER [31] using as template the structure of the complex
21 AgIMPDH-GDP (PDB code 4Z87). The structures were iteratively refined by
22 alternating manual modelling with COOT [32] with the automated refinement
23 utility of the PHENIX crystallographic software suite [33]. Rigid body, gradient-
24 driven positional, restrained individual isotropic B-factor and TLS were used for
25 structure refinement.
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32 Electron microscopy. Samples were analyzed by electron microscopy after
33 being adsorbed to glow-discharged carbon coated grids and stained with 2%
34 uranyl acetate. Grids were observed using a JEOL JEM-1230 transmission
35 electron microscope operated at 100 kV and a nominal magnification of 40.000.
36 Images were taken under low dose conditions with a CMOS Tvips TemCam-
37 F416 camera, at 2.84 Å per pixel. Image processing was performed using
38 SCIPION package [34]. The contrast transfer function of the microscope for
39 each micrograph was estimated using CTFFIND4 [35]. Helical segments were
40 picked from the micrographs using the Xmipp3 manual-picking routine
41 implemented inside SCIPION.
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51 In the initial step, images of the helical segments were extracted and
52 horizontally pre-aligned using a multi-reference pattern-free alignment protocol
53 [36]. After pre-alignment images were two-dimensional classified using RELION
54 software [37] limiting in-plane rotations, allowing bimodal search and using a
55 rectangular mask to focus the classification process in the helix. Classes
56 containing the straightest particles were selected for 3D reconstruction. Initial
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1 model was built by assigning random rotation angles to the images and 3D
2 reconstructed. This procedure generates featureless tubular structures suitable
3 as a starting model for 3D classification in RELION; this classification was
4 performed with no symmetry application to avoid initial bias in the
5 reconstructions. Afterwards, the selected classes were also refined without the
6 application of helical symmetry. When the angular assignment of the images
7 reached convergence, symmetry parameters of the volumes were determined
8 using the 'xmipp_volume_find_symmetry' utility and applied. Finally, a new cycle
9 of refinement, applying symmetry, was carried out to generate the final
10 structures.
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19 Figures of protein structures, electron density maps and electron
20 microscopy maps were rendered by using PyMOL (The PyMOL Molecular
21 Graphics System, Version 2.0 Schrödinger, LLC).
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25 *Immunofluorescence*. HeLa cells were grown onto poly-L-Lysine coated glass
26 coverslips and fixed with 2.5% paraformaldehyde for 30 minutes at room
27 temperature, washed 3 times with PBS, permeabilized during 10 minutes with
28 Triton 0.1% X100 and blocked with 1% BSA for another 10 minutes. The
29 primary antibody (mouse anti-HA 12CA5, Roche) was used at a 1:5000 dilution
30 and incubated for 2 hours at room temperature. The secondary antibody (Alexa-
31 488 anti-mouse) was used at a 1:2000 dilution and incubated overnight at 4°C.
32 Cells were then washed with 1% BSA and the nuclei were stained with DAPI.
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40 Mice retinas were fixed by immersion in 4% paraformaldehyde for 4
41 hours at 4°C. The tissue was cryo-protected in 30% sucrose, embedded in OCT
42 and 16 µm sections were obtained. Sections were washed in PBS, blocked with
43 5% BSA, 2% goat serum and 0.1% Triton X100. Primary antibody (IMPDH, H-
44 300, Santa Cruz, sc-50510) was incubated at a 1:30 dilution overnight at 4°C
45 and detected with a secondary antibody anti-rabbit Alexa-488 used at a 1:300
46 dilution, for 1 hour at room temperature. Nuclei were stained with DAPI.
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56 **Accession numbers**

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The atomic coordinates and the structure factors described in this work have been deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank under the codes PDB ID: 6I0O (HsIMPDH2-GTP) and PDB ID: 6I0M (HsIMPDH2-GDP). The electron microscopy-derived maps have been deposited in the Electron Microscopy Data Bank under the accession codes EMD ID: 4402 (HsIMPDH1-GDP) y EMD ID: 4403 (HsIMPDH1-ATP).

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Figure captions

Figure 1. *The conformational switch of HsIMPDH controls its catalytic activity.*

A-B. Experimental SAXS profiles of HsIMPDH1 (**A**) and HsIMPDH2 (**B**) wild-type and mutant Y12A in the absence and presence of the indicated purine nucleotides. **C-D.** Plots representing the fraction of compact octamers, as determined by SAXS, (empty symbols and dashed lines) and the catalytic activity ($V_{\max}^{\text{app}} / V_{\max}$; filled symbols and continuous lines) *versus* GDP concentration for HsIMPDH1-Y12A (**C**) and HsIMPDH2-Y12A (**D**). Squares: 3 mM ATP fixed concentration *versus* increasing concentrations of GDP. Circles: increasing concentrations of GDP in the absence of ATP. Data points represent the mean value and the standard errors. Lines represent the non-linear regression analysis of the experimental data, according to a standard allosteric sigmoidal equation (GraphPad Prism). At the fixed concentrations of ATP (3 mM) and GDP (3 mM) used for these experiments, HsIMPDH1-Y12A and HsIMPDH2-Y12A proteins remained as octamers with no detectable fraction of tetramers, as determined by SAXS.

Figure 2. *Retinopathy-associated missense mutations disrupt GTP/GDP-mediated allosteric inhibition in vitro.* Normalized catalytic activity ($V_{\max}$ derived from the Michaelis-Menten analysis of the experimental data) of HsIMPDH1-WT and retinopathy-associated mutants in the absence or presence of purine nucleotides. Error bars represent standard errors.

Figure 3. *In vitro reconstitution of human IMPDH cytoophidia assembly.* **A.** Turbidity measurements, indicating the formation of large polymers of HsIMPDH2, at different Ficoll-70 concentrations in the presence or absence of purine nucleotides. **B.** Electron microscopy analysis of the samples in the absence (left panels) and presence (right panels) of 150 g/L Ficoll-70. Upper panels: no nucleotide; middle panels: 3 mM ATP; lower panels: 5 mM GDP. Scale bars are 100 nanometer long. The red rectangle in the upper right panel shows bundles of twisted and curved filaments that intricate into the three dimensions without apparent regular or defined structure, while the blue rectangle shows ordered bundles of parallel protofilaments that were sporadically observed. **C.** Turbidity measurements of purified IMPDH from different organisms. HsIMPDH1, *Homo sapiens* isoform 1; HsIMPDH2: *Homo*

sapiens isoform 2; AglIMPDH: *Ashbya gossypii*; EcIMPDH: *Escherichia coli*; BslIMPDH: *Bacillus subtilis*. Error bars represent standard errors.

Figure 4. *Retinopathy-associated missense mutations alter IMPDH cytoophidia assembly in vitro.* Turbidity measurements, indicating the formation of large polymers *in vitro*, at 150 g/L Ficoll-70 of HsIMPDH1 wild-type and retinopathy-associated mutants in the absence or presence of purine nucleotides. Data were taken 10 minutes after dilution of the proteins into a solution containing Ficoll-70 and the indicated nucleotides. Error bars represent standard errors.

Figure 5. *Human IMPDH cytoophidia are catalytically active and resistant to GDP allosteric inhibition.* **A-B.** Michaelis-Menten representation of the catalytic activity of HsIMPDH1 (**A**) and HsIMPDH2 (**B**) wild-type (squares) and Y12A mutant (circles) in a dilute solution (solid Figures and continuous green lines) and in the presence of 150 g/L Ficoll-70 (empty Figures and discontinuous red lines). **C-D.** Plots representing normalized solution turbidity (dotted lines) and catalytic activity ($V_{\max}^{\text{app}} / V_{\max}$; solid Figures and continuous green line in the absence of Ficoll-70, empty Figures and dashed red line for the short incubation with Ficoll-70 and empty Figures and dashed blue line for the long incubation with Ficoll-70) at different GDP concentrations for HsIMPDH1-WT (**C**) and HsIMPDH2-WT (**D**). In our experimental setup, the measurements were performed approximately 30 minutes after GDP addition. Lines represent the non-linear regression analysis of the experimental data, according to a standard allosteric sigmoidal equation (GraphPad Prism). Error bars represent standard errors.

Figure 6. *The nucleotide controlled-conformational switch of human IMPDH.* Upper panels: the three different octamer conformations observed in eukaryotic IMPDH enzymes and their respective PDB codes. IMPDH protein is shown in cartoons and nucleotides in sticks. The approximate longitudinal dimensions of the octamers are indicated on their side. Middle panels: close-up view of two interacting Bateman domains from adjacent tetramers within the octameric arrangement. The black dashed lines show the approximate longitudinal axis of helices comprising residues 194-204. Lower panels: schematic representation of the assembly of human IMPDH into different polymers.

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FIGURE 1

A

B

C

D

FIGURE 2

FIGURE 3

A

B

C

FIGURE 4

FIGURE 5

A

B

C

D

FIGURE 6

**APO
(ACTIVE)
1NF7**

~115 Å

**ATP
(ACTIVE)
5MCP**

**GTP
(INHIBITED)
6I0O**

~95 Å

Supplementary Material (To be Published)

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