Transient Kinetic Investigation of GTP Hydrolysis Catalyzed by Interferon-γ-induced hGBP1 (Human Guanylate Binding Protein 1)*

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Within the family of large GTP-binding proteins, human guanylate binding protein 1 (hGBP1) belongs to a subgroup of interferon-inducible proteins. GTP hydrolysis activity of these proteins is much higher compared with members of other GTPase families and underlies mechanisms that are not understood. The large GTP-binding proteins form self-assemblies that lead to stimulation of the catalytic activity. The unique result of GTP hydrolysis catalyzed by hGBP1 is GDP and GMP. We investigated this reaction mechanism by transient kinetic methods using radioactively labeled GTP as well as fluorescent probes. Substrate binding and formation of the hGBP1 homodimer are fast as no lag phase is observed in the time courses of GTP hydrolysis. Instead, multiple turnover experiments show a rapid burst of Pi formation prior to the steady state phase, indicating a hydrolysis. As no lag phase is observed in the time courses of GTP Substrate binding and formation of the hGBP1 homodimer are using radioactively labeled GTP as well as fluorescent probes. Gated this reaction mechanism by transient kinetic methods ranging from protein translation, signal transduction, and cellular functions that can be grouped in two classes (2). Dynamin and dynamin-like proteins play a key role in membrane scission and fusion, whereas interferon-induced proteins like Mx and hGBP1 (human guanylate binding protein 1) are involved in antiviral defense (3, 4). Being a member of this superfamily is not decided only by the molecular size of the proteins, ranging between 65 and 100 kDa, but also by common biochemical properties and structural features. In contrast to small GTPases like Ras, Rab, Rho, etc. and α-subunits of heterotrimeric G proteins, they bind the guanine nucleotides GDP and GTP not very tightly thus showing $K_{d}$ values in the micromolar range (5). This low affinity is caused by fast dissociation rates as compared with small GTPases. Also GTP hydrolysis is faster and, more strikingly, is enhanced by self-association of the large GTPases. For dynamin and Mx, the formation of helical assemblies after binding of GTP is described (6–9), whereas hGBP1 forms homodimers when bound to GTP (10, 11).

The most conspicuous difference to other GTPases is the C-terminal subdomain takes part in the activation of GTP hydrolysis and is therefore named GTPase effector domain. How exactly the extra domains work in respect to enhanced GTP hydrolysis and nucleotide dissociation and how far they might replace regulatory proteins that are observed for other GTPases have not been resolved (12–16).

Mutations of residues critical for catalysis of GTP hydrolysis were identified in many GTPases resulting in malfunction and diseases. The most prominently known are point mutations in small GTP-binding proteins like Ras and Rho leading to cancer (17), but also mutations in large GTPases like dynamin, atlastin, and OPA1 leading to impairment of GTP hydrolysis may result in disease (18–21). Although all large GTPases are extraordinary in respect to the role and the mechanism of GTP hydrolysis being linked to self-assembly, hGBP1 is even more unique as the result of catalyzed GTP hydrolysis is phosphate and GMP

GTP-binding proteins control a multitude of cellular functions ranging from protein translation, signal transduction, and regulation of the cytoskeleton to endocytosis and immunological response. Timed and specific action of these proteins is achieved by adopting different conformations when in complex with either GDP or GTP (1). Only in one of the two states, in most cases the GTP-bound form, is the GTPase capable of fulfilling its function by specific interaction with other proteins.

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to the major part and only a small fraction of GDP (22). Earlier we have characterized affinity and binding kinetics of all three guanine nucleotides and have shown homodimer formation of hGBP1 only upon binding to GTP analogs (11, 23, 24). In contrast, the GDP as well as the GMP form of hGBP1 are monomers, and GDP hydrolysis is not detected. This study addresses the mechanism of GTP hydrolysis by a transient kinetic approach. Using rapid flow techniques allows us to resolve successive steps in the course of GTP hydrolysis.

EXPERIMENTAL PROCEDURES

Protein Preparation—hGBP1 with an N-terminal His$_6$ tag was synthesized from a pQE9 vector (Qiagen, Hilden, Germany) in Escherichia coli strain BL21(DE3) and purified as described previously (23). The concentration of hGBP1 was calculated from the absorbance at 276 nm in 20 mM potassium phosphate, pH 6.5, using a molar absorption coefficient of 45,400 m$^{-1}$ cm$^{-1}$ (23).

Nucleotides—GTP was purchased from Sigma and was purified by ion exchange chromatography as described by Lenzen et al. (25). Mant-nucleotides were synthesized according to Ref. 25. The purity of the nucleotides was greater than 98% as verified by high pressure liquid chromatography (25). Nucleotide concentrations were determined from the absorbance at 253 nm using the absorption coefficients 13,700 and 22,600 m$^{-1}$ cm$^{-1}$ for nonlabeled and mant-nucleotides, respectively (26).

Rapid Quench Flow Kinetics—Transient enzyme kinetics were investigated with the help of the rapid quench flow technique using an RQF-3 instrument (KinTek Corp., Austin, TX). To obtain time courses of GTP hydrolysis, solutions of (nucleotide-free) hGBP1 and GTP containing 10 nCi/mg [α-32P]- or [γ-32P]GTP (Amersham Biosciences) were rapidly mixed (15 μl each) in a first mixing device. The reactions were stopped after different time intervals by using 1 M perchloric acid as the quenching solution in the second mixing step. The pH value of quenched reaction mixtures was raised to about 5 by addition of potassium acetate. The solutions were centrifuged (3 min at 15,000 g) to remove precipitated potassium perchlorate and protein, and the supernatant was subjected to further analysis.

Stopped-flow measurements were performed in an SFM400 apparatus with MOS-200 optics (Bio-Light, Grenoble, France). In competitive binding experiments, hGBP1 was mixed with solutions of constant mant-GMP and increasing GTP concentrations. Nucleotides were in at least 5- or 10-fold excess over protein, providing pseudo-first order conditions in case the dissociation rates of both nucleotides can be neglected (27, 28). Dissociation rate constants of GDP and GMP were measured by displacing the (nonlabeled) nucleotides from the preformed complexes by a 20- or 40-fold excess of mant-GDP. Fluorescence was excited at 295 nm and detected after passing through a 400 nm cut-off filter. In both types of experiments fluorescence resonance energy transfer between tryptophan residues in hGBP1 and the mant-fluorophor on the nucleotide was used because direct excitation of the mant-group leads to high fluorescence background.

Kinetics of P$_i$ Release—P$_i$ release was studied according to the method of Brune et al. (29), which is based on the fluorescence change of a coumarin-labeled phosphate-binding protein (PBP) from E. coli. The A197S mutant of PBP was prepared and labeled with N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC) as described by Brune et al. (30). Following this procedure we obtained protein, which was labeled to more than 93%, as determined from the absorbance at 280 and 430 nm. The P$_i$ responsiveness of our preparation was analyzed by fluorescence titration using an LS-55 spectrophotometer (PerkinElmer Life Sciences). MDCC-PBP at 125 μM was titrated with a potassium phosphate solution containing the same MDCC-PBP concentration. Excitation and emission wavelength were 430 nm and 465 nm, respectively. Kinetics of P$_i$ release were measured under single turnover conditions using the SFM400 stopped-flow instrument. The concentrations of hGBP1 and GTP were the same as in the quench flow experiments. MDCC-PBP was added to both nucleotide and hGBP1 solutions. The fluorescence was excited at 430 nm and monitored after passing through a 455 nm cut-off filter. The P$_i$ concentration was obtained from the fluorescence signal after normalization to the P$_i$ concentration calculated from a parallel rapid quench flow experiment. To minimize phosphate contamination, the fluorescence cuvettes and the stopped-flow apparatus were incubated for 30 min with 400 μM 7-methylguanosine and 1 unit/ml purine nucleoside phosphorylase before the measurements (29). All experiments were carried out at least in duplicate in 50 mM Tris-HCl, pH 8.0, 5 mM MgCl$_2$, 2 mM dithioerythritol at 25 °C unless indicated otherwise.

Data Analysis—Analysis of single transients was performed by nonlinear regression, using the program Grafit (Erithacus Software). Single-turnover kinetics of GTP hydrolysis lead to three nucleotide time courses that were analyzed simultaneously by a global fit using the program Scientist (MicroMath). For this kind of analysis the differential equations derived from Scheme 2 were used yielding the values for $k_1$, $k_2$, and $k_3$. Correspondingly, and with the same program, the kinetics of the multiple turnover experiments were analyzed according to Scheme 3, which results in the value for $k_4$. Here, the constants as indicated in Scheme 3 were used as fixed values, and for the fast association and dissociation rate constant values of 100 μM$^{-1}$ s$^{-1}$ and 100 s$^{-1}$ were used, respectively.

[2] The abbreviations used are: mant-, N-methyl-anthraniloyl; MDCC, N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide; PBP, phosphate-binding protein.
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\[
\text{Scheme 1:} \quad \frac{[\text{E} \cdot \text{GTP}]_2}{k_1} \xrightarrow{[\text{E} \cdot \text{GDP} \cdot \text{P}]_2} \frac{2 \text{E + products}}{k_f}
\]

obtained for the rate constants are \(k = 0.74 \text{s}^{-1}\) and \(s = 0.25 \text{s}^{-1}\). This type of kinetics demands a minimum of two reaction steps as shown in Scheme 1. According to Scheme 1, there are three constants representing the rate of the first step, \(k_1\), and all of following steps (at least one), \(k_f\), and the concentration of catalytically active enzyme, \([E]_0\). They define the three parameters of Equation 1 according to Equations 2–4 (31).

\[
A = \frac{[E]_0}{[E]_0(k_1 + k_f)^2} \quad \text{(Eq. 2)}
\]

\[
k = k_1 + k_f \quad \text{(Eq. 3)}
\]

\[
s = \frac{[E]_0}{[E]_0} \quad \text{(Eq. 4)}
\]

Analysis of the phosphate transient in Fig. 1 on the basis of Scheme 1 by using Equations 1 to 4 yields the rate constants \(k_1 = 0.50 \text{s}^{-1}\) and \(k_f = 0.27 \text{s}^{-1}\). Thus the first step is not much faster than the following leading to a burst amplitude smaller than 1. Surprisingly, the fit results in a value larger than 1 for the active enzyme fraction, \([E]_0/[E]_0\), which could be explained by a systematic error on the protein and/or nucleotide concentration.

At this stage of kinetic analysis only \(\gamma\)-phosphate is detected. Further potential steps in the catalytic mechanism like GDP hydrolysis and product release are dismantled in \(k_f\). With this type of experiment and the use of \([\gamma\text{-}^{32}\text{P}]\text{GTP}\), formation of GMP was not possible to detect. Nevertheless, it is worthwhile to mention that no formation of pyrophosphate was observed in the course of these experiments. As a positive control in TLC, radioactive pyrophosphate generated from \([\gamma\text{-}^{32}\text{P}]\text{GTP}\) by incubation with phosphodiesterase was used.

Single-turnover Kinetics—In a next step we studied the hydrolysis of GTP under single-turnover conditions where the kinetics of nucleotide release are not relevant. To this end we used \([\alpha\text{-}^{32}\text{P}]\text{GTP}\), which allows us to follow the transients of all nucleotides. The reaction is started by mixing equal volumes of 150 \(\mu\text{M}\) hGBP1 and 70 \(\mu\text{M}\) \([\alpha\text{-}^{32}\text{P}]\text{GTP}\) in a rapid quench flow apparatus. Typical results of this kind of experiment are shown in Fig. 2.

As already known not only GDP but also GMP is formed by hGBP1-catalyzed hydrolysis of GTP. In the experiments of the previous section only formation of phosphate ions was observed, and no pyrophosphate was seen. Here it is most evident that GDP is generated in a first step followed by cleavage of another phosphate ion leading to the formation of GMP. At the beginning of the time trace of GMP a clear lag phase is observed in Fig. 2, which is the result of the successive phosphate cleavage mechanism. In particular, the experiment at 37°C in Fig. 2B shows that GDP is formed as an intermediate, which is for the most part hydrolyzed further and yields GMP as the major product.

The data in Fig. 2 can be explained by Scheme 2, which includes two successive hydrolysis steps and an inactivation
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step that we assume to be dimer dissociation. Competition of the second step of hydrolysis and the inactivation accounts for the final ratios of the products GMP and GDP, which are 72:28 at 25 °C and 95:5 at 37 °C. Global fit analysis according to Scheme 2 and as described under “Experimental Procedures” yields the rate constants

$$k_1 = 0.43 \text{s}^{-1}, \quad k_2 = 2.2 \text{s}^{-1}, \quad \text{and} \quad k_3 = 0.87 \text{s}^{-1} \text{ at } 25 \text{ °C and } k_1 = 3.9 \text{s}^{-1}, \quad k_2 = 8.03 \text{s}^{-1}, \quad \text{and} \quad k_3 = 0.40 \text{s}^{-1} \text{ at } 37 \text{ °C.}$$

Most notably, the first step is slower than the following, which cannot explain the phosphate burst behavior.

Kinetics of Substrate Binding and Product Release

Substrate Binding Kinetics Using Stopped-flow—Elucidation of the mechanism of GTP hydrolysis presupposes knowledge about the binding kinetics of the substrate. As we have used nucleotide analogs carrying a fluorescence label in our earlier studies, we determined here the binding kinetics of GTP, i.e. the genuine substrate that we also used for the investigation of the hydrolysis above. For this purpose we followed the binding kinetics after mixing in a stopped-flow apparatus two equal volumes of 1.0 mM hGBP1 and 5.4 mM fluorescent mant-GMP, and from top to bottom 0, 3, 9, 15, and 24 mM GTP. The pseudo-first order rate constants were determined by single exponential curve fitting. B, plot of the observed rate constants versus GTP concentration. The two sets of experiments were carried out at 0.5 mM hGBP1 and 2.7 mM mant-GMP (○) or 6 mM mant-GMP (●). The linear fits yield $k_{on}^{GTP}$ (slope) and $k_{on}^{mant}$ (intercept) according to Equation 5.

$$k_{obs} = k_{on}^{GTP} \cdot [GTP] + k_{on}^{mant} \cdot [mant-GMP]$$

(Eq. 5)

Fig. 3A shows typical fluorescence time traces that yield the $k_{obs}$ values by fitting an exponential equation. The results are shown in Fig. 3B together with the linear fit. The ordinate yields the
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FIGURE 4. Cold substrate chase kinetics. In a rapid quench flow apparatus, the rate of association of 2.3 nm [γ-32P]GTP and 20 (●) or 40 μM (○) hGBP1 was measured (concentrations after mixing). The solid lines represent the fitted single exponential curves, yielding the observed rate constants $k_{\text{obs}} = 45$ s$^{-1}$ and $k_{\text{obs}} = 88$ s$^{-1}$ at 20 and 40 μM hGBP1, respectively.

$k_{\text{on}}$ value for mant-GMP, which is 4.5 μM$^{-1}$ s$^{-1}$ in the first set of experiments and 5.7 μM$^{-1}$ s$^{-1}$ in the second. These values are in good agreement with our earlier measurement of this value, namely 5.3 μM$^{-1}$ s$^{-1}$ at 20°C (24). A value of $k_{\text{off}}^\text{GTP} = 3.1$ μM$^{-1}$ s$^{-1}$ (second set 3.0 μM$^{-1}$ s$^{-1}$) is obtained from these data for the association of hGBP1 and its substrate GTP. This is close to the $k_{\text{on}}$ value measured for mant-GTP (2.6 μM$^{-1}$ s$^{-1}$ at 20°C (32)), and it shows that substrate binding is very fast compared with hydrolysis at the concentrations we have used above.

Substrate Binding Kinetics Using Cold Substrate Chase—We determined the kinetics of GTP binding to hGBP1 by another independent method that does not necessitate the use of any fluorescent compounds. Cold substrate chase experiments allow quantification of the amount of bound GTP at individual time points by taking advantage of the enzymatic cleavage reaction. In these measurements mixtures of hGBP1 and [γ-32P]GTP were aged for short periods of time in a rapid quench flow device, and the binding reaction was stopped by the addition of a large excess of nonradioactive (cold) GTP. The mixtures were incubated for a few reciprocal turnover numbers (see above), i.e. 20 s, just enough to completely cleave the bound radioactive GTP. Then they were quenched in acid and analyzed for radioactive phosphate. The fraction of radioactive GTP, which has bound to the enzyme before the addition of cold substrate, is converted to product, whereas free labeled substrate is greatly diluted out and no longer participates in the binding and cleavage reactions. Thus, the amount of radioactive phosphate is proportional to the concentration of the enzyme-substrate complex formed during the first incubation period. Fig. 4 shows typical cold GTP chase experiments reflecting the association kinetics of 2.3 nm GTP and 20 and 40 μM hGBP1, respectively. Single exponential curve fitting yields the pseudo-first order rate constants $k_{\text{obs}}$, which are 45 and 88 s$^{-1}$ at 20 and 40 μM hGBP1, respectively. From these values the association rate constant $k_{\text{on}} = 2.2$ μM$^{-1}$ s$^{-1}$ (1.7 μM$^{-1}$ s$^{-1}$ with a different protein preparation) is calculated, which is in good agreement with the result from the competitive stopped-flow experiments above. As the dissociation rate constant of the hGBP1-GTP complex is small compared with the $k_{\text{obs}}$ values, it cannot be determined accurately from these measurements. However, 100% $P_i$ production is not reached (Fig. 4), which indicates that the GTP dissociation rate constant is of similar magnitude as the hydrolysis rate constant.

Rate of Phosphate Release—In some nucleotide cleavage reactions the rate of phosphate release was found to be rate-limiting and responsible for the phosphate burst kinetics. For example, phosphate release in myosin ATPase turned out to be a crucial step triggering large conformational changes of the enzyme (33, 34). To address this issue we used a PBP that allows us to monitor in real time the increases of phosphate concentration because of dissociation from hGBP1 complexes. This assay is based on PBP covalently labeled with the fluorescent coumarin derivative MDCC, which binds phosphate ions rapidly ($k_{\text{on}} > 100$ μM$^{-1}$ s$^{-1}$) and tightly ($K_d = 0.1$ μM) accompanied by a large increase of its fluorescence intensity (29). Fig. 5A shows a phosphate titration of our MDCC-PBP preparation (at 125 μM) demonstrating a linear response and an active site fraction of 95% under our experimental conditions.

Phosphate release kinetics were studied with the help of a stopped-flow apparatus at 75 μM hGBP1 and 35 μM GTP. In addition, MDCC-PBP was present at 125 μM. The recorded fluorescence change is shown in Fig. 5B. From the single exponential fit to the data, a value of 0.41 s$^{-1}$ was obtained for the rate constant matching the value of $k_1$ measured above. Parallel to this experiment, a single-turnover experiment at the same GTP and hGBP1 concentrations was performed by rapid quench flow as described above. [α-32P]GTP was used and no was MDCC-PBP added, and the time courses of nucleotide concentrations were assayed as described. Quantitative comparison of phosphate and nucleotide concentrations necessitates multiplication of the GMP concentration by a factor of 2 as two phosphate ions stem from this nucleotide. The sum of the concentrations of GDP and GMP (times two) are shown in Fig. 5C together with the experimental fluorescence change from Fig. 5B. Here in Fig. 5C the maximum fluorescence value is normalized to a phosphate concentration of 59.5 μM (= 1.7 × 35 μM) as 70% GMP is formed at this temperature. The time course of nucleotide production and the fluorescence trace representing the released phosphate concentration overlap nicely. The same experiments were done at 37°C where even more GMP is produced. Again, the kinetics of phosphate release represented by the fluorescence trace and the sum of GDP and two times GMP concentrations were almost perfectly overlaid (data not shown). Both the match of the nucleotide and fluorescence curves and the rate of phosphate release equaling the rate of GTP hydrolysis demonstrate that phosphate release is faster than nucleotide cleavage and that the rate of phosphate release cannot be responsible for the phosphate burst behavior.

Rate of Nucleotide Release—To explain the phosphate burst behavior in multiple turnover kinetics and to identify a slow, rate-limiting step at the end of the reaction cycle, we measured the rate constants of GDP and GMP dissociation. From earlier studies we already know the rate constants for the dissociation...
of mant-GDP and mant-GMP at 25 °C, which are 14 and 2.1 s⁻¹, respectively (23). Here we wanted to rule out any influence of the mant group on the dissociation kinetics, and we performed displacement experiments. The preformed complex of hGBP1 and unlabeled GMP (10 μM hGBP1 and 30 μM GMP) was mixed in a stopped-flow experiment with mant-GDP at a 20- or 40-fold molar excess over the unlabeled nucleotide (Fig. 6). As a $k_{\text{on}}$ value of 3.4 μM⁻¹ s⁻¹ was determined earlier for mant-GDP (23), under these conditions the observed rate constant corresponds to the dissociation rate constant of hGBP1 and GMP. For both mant-GDP concentrations the same value was obtained, $k_{\text{off}}^{\text{GMP}} = 20$ s⁻¹. In the same way the dissociation rate constant of the hGBP1-GDP complex was determined, $k_{\text{off}}^{\text{GDP}} = 26$ s⁻¹, where the two values obtained with 20- and 40-fold molar excess mant-GDP were less than 5% different from their mean value. These results demonstrate that the dissociation rates observed for GMP and GDP are 10- and 2-fold faster than for the mant-nucleotides, respectively. In contrast, our experiments on the GTP-binding rate have shown that the mant label does not strongly influence the association of nucleotide and hGBP1.

**DISCUSSION**

Our earlier biochemical and structural studies have shown that GTP binding to hGBP1 is followed by dimer formation, which leads to stimulation of catalytic hydrolysis activity (11, 23, 24). The reaction products are phosphate ions, 5–15% GDP and 85–95% GMP at 37 °C. Our recent x-ray structures show an
arginine residue placed into a position close to the phosphate moiety of the nucleotide after dimerization and a shift of the nucleotide toward the catalytic center after the first cleavage step (10). This study addresses the molecular mechanism of nucleotide hydrolysis catalyzed by hGBP1 by investigation of the transient kinetics. The experiments addressing the hydrolysis rates were focused on conditions where dimer formation of the GTP complex is saturated. This means that our results characterize the catalytic properties of the hGBP1 dimer. A crucial experiment in this study is the multiple turnover kinetics shown in Fig. 1. Clearly, a phosphate burst phase is observed that indicates that GTP cleavage is followed by some slower reaction. Classical studies on the mechanisms of enzymatic nucleotide hydrolysis have shown that conformational changes of the enzyme as well as the dissociation of the phosphate or the nucleotide products may represent a rate-limiting step at a later stage of the catalytic reaction cycle. We tried to find out which of these possibilities applies for the mechanism of hGBP1-catalyzed GTP hydrolysis, and we investigated the occurrence of potential intermediate steps.

The observation of a product burst and the absence of a lag phase immediately rules out that reaction steps before the chemical cleavage step are slow and limiting the steady state phase. Nevertheless, we used the genuine substrate, GTP, in order to define the rate of association. The two experimental methods applied, competitive stopped-flow kinetics and cold substrate chase, yielded \( \kappa_{on} \) values of 3 and 2 \( \mu \text{M}^{-1} \text{s}^{-1} \), respectively. With the concentrations used in all the other experiments, this means that GTP binding is much faster than hydrolysis. In addition, no evidence was found for any rate limitation because of formation of the hGBP1 dimer after GTP binding. Summarizing, substrate binding, and dimer formation are fast under the experimental conditions used here for the study of the hydrolysis kinetics.

To our surprise the phosphate burst followed by a slower process could not be rationalized by the rate of GDP hydrolysis or the inactivation step because both turned out to be too fast as shown by the single-turnover experiments in Fig. 2. Referring to the x-ray structures of hGBP1 in different nucleotide states, i.e. GDP-AlF\(_3\) and GMP-AlF\(_3\) representing the GTP and GDP bound forms, respectively, we come to another interesting conclusion. These structures suggest a shift of the nucleotide toward the catalytic center after GTP hydrolysis by positioning the \( \beta \)-phosphate of GDP at the same place as the \( \gamma \)-phosphate of GTP was located before (near arginine 48) (10). Now, according to the fast rate which we observe for the second hydrolysis (\( k_2 = 2.2 \text{ s}^{-1} \)), the movement of the nucleotide within the binding pocket does not represent a slow step limiting the steady state rate. Conformational changes of the protein in order to shift the nucleotide position are fast. In fact, 2.2 \( \text{ s}^{-1} \) represents the lower limit because we cannot decide if \( k_3 \) is controlled by the nucleotide shift or the cleavage event.

The rate of product release may be responsible for the burst behavior and control the steady state rate. Displacement of GDP and GMP, respectively, from the hGBP1 complex by competing with a large molar excess of mant-nucleotide yielded the dissociation rate constants, \( \kappa_{off} \), for the two products GDP and GMP. The large values of 26 and 20 \( \text{s}^{-1} \), respectively, cannot account for the observed steady state rate. The dissociation of phosphate, the other product of nucleotide hydrolysis, was monitored by the use of a fluorescent phosphate-binding protein (29). The observed time course was compared with the time course of phosphate cleavage calculated from the measured GDP and GMP concentrations. The two time courses in Fig. 5C were almost perfectly overlaid, even within the initial lag phase. This observation suggests that phosphate dissociation is faster than the precedent cleavage step. Obviously, the covering of the nucleotide, including the phosphate moiety by the protein as observed in the x-ray structures, does not represent any hindrance for the phosphate ion to escape. In conclusion, the steady state rate is not controlled by the release rate of any product.

After all the rate constant values were determined and estimated, so far none of the potential reaction steps following GTP hydrolysis can account for the phosphate burst kinetics. This observation can be rationalized by a slow step after reaching the GMP state represented by \( k_4 \) in Scheme 3. By fitting the \( P_i \) burst experiments (Fig. 1) according to Scheme 3 by fixing the rate constants to the values determined by the other experiments, a value of \( k_4 = 0.26 \text{ s}^{-1} \) is obtained. A likely explanation is the dissociation of the dimer or a conformational change associated with this. In contrast to the GTP state, dimer dissociation is irreversible for both the GDP and GMP states, because no dimers are observed with other methods when hGBP1 is bound to one of these two nucleotides. Of all dimer species the GMP-bound state has the longest lifetime of about 4 s. Thus the GMP-bound state is populated significantly and also long enough to mediate distinct but so far unknown functions. An alternative possibility not reflected in Scheme 3 is a slow dissociation of GDP and GMP from the hGBP1 dimer governing the values of \( k_3 \) and \( k_4 \), respectively. We assume this to be less likely as the structure of the protein dimer suggests that protein dissociation should precede nucleotide dissociation.

Another aspect of the phosphate burst kinetics was not discussed so far, namely the size of the amplitude according to Equation 1. Its value is defined by the two rate constants, \( k_3 \) and \( k_\phi \) and the active site fraction, \( [E]_\mu/[E]_0 \) (Equation 2). The best fit to the data leads to a value of \( [E]_\mu/[E]_0 = 1.4 \), i.e. larger than 1, which we explain by errors on the concentrations of the enzyme and the nucleotide. Intriguingly, \( [E]_\mu/[E]_0 \) fixed to values smaller than 1 does not allow reasonable fitting of the phosphate burst experiments. For example, fixing a value of \( [E]_\mu/[E]_0 = 0.5 \) in Fig. 1 leads to values of \( k_3 \) and \( k_\phi \), which cannot...
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properly account for the kinetic behavior as demonstrated by the resulting dotted curve. In conclusion, both halves of the hGBP1 dimer are catalytically active and indistinguishable.

It is notable that all kinetic constants defining the core mechanism of nucleotide hydrolysis catalyzed by hGBP1 are close together in their values. On the one hand this makes analysis difficult, and as a matter of fact may impose some uncertainty on the values we determined. On the other hand it is an interesting finding as it is so different from other GTPases. Small GTPases are slow in their cleavage and nucleotide dissociation steps. Each step can be strongly accelerated by specific interaction partners. Phosphate release is the slowest step within the catalytic cycle of myosin ATPase that is accelerated by the interaction with actin (33). We had anticipated that the catalytic mechanism of hGBP1 is similar to dynamin as it belongs to the same family, and it forms dimers. However, although phosphate release and nucleotide dissociation are fast in dynamin, it is clearly the cleavage step that limits the hydrolysis rate of this enzyme (0.006 s$^{-1}$ at 20 °C), and no product burst is observed (35). This step is much faster with hGBP1, and some event later in the cycle is rate-limiting, which is not yet resolved. Interestingly, GTPase activity of dynamin is strongly increased by formation of larger microtubule or lipid nanotubule-bound assemblies to a value similar to hGBP1 hydrolysis activity described here, and currently there are no transient kinetic data about the rate-limiting step of GTP hydrolysis under these conditions (12, 13, 36, 37). For hGBP1, a further increase of GTPase activity appears to be difficult as there are many steps to be accelerated at the same time in order to reach efficient activation. In the physiological setting of an interferon-induced cell, the concentration of hGBP1 is about in the same range as the dissociation constant of the GTP-bound protein dimer. Therefore, one can expect hGBP1 to reach full GTPase activity when concentrated at a certain cellular compartment such as the Golgi complex where it has been observed after stimulation (38).

In contrast to the difficulty to further increase the catalytic activity, the large number of reaction steps with similar rates bears a high potential for intervention by an inhibitor of hGBP1 GTPase activity. This is the more likely scenario anyway as the catalytic activity of hGBP1 is very high compared with other GTPases. Also the product ratio could be controlled by an interaction partner by influencing one of the rate constants.

In summary, we investigated the molecular mechanism of GTP hydrolysis catalyzed by the hGBP1 dimer. Both molecules are catalytically active and cleave off phosphate ions from the nucleotide in two successive steps. Intriguingly, the second step is even faster than the first ($k_1 = 0.45 \text{ s}^{-1}$), implying that the shift of the nucleotide within the catalytic center is rapid, at least $k_2 = 2.2 \text{ s}^{-1}$. Nevertheless, GDP hydrolysis is competed by inactivation of the catalytic machinery, most probably by dissociation of the hGBP1 dimer with a rate constant of $k_3 = 0.87 \text{ s}^{-1}$ leading to the observed product ratio of GMP and GDP. Likewise, we suggest that dissociation of the GMP-bound dimer is slow, $k_4 = 0.26 \text{ s}^{-1}$, giving rise to the observed burst behavior and controlling the steady state rate to the major part. We suggest that the assembled forms of the GDP- and GMP-bound states of hGBP1 experience a life time of a few seconds each and are only accessible through precedent binding of GTP. Further studies will help to relate these findings about the catalytic mechanism to the biological function of hGBP1.

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