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Analysis of Guanine Nucleotide Binding and Exchange Kinetics of the *Escherichia coli* GTPase Era

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Era is an essential *Escherichia coli* guanine nucleotide binding protein that appears to play a number of cellular roles. Although the kinetics of Era guanine nucleotide binding and hydrolysis have been described, guanine nucleotide exchange rates have never been reported. Here we describe a kinetic analysis of guanine nucleotide binding, exchange, and hydrolysis by Era using the fluorescent mant (*N*-methyl-3′-*O*-anthraniloyl) guanine nucleotide analogs. The equilibrium binding constants (K_D) for mGDP and mGTP (0.61 ± 0.12 μM and 3.6 ± 0.80 μM, respectively) are similar to those of the unmodified nucleotides. The single turnover rates for mGTP hydrolysis by Era were 3.1 ± 0.2 mmol of mGTP hydrolyzed/min/mol in the presence of 5 mM $MgCl_2$ and 5.6 ± 0.3 mmol of mGTP hydrolyzed/min/mol in the presence of 0.2 mM $MgCl_2$. Moreover, Era associates with and exchanges guanine nucleotide rapidly (on the order of seconds) in both the presence and absence of Mg^{2+}. We suggest that models of Era function should reflect the rapid exchange of nucleotides in addition to the GTPase activity inherent to Era.

The *Escherichia coli era* gene encodes an essential protein (10, 17, 30) that binds GTP and GDP specifically (1, 5, 17). Era hydrolyzes GTP to GDP, although the published rates of hydrolysis in vitro range from 0.3 to 17.5 mmol of GTP/min/mol of Era (5, 14, 17). Essential Era homologues have been found in various other bacteria, all of which are capable of complementing an *E. coli era* deletion mutant (2, 22, 24, 31–33). Moreover, Era homologues have been identified in eukaryotes, including *Caenorhabditis elegans*, mice, and humans (4), suggesting that the essential functions of Era are conserved.

Era displays similarity to $p21^{Ras}$ in its N terminus, although sequence alignments between these two proteins indicate that Era lacks homology in the G2 region of the guanine nucleotide binding domain and has no homology to Ras in the C-terminal sequences (1). The crystal structure of *E. coli* Era has been solved to 2.4 Å (7) and revealed a two-domain structure with the GTP binding domain at the N terminus and a predicted KH-like RNA binding motif in the C terminus.

Although its precise cellular function is unknown, Era has been implicated in a wide array of cellular functions. Era mutants display a cell cycle arrest phenotype and suppress temperature-sensitive chromosome partitioning mutations in *dnaG*, suggesting that Era may play a role in DNA replication or chromosome partitioning (3, 4). An Era mutant lacking the G2 domain has been shown to be defective in employing certain tricarboxylic acid intermediates as the sole carbon source, implying a role for Era in carbon metabolism (21, 29). Era has also been linked to the phosphoenolpyruvate:sugar phosphotransfer system (PTS), as certain *era* mutations can be suppressed by mutations in PTS-related genes (23).

The most clearly elucidated cellular function for Era identified to date involves the interaction of Era with the translational machinery. Genetic experiments have shown that elevated copies of the 16S rRNA methyltransferase gene of *E. coli* (*ksgA*) suppress a cold-sensitive mutation in *era* (16). *Streptococcus pneumoniae* Era was shown to bind specifically to synthetic RNA, and point mutations in the proposed C-terminal RNA binding domain disrupt this interaction (12). Subsequently, it has been demonstrated that the 16S rRNA of *S. pneumoniae* copurifies with Era (18) and that *E. coli* Era binds both 16S rRNA and the 30S ribosomal subunit directly, in the absence of guanine nucleotides (28). A deletion of the C-terminal region of *S. pneumoniae* Era abrogates its ability to complement an *E. coli* Δ*era* strain (31), suggesting that RNA binding by Era is critical for cell viability.

Despite the fact that over a decade has passed since the first biochemical characterization of an Era protein (1), guanine nucleotide exchange rates have never been published for Era. In this report, we describe a kinetic analysis of guanine nucleotide binding, exchange, and hydrolysis by Era using mant guanine nucleotide analogs. The mant group (*N*-methyl-3′-*O*-anthraniloyl) is a fluorophore whose fluorescence is sensitive to the hydrophobicity of its environment and therefore can be used to differentiate between bound and free nucleotide on a very rapid time scale (11, 13, 19, 20, 25–27). We demonstrate here that for Era the equilibrium binding constant (K_D) and hydrolysis values for mant guanine nucleotides are similar to those of the unmodified nucleotides. Moreover, unlike Ras (9), Era exchanges guanine nucleotide rapidly (on the order of seconds) at a rate similar to that of the *Caulobacter crescentus* protein CgtA (15). We suggest that models of Era function should reflect the rapid exchange of nucleotides in addition to the GTPase activity inherent to Era.

MATERIALS AND METHODS

Gene amplification and cloning. The gene encoding the *E. coli* protein Era was amplified using colony PCR with *E. coli* W3110 cells (F^- λ^- *mcrA mcrB*) as the source of template DNA and Advantage cDNA polymerase (Clontech) on a PTC-100 programmable thermal controller (MJ Research, Inc.). The primers Era5 (5′GCGAGCTCATGAGCATCGATAAAAGTTA3′) and Era3 (5′TTAC TCGAGGAGTTACTCTTAAAGATCG3′) create unique *Sac*I and *Xho*I sites, respectively. The 0.9-kb Era PCR product was digested with *Sac*I and *Xho*I and ligated into *Sac*I/*Xho*I-digested pET28a (Novagen) to form an in-frame fusion to the N-terminal His tag sequence. The correct clone (pJM1122) was identified by restriction mapping and verified by dideoxy sequencing. Era protein expressed from pJM1122 included the N-terminal tag sequence MGSSHHHHHHSSG LVPRGSHMASMTGGQQMGRGSEFEL preceding its initiator methionine.

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Protein expression and purification. A 1-liter culture of *E. coli* BL21(DE3) cells [*hsdS gal*(λ*c*I*ts*857 *ind*1 *S*am7 *nin*5 *lac*UV5-T7 gene*1*)] containing pJM1122 was grown in Luria-Bertani medium containing 30 μg of kanamycin per ml at 37°C with continuous shaking until the optical density at 600 nm reached 0.6 to 0.7. The cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for an additional 3 h under the same conditions. Cells were pelleted, resuspended in 30 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride), and lysed by two passages through a French pressure cell (American Instrument Company); the lysate was cleared by centrifugation (12,000 rpm, 30 to 60 min). The cleared lysate was passed through a 0.45 μm-pore-size filter and applied to an 8-ml Ni-nitrilotriacetic acid (NTA) column (Qiagen). The column was washed with 10 to 15 bed volumes of lysis buffer, and the protein was eluted by a linear gradient of 100 to 150 mM imidazole in lysis buffer. Fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% SDS-polyacrylamide gel, and proteins were detected by Coomassie blue stain. The relevant fractions were pooled, dialyzed against core buffer (10% glycerol, 50 mM Tris-Cl [pH 8.0], 1 mM dithiothreitol), and stored in aliquots at −80°C. The mass of the purified Era was determined by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Protein and Carbohydrate Structure Facility, University of Michigan).

UV cross-linking. For UV cross-linking, 250 pmol of purified Era and 2 pmol (10 μCi) of [γ-^{32}P]GTP were incubated on ice in binding buffer (10% glycerol, 50 mM Tris-Cl [pH 8.0], 50 mM KCl, 2 mM dithiothreitol, 10 μM ATP, 5 mM $MgCl_2$) for 5 min. Samples were mixed with 400× competitor nucleotide (800 pmol) or an equal volume of water and incubated for a further 5 min. Bound [γ-^{32}P]GTP was cross-linked to Era by UV treatment (254 nm, 1 J/cm^2). Radiolabeled Era-GTP complexes were separated on a 15% SDS-polyacrylamide gel. The gel was vacuum dried and exposed to X-ray film.

Fluorescence assays. The guanine nucleotide binding properties of the purified Era were confirmed, and the kinetic parameters (k_{off}, k_{on}, and K_D) were determined using the fluorescent GDP and GTP analogs 2′ or 3′ mant-GDP and mant-GTP (mGDP and mGTP). The mGDP and mGTP were synthesized as described previously (15). The binding buffer for all assays consisted of 10% glycerol, 50 mM Tris-Cl (pH 8.0), 50 mM KCl, 2 mM dithiothreitol, and 10 μM ATP. $MgCl_2$ was added to a final concentration of 5 or 0.2 mM; samples without exogenous $MgCl_2$ also contain 1 mM EDTA. All assays were performed at 37°C. All curve fittings were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, Calif. [www.graphpad.com]).

Mant guanine nucleotide binding. The intensity of fluorescence of the mant moiety is a function of the hydrophobicity of its environment. Binding of Era to guanine nucleotides was indicated by the increase in fluorescence of the mant analogs in the presence of protein. For the purposes of these assays, 0.1 μM mGDP or mGTP in binding buffer was set as the baseline, and the excitation spectrum from 310 to 410 nm was recorded; 5 μM Era was added, and the excitation spectrum was recorded. Relative fluorescence was calculated as the fluorescence signal of free or Era-bound mant nucleotide at any excitation wavelength divided by the fluorescence signal of free mant nucleotide at an excitation wavelength 361 nm.

Stopped-flow measurement of dissociation rate constant. Protein (3 μM) was prebound to 0.3 μM mGDP or mGTP in binding buffer. This solution was mixed with 45 μM GDP or GTP in binding buffer using an RF5301PC spectrofluorophotometer (Shimadzu) equipped with an SFA-20 rapid kinetics stopped-flow accessory (Hi-Tech Scientific, Salisbury, United Kingdom). Decrease of mGDP or mGTP fluorescence upon addition of GDP or GTP was recorded over time (excitation, 10- or 15-nm slit at 361 nm; emission, 20-nm slit at 446 nm). Five to ten separate curves were averaged for each condition, and the resulting average curves were fitted to a single or double exponential decay equation of the form $F = A_0 + A_1e^{-k_1t} + A_2e^{-k_2t}$. Rates shown represent the average of k_{off} values from three separate experiments.

Stopped-flow measurement of association rate constant and equilibrium binding constant. Era (1 μM) and 1 μM mGDP or mGTP in binding buffer containing various concentrations of $MgCl_2$ (as described above) were mixed in an Applied Photophysics SX18MV stopped-flow spectrophotometer, and the increase in fluorescence intensity upon binding of mGDP or mGTP to Era was monitored over time (excitation, 281 nm, 2.3-nm slit; emission, KV399 long-pass filter). Five to ten separate curves were averaged for each concentration of mant nucleotide, and the average curves were fitted to both a double and a triple exponential association equation of the form $F = A_0 + A_1(1 - e^{-k_1t}) + A_2(1 - e^{-k_2t})$. The association rate constants, k_{on}, as well as their amplitudes, A, are derived from the equation. Average association rate is defined as $(k_{fast} \cdot A_{fast} + k_{slow} \cdot A_{slow})/(A_{fast} + A_{slow})$.

The equilibrium binding constant, K_D, was obtained by plotting total amplitude (i.e., $A_T = A_1 + A_2$) versus concentration of total mant nucleotide for association reactions between 1 μM Era and 0.5 to 20 μM mant nucleotide mGDP or mGTP. The K_D for mGDP was corrected for depletion of total nucleotide due to binding. The resulting data sets were fit to hyperbolic functions and the K_D derived from the equations.

Hydrolysis of mGTP. Era (5 μM) was preincubated for 5 min at 37°C in binding buffer containing 0.2 or 5 mM $MgCl_2$; 0.1 μM mGTP was added, and fluorescence intensity was recorded over time (excitation, 3- or 5-nm slit at 361 nm; emission, 15- or 20-nm slit at 446 nm). The resulting decrease in fluorescence was fitted to a single exponential decay equation of the form $F = A_0 + Ae^{-kt}$, the half time of hydrolysis, $T_{1/2}$, is calculated as ln2/k, and the single-turnover hydrolysis number and rate are described by the expression [mGTP]/$2T_{1/2}$/[Era] and ln2/$T_{1/2}$, respectively.

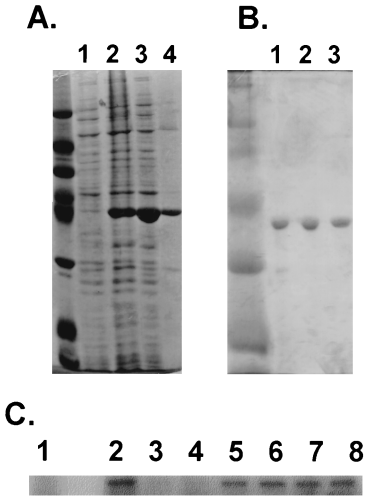


FIG. 1. Purification of His-tagged Era and specific binding to GTP and GDP. (A) Coomassie blue-stained gel containing uninduced (lane 1) and induced (lane 2) *E. coli* cells and the soluble (lane 3) and insoluble (lane 4) fractions obtained after lysis through a French pressure cell. (B) Silver-stained gel containing the Ni-NTA-purified Era (lane 1) and the fractions after further purification through anion exchange (lane 2) and gel filtration (lane 3). (C) Autoradiogram of Era-[γ-^{32}P]GTP complexes separated by SDS-PAGE. For this assay, 250 pmol of Era and 2 pmol (10 μCi) of [γ-^{32}P]GTP were prebound in binding buffer containing 5 mM $MgCl_2$. Without UV cross-linking (lane 1), no Era-[γ-^{32}P]GTP complexes are observed. UV cross-linking resulted in Era-[γ-^{32}P]GTP complexes detected without competing nucleotide (lane 2) or in the presence of 800 pmol GTP, GDP, GMP, ATP, CTP, or UTP (lanes 3 to 8). Era-[γ-^{32}P]GTP binding is inhibited by excess GTP and GDP (lanes 3 and 4, respectively).

RESULTS

Era was purified as a soluble stable protein. An N-terminally polyhistidine-tagged Era protein was stably overexpressed in and purified from *E. coli* BL21(DE3) cells. The expressed Era protein comprised approximately 40% of the total cellular protein, and approximately 75% of the Era was found in the soluble fraction after cell lysis (Fig. 1A). The protein was purified by affinity chromatography through Ni-NTA resin (Qiagen) and was eluted in a single peak with a molecular mass of 37,990 Da as determined by MALDI-TOF mass spectrometry. This mass was within 0.31% of the expected value (37,873 Da [data not shown]), and the Era constituted ≥95% of the total protein (Fig. 1B, lane 1). In contrast to Chen et al. (6), we experienced no difficulties in purifying soluble, stable His-tagged Era.

His-tagged Era binds GTP and GDP specifically. To ensure that our Era preparation bound specifically to GTP and GDP, we performed UV cross-linking of Era to [γ-^{32}P]GTP in the presence and absence of nonradioactive competitor nucleotides. Figure 1C shows the results from such an assay. In the absence of competitor nucleotide, Era binds [γ-^{32}P]GTP (lane 2) and this binding is unaffected by the presence of excess GMP, ATP, CTP, or UTP (lanes 5 to 8). The presence of GTP or GDP (lanes 3 and 4), however, reduces the binding of Era to [γ-^{32}P]GTP to the level observed in the non-cross-linked sample (lane 1). Thus, we conclude that our His-tagged Era is specific for binding to GTP and GDP.

mGDP and mGTP are functionally equivalent analogs of GDP and GTP. As an alternative to the use of radionucleotides

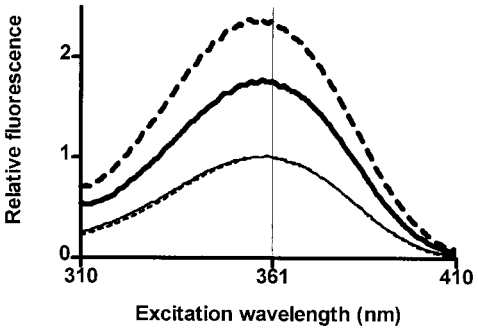
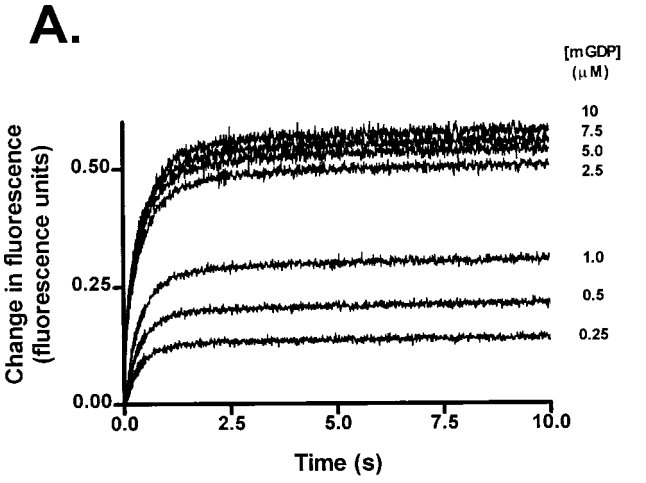


FIG. 2. Excitation spectra for mGDP and mGTP alone or bound to Era. Excitation spectra from 310 to 410 nm were recorded at an emission wavelength of 446 nm in the presence of 5 mm $MgCl_2$. The light lines (superimposed) represent the fluorescence signal from 0.1 μM mGDP or mGTP in the absence of Era; the fluorescence intensity of the mant moiety is identical when coupled to GDP or GTP. Upon addition of Era (5 μM), the fluorescence intensities of both mGDP and mGTP increase. The fluorescence signal from Era-mGDP is shown by the heavy solid line, and the fluorescence signal from Era-mGTP is shown by the heavy dotted line.

and a method appropriate for the rapid kinetics of Era, our laboratory has made use of the fluorescent GDP and GTP analogs mGDP and mGTP. Use of fluorescent analogs allows us to monitor the kinetics continuously in real time. Moreover, the mant moiety (*N*-methyl-3′-*O*-anthraniloyl) is a fluorophore whose fluorescence intensity varies in response to the hydrophobicity of its environment. Changes in fluorescence signal, therefore, can be used to differentiate between bound and free nucleotide states, serving as an indicator of binding of guanine nucleotide to a protein (11, 13, 19, 20, 25–27).

Figure 2 shows typical excitation spectra from mGDP and mGTP alone and bound to Era in the presence of 5 mM $MgCl_2$ monitored at an emission wavelength of 446 nm. The light line represents the mGDP and mGTP in the absence of Era; the fluorescence intensity of the mant moiety is the same regardless of whether it is coupled GDP or GTP, and so the traces are superimposed. The intensity of the fluorescence signal increases significantly when either the mGDP or mGTP is bound to Era (Fig. 2, compare heavy lines to light line), with the peak emission intensity for both the free and bound mant nucleotides occurring at an excitation wavelength of 361 nm. At this wavelength, the maximal fluorescence emission of mGDP-Era was 1.7 times that of mGDP alone, and for mGTP-Era it was 2.4 times that of mGTP alone. No increase in mATP fluorescence was observed upon addition of Era (data not shown), further indicating that Era does not bind ATP.

To demonstrate that the mant nucleotide analogs provide an accurate representation of Era interaction with GDP and GTP, the equilibrium binding constants for Era-mGDP or Era-mGTP in the presence of 5 mM $MgCl_2$ were obtained. The increase in fluorescence intensity upon binding of mGDP or mGTP to Era was monitored as a function of time for a range of mant nucleotide concentrations. Figure 3 shows the association curves for 0.5 μM Era with 0.25 to 10 μM mGDP (Fig. 3A) or mGTP (Fig. 3B). Both the rate of association of Era and mant nucleotide and the amplitude of the fluorescence signal increase with nucleotide concentration. Association rate constants (see below, "Association rate of Era and mant nucleotides is also rapid") and the amplitude of the fluorescence signal were obtained by fitting the data to a double exponential association curve. K_D curves (Fig. 4) were obtained by plotting the total amplitude of the association signal against the final concentration of mant nucleotide, and the data were fit to a



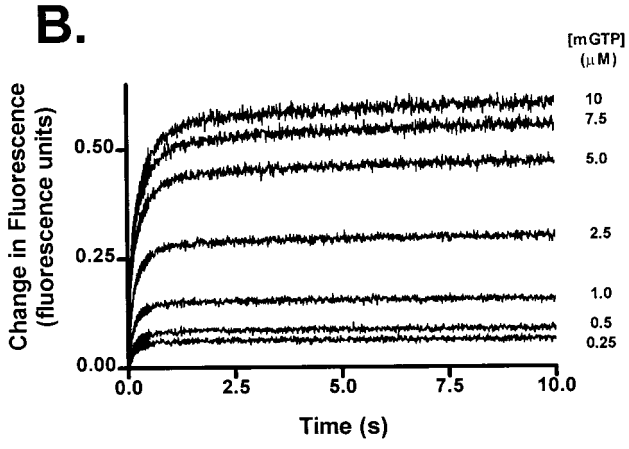


FIG. 3. Association curves for binding of Era to mGDP and mGTP. Era (1 μM) and 0.5 to 20 μM mGDP (A) or mGTP (B) in binding buffer containing 5 mM $MgCl_2$ were mixed in a stopped-flow apparatus, and the increase in fluorescence intensity was recorded over time. Data were fitted to a double exponential equation, and the rate constants and amplitude values were calculated. Curves shown represent the average of three separate data sets.

one-site binding equation. Era binds to mGDP with a slightly higher affinity than mGTP; the K_D for Era binding to mGDP is 0.61 ± 0.12 μM (Fig. 4A), and the K_D for mGTP is 3.6 ± 0.80 μM (Fig. 4B). These values agree reasonably well with the published K_D, obtained using radionucleotides, of 1.0 and 5.5 μM for GDP and GTP, respectively (5). Based on these data, we conclude that with respect to Era, mGDP and mGTP have binding properties similar to those of GDP and GTP.

Exchange of guanine nucleotides by Era is rapid. To determine the rate of guanine nucleotide exchange by Era, we monitored the difference in fluorescence intensity between Era-bound and free mant nucleotide. Era was prebound to mGDP or mGTP and then rapidly mixed with an excess of nonfluorescent GDP or GTP. The rate of mant nucleotide release was monitored as a decrease in fluorescence intensity over time. No differences were observed regardless of whether GDP or

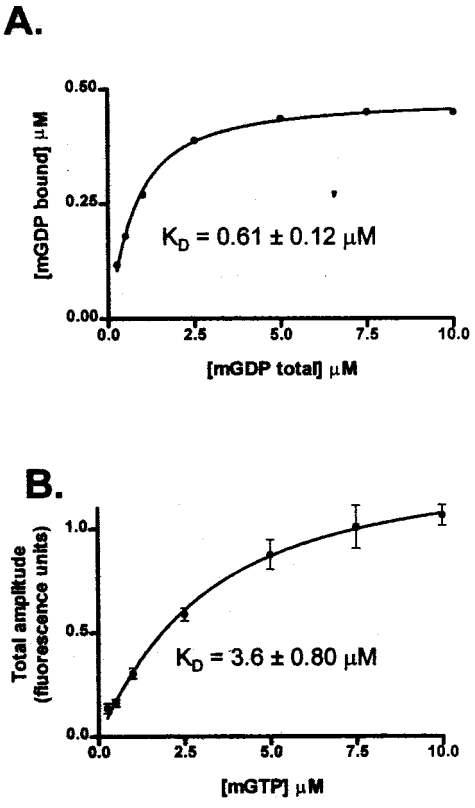


FIG. 4. Equilibrium binding constants for Era and mGDP or mGTP. The equilibrium binding constant, K_D, was obtained for mGDP and mGTP by plotting total amplitudes from the association curves (Fig. 3) against final concentration of mant nucleotide and fitting to a one site binding equation; due to the low K_D value, the mGDP data were subsequently corrected for depletion of nucleotide due to binding. (A) Single set of data for mGDP K_D; (B) average of three data sets for mGTP K_D.

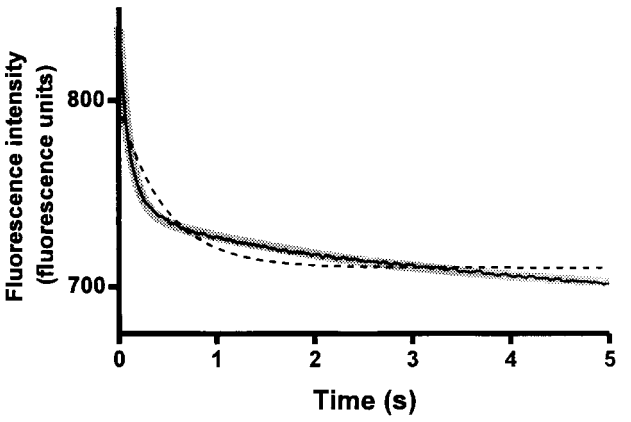


FIG. 5. Guanine nucleotide exchange by Era. Data from a typical mant nucleotide exchange assay are shown, in this case exchange of mGTP for GDP in the presence of 5 mM $MgCl_2$. Era (3 μM) was prebound to 0.3 μM mGTP in binding buffer. This solution was mixed with a 45 μM solution of GDP in a stopped-flow apparatus, and the decrease in fluorescence intensity was recorded over time. Data (black solid line) were fitted to a single (black dotted line) or double (gray line) exponential equation. The exchange rates, k_{off}, and amplitude values were calculated from the double exponential equation.

GTP was used as the competing nucleotide (data not shown). Figure 5 shows a typical trace for an exchange assay, in this case the exchange of mGTP for GDP by Era in the presence of 5 mM $MgCl_2$. The fluorescence intensity decays exponentially on the order of seconds. Thus, the rate of guanine nucleotide exchange by Era is extremely rapid in the presence of Mg^{2+}, unlike that of Ras (Fig. 5 and reference 9).

Interestingly, however, the data for all exchange assays were better described by a double exponential decay curve than a single exponential decay curve (Fig. 5). In an attempt to explain the presence of the second rate component, our Ni-NTA-purified Era was further purified (to ≥98%) using anion-exchange and gel filtration columns (Fig. 1B, lanes 2 and 3), and guanine nucleotide exchange was again assayed. After each column step, two rate components were still present and the rates were identical to those obtained with the original Era preparation (data not shown). In light of recent data indicating that Era can be found in an rRNA-associated form (18, 28), Ni-NTA-purified Era was also preincubated with RNase A and assayed for exchange rate. Again, both rate components were present and the rates were identical to those obtained with untreated Era (data not shown). We conclude that the presence of a second guanine nucleotide exchange rate component is inherent to Era and not the product of a contaminant or an rRNA component.

The rate of guanine nucleotide release by Era is extremely rapid. In the presence of 5 mM $MgCl_2$, both the fast and slow rate components for mGDP and mGTP exchange alike are on the order of seconds (Table 1). The fast rate component for release of mGTP is 3.6 times more rapid than that of mGDP release; however, the slow rate components for release of either nucleotide are similar. The contributions of the fast and slow rate components are almost equivalent for mGDP exchange, whereas for mGTP exchange the fast rate component accounts for approximately two-thirds of the total amplitude (Table 1).

To investigate the effects of lower concentrations of Mg^{2+}, a known cofactor for guanine nucleotide binding by Ras-like guanine nucleotide binding proteins, on guanine nucleotide exchange by Era, exchange reactions were carried out in the presence of 0.2 or 0 mM $MgCl_2$. Both the fast and slow rates of exchange of mGDP by Era increase moderately at lower concentrations of $MgCl_2$ (Table 1), with the slow component being somewhat more affected than the fast component (2.5- to 2.8-fold and 1.5- to 2.2-fold increases, respectively). With the decrease in Mg^{2+} levels, however, the contribution of the fast rate component also decreases, from approximately one-half to one-third of the total amplitude (Table 1). Thus, the average exchange rate for mGDP increases approximately twofold in the absence of Mg^{2+}.

Changes in mGTP exchange rates at lowered levels of $MgCl_2$ are more variable. The rate of fast exchange of mGTP increases somewhat in the absence of $MgCl_2$, although the contribution to the total amplitude is unchanged (Table 1). In the presence of 0.2 mM $MgCl_2$, the fast exchange rate decreases to half of that observed in the presence of 5 mM $MgCl_2$, and the contribution of the fast rate also decreases to approximately one-third to one-half of the total (Table 1). The slow exchange rate for mGTP increases modestly in the presence of 0.2 mM $MgCl_2$ but is approximately the same in the absence of $MgCl_2$, compared to 5 mM $MgCl_2$ (Table 1). Thus, the average exchange rate for mGTP appears to increase modestly at the extremes of $MgCl_2$ concentration relative to the rate in the presence of 0.2 mM $MgCl_2$. Interestingly, in the presence of 0.2 mM $MgCl_2$, both the fast and slow exchange rates for mGDP and mGTP and their contributions to the total amplitude are almost identical (3.7 and 0.27 s^{-1}, respectively, for mGDP; 4.0 and 0.34 s^{-1} for mGTP [Table 1]). Similar results were ob-

TABLE 1. Dissociation rate constants for Era-mGDP and Era-mGTP[a]

Substrate	Era-mGDP			Era-mGTP		
	k_{off} (s^{-1})		Contribution of k_{off} fast (%)	k_{off} (s^{-1})		Contribution of k_{off} fast (%)
	Fast	Slow		Fast	Slow	
$MgCl_2$						
5 mM	2.5 ± 0.2	0.27 ± 0.08	55 ± 12	8.8 ± 0.2	0.34 ± 0.01	68 ± 11
0.2 mM	3.7 ± 0.6	0.68 ± 0.05	32 ± 1	4.0 ± 1.1	0.60 ± 0.04	42 ± 3
1 mM EDTA	4.5 ± 0.9	0.74 ± 0.06	31 ± 2	14.8 ± 0.4	0.26 ± 0.03	75 ± 3

[a] Error parameters are derived from the average of three separate data sets.

tained with either mGDP or mGTP under all conditions when GTP was used as the nonfluorescent competitor (data not shown).

Association rate of Era and mant nucleotides is also rapid. To further analyze the kinetic properties of Era interaction with guanine nucleotides, we assayed the rate of binding of Era and mant guanine nucleotides. Era and mGDP or mGTP were rapidly mixed, and the association rate was monitored as an increase in fluorescence intensity over time. The fluorescent analogs enabled us to monitor this process continually in real time, thereby obtaining k_{on} values that are too rapid to be detected in radionucleotide assays.

Figure 6 shows the trace from a typical association reaction, in this case between 0.5 μM Era and 0.5 μM mGDP in the presence of 5 mM $MgCl_2$. The fluorescence intensity increases exponentially on the order of milliseconds (Fig. 6, note split time scale), indicating that guanine nucleotide binding by Era is rapid. The data were fitted to a double exponential association curve, and the fast and slow k_{on} values were determined (Table 2). It should be noted that the association data sets could also be fitted to a triple exponential association equation, suggesting that the binding of guanine nucleotides to Era is an extremely complex process (data not shown).

The rates of association of Era-mGDP and Era-mGDP are both rapid, but they vary greatly in the presence of 5 mM $MgCl_2$ (Table 2). Both the fast and slow association rates for Era-mGTP are greater than those of Era-mGDP association, 1.7- and 1.2-fold, respectively. The contribution of the fast rate component to the total also differs, comprising one-half of the total signal for mGDP but almost three-fourths of the total signal for mGTP (Table 2).

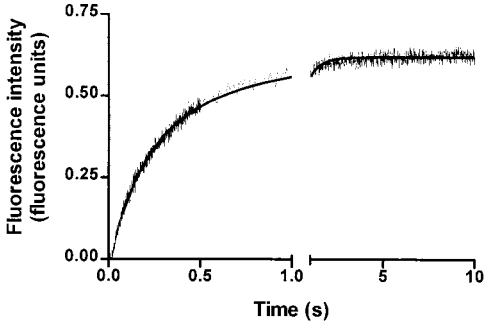


FIG. 6. Association of mant nucleotides with Era. Data from a typical mant nucleotide association assay, in this case 0.5 μM Era and 0.5 μM mGDP in the presence of 5 mM $MgCl_2$. Era (1 μM) and 1 μM mGDP in binding buffer were mixed in a stopped-flow apparatus, and the increase in fluorescence intensity was recorded over time. Data (light line) were fitted to a double exponential equation (heavy line), and the association rate constants, k_{on}, and amplitude values were calculated.

As with guanine nucleotide exchange, we assessed the effects of lower concentrations of Mg^{2+} on Era-guanine nucleotide association. Both the fast and slow Era-mGDP association rate components are similar at the extremes of $MgCl_2$ concentration (Table 2), but these rates are 1.3- to 2.4-fold slower than those observed in the presence of 0.2 mM $MgCl_2$. The contribution of the fast rate component to Era-mGDP association, however, does not follow the same pattern, accounting for approximately half of the total signal in the presence of 5 mM $MgCl_2$ but one-fourth to one-third at the lower concentrations (Table 2).

The fast rate constants for Era-mGTP association are similar at all tested $MgCl_2$ concentrations (Table 2). The contribution of the fast rate is also relatively unaffected by changes in the concentration of $MgCl_2$, increasing only moderately with a decrease in $MgCl_2$ concentration (Table 2). The Era-mGTP slow association rate, however, is somewhat faster (≈2-fold [Table 2]) in the presence of 5 mM $MgCl_2$ than 0.2 mM $MgCl_2$ or 1 mM EDTA.

It is interesting that association and dissociation rates for mGTP are affected differently by changes in the concentration of $MgCl_2$ (Table 2). Association of Era-mGTP occurs at approximately the same rate at all concentrations of $MgCl_2$ tested. The dissociation rate of Era-mGTP, however, is more rapid at the extreme concentrations than in the presence of 0.2 mM $MgCl_2$. Furthermore, whereas the dissociation rates for Era-mGDP and Era-mGTP vary greatly in the absence of $MgCl_2$, the association rates for these complexes are extremely similar under the same condition. These data suggest that the Mg^{2+} cofactor affects Era association and dissociation of guanine nucleotides differently.

Hydrolysis of mGTP by Era occurs on a time scale of minutes. In the presence of 0.2 or 5 mM $MgCl_2$, the fluorescence intensity of mGTP is significantly higher than that of mGDP when bound to Era. We took advantage of this difference to determine the rate of hydrolysis of mGTP by Era. Because the fluorescence signal depends on mant nucleotide binding to protein, fluorescence is not detectable at very low levels of Era or mGTP, whereas at high mGTP or mGDP levels the fluorescence intensity increase of mant nucleotide bound to Era is overwhelmed by background fluorescence from free mant nucleotide. Thus, the fluorescent system does not allow the standard V_{max} and K_m measurements for hydrolysis of mGTP by Era. We were, however, able to approximate single turnover hydrolysis reactions for Era. Era (5 μM) was added to 0.1 μM mGTP; given a K_D of approximately 2.8 μM observed for Era-mGTP, approximately two-thirds of the Era was bound to nucleotide under these conditions. Fluorescence was monitored over time, and the decrease in intensity as the bound nucleotide was converted from mGTP to mGDP was monitored.

TABLE 2. Association rate constants for Era-mGDP and Era-mGTP[a]

Substrate	Era-mGDP			Era-mGTP		
	k_{on} ($\mu M\ s^{-1}$)		Contribution of k_{on} fast (%)	k_{on} ($\mu M\ s^{-1}$)		Contribution of k_{on} fast (%)
	Fast	Slow		Fast	Slow	
$MgCl_2$						
5 mM	6.3 ± 0.6	1.6 ± 0.2	51 ± 6	11.0 ± 0.8	2.0 ± 0.3	68 ± 3
0.2 mM	15.2 ± 2.9	2.0 ± 0.1	24 ± 3	10.0 ± 0.5	1.1 ± 0.2	77 ± 2
1 mM EDTA	7.2 ± 0.7	1.6 ± 0.1	33 ± 3	9.0 ± 0.5	1.1 ± 0.3	86 ± 2

[a] Error parameters are derived from the average of three separate data sets.

Figure 7 shows the results of a typical hydrolysis reaction in the presence of 0.2 mM $MgCl_2$. The data points were fitted to a single exponential decay curve. The hydrolysis rates were 0.38 ± 0.02 min^{-1} in the presence of 0.2 mM $MgCl_2$ and 0.21 ± 0.02 min^{-1} in the presence of 5 mM $MgCl_2$. Because the binding of Era to mGTP is extremely rapid, the hydrolysis rate also provides a good estimate of turnover number for Era. The hydrolysis rates yield turnover numbers for GTP hydrolysis by Era of 5.6 ± 0.3 mmol of mGTP hydrolyzed/min/mol of Era in the presence of 0.2 mM $MgCl_2$ and 3.1 ± 0.2 mmol of mGTP hydrolyzed/min/mol of Era in the presence of 5 mM $MgCl_2$. These values differ less than twofold, suggesting that altering the concentration of $MgCl_2$ does not dramatically affect hydrolysis. Moreover, the previously reported Era hydrolysis rates vary widely, and while both of the rates reported here are lower than those published for [γ-^{32}P]GTP-Era in the presence of 5 mM $MgCl_2$ (17.5 mmol/min/mol [5] and 9.8 mmol/min/mol [14]), they are higher than the hydrolysis rate obtained in the presence of 5 mM $MgCl_2$ (0.3 mmol/min/mol [17]). Fluorescent hydrolysis assays could not be performed in the presence of 1 mM EDTA because the fluorescence intensities of mGDP and mGTP bound to Era are identical (data not shown).

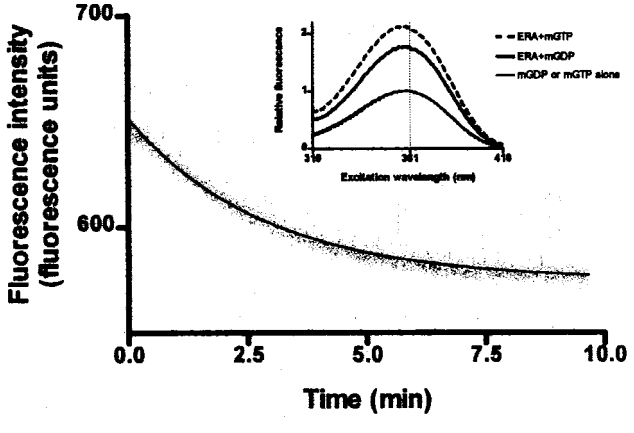


FIG. 7. Hydrolysis of mGTP by Era in the presence of 0.2 mM $MgCl_2$. Data are from a typical hydrolysis assay in which 0.1 μM mGTP was prebound to 5 μM Era in binding buffer containing 0.2 mM $MgCl_2$, and the decrease in fluorescence intensity due to hydrolysis of mGTP to mGDP was recorded over time (points). Data were fitted to a single exponential decay curve (solid line), and the single-turnover hydrolysis rate was calculated. (Inset) Excitation spectra (excitation, 310 to 410 nm; emission, 446 nm) demonstrating the difference in fluorescence intensity of Era-mGTP and Era-mGDP in the presence of 0.2 mM $MgCl_2$. The light lines (superimposed) represent the fluorescence signal from 0.1 μM mGDP or mGTP in the absence of Era; the fluorescence intensity of the mant moiety is the same when coupled to GDP or GTP. Upon addition of Era (5 μM), the fluorescence intensities of both mGDP and mGTP increase. The fluorescence signal from Era-mGDP is shown by the heavy solid line, and the fluorescence signal from Era-mGTP is shown by the heavy dotted line.

DISCUSSION

In this report, we describe an analysis of the kinetics of Era interactions with guanine nucleotides. The experiments described make use of the guanine nucleotide analogs mGDP and mGTP. The fluorescence intensity of the mant moiety is sensitive to the hydrophobicity of its environment, allowing one to distinguish between free and protein-bound nucleotide, and even between mGDP- and mGTP-bound forms of the protein, under some conditions. Moreover, observing Era-guanine nucleotide interactions through fluorescence allows the kinetics of binding and hydrolysis to be followed in real time. This has proven exceptionally valuable to the study of Era, as it provides the first opportunity to observe and quantitate the rapid guanine nucleotide exchange.

We show here that both mant guanine nucleotide binding and exchange by Era are rapid, occurring on the order of seconds, while hydrolysis occurs on the minute time scale. The kinetics of binding and exchange are complex, requiring multiple rate components to fully describe the reactions. Attempts to form kinetic models to describe this behavior have been unsuccessful thus far. Regardless of the complexity, however, the overall rapid exchange of nucleotides by Era may play a significant biological role.

In the absence of data regarding the rate of guanine nucleotide exchange, models for Era function have focused exclusively on the potential regulatory role of the inherent GTPase activity of Era. However, in vitro exchange of guanine nucleotides by Era is extremely rapid, in fact over 10-fold more rapid than the rate of hydrolysis. If Era also rapidly exchanges nucleotides in vivo, then in the absence of GTPase-activating proteins or guanine nucleotide dissociation inhibitors to speed hydrolysis or slow exchange, the exchange of guanine nucleotides would play a significant role in Era function. In light of our data, then, this suggests that the relative levels of intracellular GDP and GTP available to the rapidly exchanging Era protein could be the prime determinant of the nucleotide to which Era is bound. If, as with other Ras-like guanine nucleotide binding proteins, the GDP-bound form of Era is inactive and the GTP-bound form is active, then levels of Era activity also would depend on the composition of the guanine nucleotide pools. It is also possible that the two forms of Era, GDP and GTP bound, are both active but have different cellular functions. Era has been implicated in a variety of unrelated cellular processes (3, 4, 16, 18, 23, 28, 29, 31), and it is possible that the guanine nucleotide occupancy of Era dictates which functions Era can fulfill.

We do not suggest that hydrolysis plays no role in Era func-

tion. It is possible that the rapid guanine nucleotide exchange kinetics that we observe with Era are, in a sense, an artifact of in vitro experimentation. In vivo, Era may be associated in multiprotein complexes that either contain an as yet unidentified guanine nucleotide dissociation inhibitor or serve that role by constraining the guanine nucleotide binding domain of Era. There is evidence for Era involvement in several large protein complexes or systems, most notably the 30S ribosomal subunit (18, 28). In addition, Era itself has also been shown to aggregate at or near the cellular membrane (8). Thus, hydrolysis and not exchange may even be the predominant regulatory mechanism.

We, however, favor a model in which both hydrolysis and exchange play significant roles in Era regulation. It is possible, for example, that when cellular energy levels, and therefore GTP pools, are low, the lower affinity of Era for GTP and the rapid exchange combine to maintain Era in a GDP-bound inactive state. When cellular energy is high, a rapid guanine nucleotide exchange rate would result in GTP-bound, active Era. DNA replication and translation occur only when energy levels are high, and so the DNA replication and translational functions of Era would be relevant when cellular energy is high. Once Era is bound in a multiprotein complex, however, guanine nucleotide exchange could be sterically inhibited and hydrolysis would be required to inactivate Era.

In this report, we demonstrate that Era displays a rapid guanine nucleotide exchange rate and provide another piece of the puzzle of Era function. Models for Era action and regulation must not depend solely on GTPase activity but must also account for the ability of Era to rapidly exchange guanine nucleotides.

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ADDENDUM IN PROOF

His-tagged Era protein was subjected to thrombin cleavage, resulting in a 17-amino-acid N-terminal truncation. The fast and slow guanine nucleotide exchange rates for this protein were similar to those reported here for His-tagged Era.

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