Kinetics of the Interaction of Translation Factor SelB from *Escherichia coli* with Guanosine Nucleotides and Selenocysteine Insertion Sequence RNA

Martin Thanbichler‡, August Böck‡, and Roger S. Goody¶‖

From the ‡Lehrstuhl für Mikrobiologie der Universität München, Maria-Ward-Straße 1a, 80638 München, Germany and the ¶Max-Planck-Institut für Molekulare Physiologie, Rheinlanddamm 201, 44026 Dortmund, Germany

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The kinetics of the interaction of GTP and GDP with SelB, the specific translation factor for the incorporation of selenocysteine into proteins, have been investigated using the stopped-flow method. Useful signals were obtained using intrinsic *i.e.* tryptophan fluorescence, the fluorescence of methyllanthraniloyl derivatives of nucleotides, or fluorescence resonance energy transfer from tryptophan to the methyllanthraniloyl group. The affinities of SelB for GTP (*K*~d~ = 0.74 μM) and GDP (*K*~d~ = 13.4 μM) were considerably lower than those of other translation factors. Of functional significance is the fact that the rate constant for GDP release from its complex with SelB (15 s⁻¹) is many orders of magnitude larger than for elongation factor Tu, explaining why a GDP/GTP exchange factor is not required for the action of SelB. In contrast, the rate of release of GTP is 2 orders of magnitude slower and not significantly faster than for elongation factor Tu. Using a fluorescently labeled 17-nucleotide RNA minihelix that represents a binding site for the protein and that is part of the *fdhF* selenocysteine insertion sequence element positioned immediately downstream of the UGA triplet coding for selenocysteine incorporation, the kinetics of the interaction were studied. The high affinity of the interaction (*K*~d~ = 1 nM) appeared to be increased even further when selenocysteyl-tRNA^Sec^ was bound to SelB, but to be independent of the presence or nature of the guanosine nucleotide at the active site. These results suggest that the affinity of SelB for its RNA binding site is maximized when the charged tRNA is bound and decreases to allow dissociation and reading of codons downstream of the selenocysteine codon after selenocysteine peptide bond formation.

The specialized translation factor SelB is the key molecule for the specific incorporation of the amino acid selenocysteine into polypeptides. SelB binds guanine nucleotides; selenocysteyl-tRNA^Sec^; and a secondary structure of the mRNA, the SECIS element. In bacterial selenoprotein mRNAs, this SECIS element is located immediately downstream of the UGA codon directing selenocysteine insertion, whereas in Archaea and Eukarya, it is situated in the 3′-nontranslated region (for a review, see Ref. 1). Binding of SelB to the SECIS structure is mediated by an −17-kDa C-terminal domain of the protein (domain IVb) that maintains its binding capacity when separated from the rest of the molecule. Minimization of the SECIS element showed that a 17-nucleotide long minihelix (see Fig. 1) was still able to bind to SelB or its C-terminal domain and to promote selenocysteine incorporation in vivo. On the other hand, the N-terminal two-thirds of SelB, which display sequence similarity to elongation factor Tu, are still capable of binding selenocysteyl-tRNA^Sec^ when separated from the mRNA binding domain (2, 3).

The formation of the quaternary complex is essential for the decoding of the UGA codon with selenocysteine at the ribosome. It is assumed that within this complex, SelB attains a conformation rendering it compatible for interaction with the ribosome, which is required for triggering GTPase activity. It is further assumed that GTP hydrolysis changes the conformation such that the charged tRNA is released. Therefore, in this model, SelB has two functions: the first one is to discriminate between an UGA codon programmed for selenocysteine insertion by the SECIS element and an ordinary UGA stop codon that lacks the SECIS element (4). Furthermore, in bacterial mRNAs coding for selenocysteine, SelB also tethers the tRNA to the ribosomal A site (4, 5).

Characterization of the kinetics of the SelB interaction with its substrates and the conformational changes involved is therefore of paramount importance for understanding the decoding process. Equilibrium dialysis measurements of the interaction of the protein with guanine nucleotides had indicated that GDP binding is approximately an order of magnitude weaker that GTP binding (6). This could obviate the necessity for a guanine nucleotide release factor. It has also been observed that SelB forms a tighter complex with the SECIS element in the presence of selenocysteyl-tRNA^Sec^ than in its absence (7). This study deals with a detailed analysis of the association and dissociation kinetics of guanine nucleotides with SelB alone and in the presence of the mRNA. Additionally, the kinetics of the interaction of SelB with SECIS elements and the influence of guanine nucleotides and selenocysteyl-tRNA^Sec^ thereon were investigated.

**EXPERIMENTAL PROCEDURES**

Materials—1-t-[¹⁴C]Serine was obtained from NEN Life Science Products and had a specific activity of 160 mCi/mmol. Sodium [⁷⁵Se]selenite with a specific activity of 296 mCi/mmol and RNase P1 were purchased from Amersham Pharmacia Biotech (Freiburg, Germany). Yeast inorganic pyrophosphatase was from Sigma-Aldrich (Deisenhofen, Germany). Bovine serum albumin and baker’s yeast tRNA were obtained from Amersham Biosciences (Freiburg, Germany) and the University of Münster, Germany.

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‡To whom correspondence should be addressed. Tel.: 49-231-1206229; E-mail: goody@mpi-dg.mpg.de.

§The abbreviations used are: Sec, selenocysteine; DTT, dithiothreitol; Pipes, piperazine-N,N′-bis(2-ethanesulfonic acid); mantGTP/DIP, 3′-methylanthraniloyl-2′-deoxy-GTP/DIP; mantGTP/DIP, 2′(3′)-methylanthraniloyl-GTP/DIP; EF-Tu, elongation factor Tu; SECIS, selenocysteine insertion sequence.
Roche Molecular Biochemicals (Mannheim, Germany). The 17-nucleotide long minihelix representing the binding part of the SECIS element of the fdhF mRNA from Escherichia coli (see Fig. 1) was prepared as described previously (2) and was a generous gift from M. Kromayer. The labeled fdhF minihelix carrying a (3′-5′)-diphosphatefluoresceinyl)-5-carboxyfluorescein-GU (GCCAGC-GCGAG-3′) was synthesized by Interactiva (Ulm, Germany). Selencysteine synthase (SelE) purified as described (8) was a generous gift from K. Forchhammer. T7 RNA polymerase was isolated as described (9) and kindly provided by S. Leonhartsberger.

**Purification of SelB and SelB-(472–614)**—For purification of SelB, E. coli strain BL21(DE3) 101 was transformed with plasmid pWL194 (11) harboring selB under control of the T7 810-promoter. Transformants were grown in a 10- or 50-liter Biostat fermentor (B. Braun, Dassel, Germany) at 37 °C in a medium containing 3% peptone, 1% yeast extract, 0.4% glucose, 25 mM NaCl, 2 mM MgCl₂, 60 mM potassium phosphate (pH 7.1), and 100 μg/ml ampicillin. At an A₆₀₀ of 3.0, expression of selB was induced by addition of 100 μg isopropyl−β−thiogalactopyranoside. Subsequently, the temperature was lowered to 30 °C. After 75−150 min, the suspension was chilled to 0 °C, and the cells were harvested in a continuous flow centrifuge.

Breakage of the cells, removal of cell debris, enrichment of SelB by sedimentation with the ribosomes and subsequent extraction from the pellet with high salt buffer, and fractionated ammonium sulfate precipitation were performed as described (11). The final precipitate was dissolved in 50 ml of buffer A (100 mM potassium phosphate (pH 7.0), 2 mM magnesium acetate, 2 mM DTT, and 0.5 mM EDTA). After dialysis for 6 h against two changes (2 liters each) of the same buffer, SelB was eluted at a potassium phosphate concentration of ~165 mM. Fractions containing SelB, as monitored by SDS gel electrophoresis, were pooled and dialyzed against two changes (2 liters each) of buffer B (100 mM potassium phosphate (pH 7.0), 5 mM MgCl₂, 2 mM DTT, and 0.5 mM EDTA) for 6 and 12 h, respectively. The pool was then loaded onto a Q-Sepharose anion-exchange column (1 × 5 cm; Amersham Pharmacia Biotech), which was developed with 90 ml of a linear gradient of 0–400 mM KCl in 20 mM sodium acetate (pH 4.6)). The transcripts were subsequently precipitated by addition of 0.1 volume of sodium acetate (pH 4.6). The solution was extracted with phenol (equilibrated with 100 mM sodium acetate (pH 4.6)). The transcripts were subsequently precipitated by ethanol and stored at −20 °C.

**Preparation and Folding of the RNA Stem-Loops**—The 39-nucleotide minihelix carrying a (3′-5′)-diphosphatefluoresceinyl)-5-carboxyfluorescein-GU (GCCAGC-GCGAG-3′) was synthesized by Interactiva (Ulm, Germany). The 17-nucleotide long minihelix representing the binding part of the SECIS element from fdnG was prepared in 250 μl of ice-cold 10% trichloroacetic acid containing 0.1% L-serine. After addition of 100 μg of bakers’ yeast tRNA, the precipitate was transferred onto glass-fiber filters and washed four times with 1 ml of 10% trichloroacetic acid containing 0.1% l-serine, and three times with 1 ml of 80% ethanol. The filters were dried at 65 °C, and their radioactivity was determined by liquid scintillation counting.

**Aminoacylation of tRNA**—Aminoacylation of tRNA was performed in a reaction mixture containing 100 μl Hepes (pH 7.0), 10 mM KCl, 10 mM magnesium acetate, 1 mM DTT, 10 mM ATP, 200 μM l-serine, 85 μM tRNA, 0.1 mg/ml bovine serum albumin, 2.5 units/ml pyrophosphatase, and 0.84 ng/ml aeryl-tRNA synthetase. After incubation for 15 min at 37 °C, the reaction was stopped by addition of 0.033 volume of 3 mM sodium acetate (pH 4.6) and subsequent treatment with phenol equilibrated with 100 mM sodium acetate (pH 4.6). Seryl-tRNA was precipitated from the aqueous phase at −20 °C by addition of 0.1 volume of 3 mM sodium acetate (pH 4.6) and 1 volume of isopropyl alcohol. The precipitate was collected by centrifugation at 16000 × g for 45 min, washed with 80% ethanol, dried, and resublimed in 0.1 mM EDTA.

** Determination of the RNA Stem-Loop Concentration**—To determine their exact concentration, RNA stem-loops were hydrolyzed by RNase P1 in a reaction mixture containing 30 mM sodium acetate (pH 5.0), 1.6 mM ZnCl₂, and 8 units/ml RNase P1 in a volume of 100 μl. After incubation for 2 h at 60 °C, the solution was diluted with distilled H₂O, and potassium phosphate (pH 7.0) was added to a final concentration of 50 mM. Subsequently, the absorption of the released nucleotide monophosphates was measured at 260 nm. From the value obtained, the oligonucleotide concentration was calculated using the specific absorption coefficients of fdhF minihelix and fdnG minihelix.

**Isolation of Selenophosphate Synthetase**—Isolation of seryl-tRNA synthetase carrying plasmid pCB2013 according to the procedure described by Baron and Bock (19). Its concentration was determined by aminoacylation with l-[3H]serine under saturating seryl-tRNA synthetase conditions. The underlining specific absorption coefficients (ε₅₀₀) of the nucleotide monophosphates were 172.2 μM⁻¹ cm⁻¹ for the fdhF minihelix and 112.2 μM⁻¹ cm⁻¹ for the fdnG stem-loop. The underlying specific absorption coefficients (ε₅₀₀) of the nucleotide monophosphates were 172.2 μM⁻¹ cm⁻¹ for AMP, 112.2 μM⁻¹ cm⁻¹ for CMP, 98.8 μM⁻¹ cm⁻¹ for UMP, and 7.45 μM⁻¹ cm⁻¹ for CMP.

**Isolation of tRNA**—tRNA was purified from E. coli MC4100 (18) carrying plasmid pCB2013 according to the procedure described by Baron and Bock (19). Its concentration was determined by aminoacylation with l-[3H]serine under saturating seryl-tRNA synthetase concentrations. The aminoacylation mixture was pipetted into 250 μl of ice-cold 10% trichloroacetic acid containing 0.1% l-serine. After addition of 100 μg of bakers’ yeast tRNA, the precipitate was transferred onto glass-fiber filters and washed four times with 1 ml of 10% trichloroacetic acid containing 0.1% l-serine, and three times with 1 ml of 80% ethanol. The filters were dried at 65 °C, and their radioactivity was determined by liquid scintillation counting.

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30 min, washed with 80% ethanol, dried in vacuo, and resolubilized in 10 mM sodium acetate (pH 4.6).

Conversion of Ser-tRNA\(^{sec}\) to Selenocysteyl-tRNA\(^{sec}\)—Ser-tRNA\(^{sec}\) was converted to selenocysteyl-tRNA\(^{sec}\) in a reaction mixture containing 100 mM Pipes (pH 8.7), 10 mM KCl, 10 mM magnesium acetate, 0.5 mM ATP, 250 \(\mu\)M sodium selenite, 17 \(\mu\)M ser-tRNA\(^{sec}\), 0.1 mg/ml SeLA, and 5.9 mg/ml SeLD. After incubation at 37 °C for 35 min, 0.05 volume of 3 M sodium acetate (pH 4.6) and 0.05 volume of 200 mM DTT were added. The solution was thoroughly shaken with 1 volume of phenol equilibrated with 100 mM sodium acetate (pH 4.6) and centrifuged for 5 min at 16,000 \(\times\) g. The supernatant was then mixed with 0.1 volume of 3 M sodium acetate (pH 4.6) and 1 volume of isopropyl alcohol and incubated for 1 h on ice. The precipitate was sedimented by centrifugation for 10 min at 16,000 \(\times\) g, washed with 80% ethanol containing 5 mM DTT, dried in vacuo, and resolubilized in 10 mM sodium acetate (pH 4.6) containing 5 mM DTT. All steps were performed in a glove box under anaerobic conditions. The concentration of selenocysteyl-tRNA\(^{sec}\) was determined by performing parallel reactions using sodium \(^{75}\)Se selenite instead of the nonradioactive compound and determining the radioactivity as described above.

Transient Kinetic Experiments—Stopped-flow experiments were performed in a High Tech Scientific instrument using an excitation wavelength of 366 nm and an emission cutoff filter with an edge at 389 nm for monitoring the fluorescence from the methylnanthraniloyl group. For tryptophan fluorescence, an excitation wavelength of 289 nm and a 320-nm cutoff filter were used. For fluorescence resonance energy transfer measurements, a combination of the 289-nm excitation wavelength with the 389-nm filter were used. To monitor the fluorescence of the fluorescein group, an excitation wavelength of 494 nm and a 530-nm cutoff filter were used. Since the protein stock solutions were in 50% glycerol and relatively dilute, all solutions of reactants were adjusted to 10% glycerol content. Experiments in which the only source of glycerol was the protein stock solution, which resulted in a final glycerol concentration in the observation chamber that was ≤5%, led to very similar results for nucleotide association kinetics, but with the disadvantage that there was a significant disturbance of the optical signal at the beginning of the transient. The buffer used contained 100 mM potassium phosphate (pH 7.0), 5 mM MgCl\(_2\), 2 mM DTT, 0.5 mM EDTA, and 10% glycerol. Primary data fitting was performed with the software of the stopped-flow apparatus, and secondary analysis was with GraFit Version 3.0 (Erithacus Software, Staines, United Kingdom) and Scientist Version 2.01 (Micromath, Salt Lake City, UT).

RESULTS

Interaction of mantdGTP and GTP with SelB—Binding of GTP and GDP derivatives with a methylnanthraniloyl group in the ribose moiety to SelB resulted in a significant increase (−130%) in the fluorescence of the attached group. It was difficult to use this signal in equilibrium titration experiments since titrating a fixed concentration of the enzyme with increasing concentrations of fluorescent nucleotide resulted in a strongly increasing background signal from excess unbound nucleotide. The standard solution to this problem would be to titrate an increasing concentration of protein to a fixed concentration of fluorescent nucleotide. This was precluded by the low solubility of the protein, which prevented preparation of a highly concentrated stock solution. These problems did not prevent transient kinetic experiments using the stopped-flow method, and an example of the fluorescence transient seen on rapid mixing of SelB and mantdGTP is shown in Fig. 2A. mantdGTP was used for most of the studies reported here since it was noticed that with mantGTP (i.e. the ribose rather than the deoxyribose derivative), biphasic association curves were obtained, and it was suspected that this was due to the fact that mantGTP is a mixture of the 2'- and 3'-isomers (20). The monophasic transients observed with mantdGTP were consistent with this interpretation.

Increasing the mantdGTP concentration while keeping the SelB concentration constant led to a linear increase in the observed pseudo first-order rate constant of association, as shown in Fig. 2B. In this type of experiment, the observed rate constant is given by the following relationship (Equation 1),

\[ k_{\text{obs}} = k_{+1}(\text{mantdGTP}) + k_{-1} \]

where \(k_{+1}\) and \(k_{-1}\) are the association and dissociation rate constants, respectively in the interaction between the protein and the nucleotide. The slope of the fitted straight line in Fig. 2B defines the value of \(k_{+1}\) as \(1.67 \times 10^5 \text{ M}^{-1} \text{s}^{-1}\), whereas the intercept on the y axis is small, but appears to be of the order of \(0.1 \text{ s}^{-1}\), giving an approximate value for \(k_{-1}\).

The value of \(k_{-1}\) was determined more exactly in a displacement experiment in which an excess of unlabeled GTP was used to displace mantGTP. As shown in Fig. 3, the rate of mantdGTP release is indeed relatively slow and has the value of 0.092 s\(^{-1}\). Taking the two kinetic constants together allows us to calculate a value of 0.55 \(\mu\)M for the \(K_d\) value of mantdGTP. This is in reasonable agreement with the value of 1.7 \(\mu\)M determined by equilibrium dialysis using radioactively labeled GTP (6). Since it was of importance to determine the corresponding parameters for the natural substrate GTP, intrinsic protein...
fluorescence was also examined as a potential signal of nucleotide binding. A small (−2.5%) increase in fluorescence occurred on GTP binding, and although this was too small to be used conveniently for accurate equilibrium titrations, it could be used to monitor the association kinetics with SelB using the stopped-flow method. As can be seen from the data of Fig. 4A, the signal is noisy, but increasing the GTP concentration led to an obvious increase in the rate of the transient seen. In Fig. 4B, it is apparent that there is a linear dependence of the observed rate constant on the GTP concentration, and the slope of the plot of the rate constant against GTP concentration leads to a value of $2.16 \times 10^5 \text{M}^{-1} \text{s}^{-1}$, which is similar to that of mantdGTP from Fig. 2B. The rate constant for the association of unmodified GTP could also be determined by using it as a competitor for mantdGTP in experiments of the type described for Fig. 5. Here, the GTP concentration was varied, and the mantdGTP concentration was held constant. The slope of the straight-line fit to the data points defines the rate constant for GTP association with SelB, which, in approximate agreement with the results of Fig. 4B, has a value of $1.93 \times 10^5 \text{M}^{-1} \text{s}^{-1}$.

The rate constant for GTP release was determined by displacement with excess mantdGTP. Since this experiment could not be performed with a very large excess of mantdGTP (because of the high background from unbound mantdGTP), it was performed at two different concentrations of mantdGTP, both in moderate excess over GTP, which was preincubated with SelB. Since the measured rate constant in both cases was identical within experimental error, we conclude that this represents the value $k_{-1}$ for GTP ($0.16 \text{ s}^{-1}$). Again, the calculated $K_d$ value (0.74 $\mu$M) is in good agreement with that determined previously by equilibrium dialysis (6).

Interaction of mantdGDP and GDP with SelB—GDP and mantGDP displayed quantitatively different kinetics of the interaction with SelB when compared with GTP or mantGTP. Initial stopped-flow experiments showed that the association reaction was faster than for GTP or mantGTP. Since the signal quality at the high concentrations of mantGDP needed for these studies was not sufficient to detect the transition from monophasic behavior seen with mantGTP, the more readily available mantGDP (and not mantdGTP) was used for these experiments. Fig. 6A shows the dependence of the observed rate constant for association of mantGDP on the concentration.

From the approximately linear dependence, values of $1.79 \times 10^6 \text{M}^{-1} \text{s}^{-1}$ for the association rate constant and $14.25 \text{ s}^{-1}$ for the dissociation rate constant were obtained. The rate constant for association of unmodified GDP was obtained by examining the competing influence of GDP on the mantGDP association, as described above for GTP, and the results are shown in Fig. 6B. The value obtained from the slope of the fitted straight line ($1.12 \times 10^6 \text{M}^{-1} \text{s}^{-1}$) is similar to that for mantGDP and confirms that the association rate constant is considerably greater than for GTP.

A further indication of the more rapid equilibration of GDP compared with GTP was obtained from experiments in which GDP was used as a competitor of mantdGTP binding in association reactions. In contrast to the behavior shown in Fig. 5, where GTP competed with mantdGTP binding, the first-order rate constant for mantdGTP binding decreased as the GDP concentration was increased (data not shown). As discussed previously (21), this type of behavior arises when the competi-
ing ligand is in rapid equilibrium with its bound form on the time scale of the observed transient.

More accurate estimates of the dissociation rate constants for mantGDP and GDP were obtained from displacement experiments, as shown in Fig. 7. Displacement of mantGDP by GDP could be measured easily since a large excess of the nonfluorescent unmodified nucleotide could be used. The value of the dissociation rate constant obtained (13.9 s\(^{-1}\)) is consistent with the magnitude of the intercept on the y axis in Fig. 6A (14.25 s\(^{-1}\)). GDP dissociation was more difficult to measure due to the large background fluorescence of a high concentration of mantGDP as displacing agent. The experiment was repeated at two different concentrations of displacing agent, with little effect on the measured rate constant, which was \(-15\) s\(^{-1}\).

The calculated \(K_d\) values for GDP and mantGDP are 13.4 and 7.8 \(\mu\)M, respectively. The results of the kinetic experiments with GTP, GDP, and their fluorescent derivatives are summarized in Table I.

**Interaction of RNA Stem-Loops with SelB**—On interaction of SelB with the 17-mer \(fdhF\) minihelix, there was a small quenching of the intrinsic protein fluorescence. However, the size of the signal change was too small to allow reliable kinetic or titration experiments to be performed.

Detailed investigations of the interactions of the two RNA stem-loop structures directing selenocysteine insertion in \(E.\ coli\) (Fig. 1) with SelB were possible using a fluorescently labeled construct. The \(fdhF\) minihelix bearing a fluorescent group at the 5'-end displayed a significant increase in fluorescence intensity on interaction with the protein. The signal was stable enough to allow a titration of SelB to a constant concentration of labeled \(fdhF\) minihelix RNA. This resulted in a maximal increase of \(-7\%\) in fluorescein fluorescence. The shape of the curve obtained (Fig. 8A) already indicates a high affinity for the interaction since at the concentration used (181 nM fluorescent \(fdhF\) minihelix), the almost linear approach to the plateau reached at saturation suggests that the \(K_d\) value must be much less than 181 nM. Fitting the data to a quadratic equation leads to a \(K_d\) value of 0.7 nM. However, this value is not regarded as highly reliable since it will be determined by just a few points as saturation is closely approached. The signal was not stable enough to allow titrations at significantly lower concentrations of RNA. However, the kinetic studies described below confirm that the \(K_d\) value is in the range of 1 nM.

To test whether the fluorescent label on the RNA has an influence on the interaction with SelB, a competitive titration was performed in which fluorescent \(fdhF\) minihelix was displaced from its preformed complex with SelB by a large excess of unlabeled \(fdhF\) minihelix. As shown in Fig. 8B, the labeled RNA could be completely displaced. This provides evidence that the binding is purely competitive and excludes the possibility of nonspecific binding of the labeled RNA. Fitting the data using the program Scientist led to the conclusion that the affinity of SelB for the \(fdhF\) minihelix is almost unaffected by the modification. Using a \(K_d\) value of 1.26 nM for fluorescent \(fdhF\) minihelix, the \(K_d\) for the unmodified minihelix was calculated to be 1.36 nM. Fig. 8B also shows that displacement by the other stem-loop construct examined (\(fdnG\)) led to identical results, and the derived \(K_d\) value was 1.38 nM. We thus conclude that the interaction of the stem-loop structures with SelB occurs with nanomolar affinity and is unaffected by a large fluorescent label at the 5'-end of the structure.

In further experiments, the fluorescently labeled RNA was used to investigate the kinetics of the interaction with SelB.

**FIG. 5.** Competitive binding kinetics of SelB with mantdGTP in presence of varying concentrations of GTP. The concentrations of SelB and mantdGTP were 0.6 and 6 \(\mu\)M, respectively. Fluorescence was detected as described in the legend Fig. 2. The fitted line corresponds to a value of \(1.93 \times 10^2\) M\(^{-1}\) s\(^{-1}\) for \(k_{-1}\).

**FIG. 6.** Kinetics of association of SelB with mantGDP and GDP. A, dependence of the pseudo first-order rate constant for association of SelB (0.75 \(\mu\)M) with mantGDP on the nucleotide concentration. Fluorescence was detected as described in the legend to Fig. 2A. The slope of the fitted straight line corresponds to a value of \(1.79 \times 10^2\) M\(^{-1}\) s\(^{-1}\) for \(k_{+1}\) whereas the y axis intercept gives a value of 14.25 s\(^{-1}\) for \(k_{-1}\). B, competitive binding kinetics of SelB (0.6 \(\mu\)M) with mantGDP (5 \(\mu\)M) in the presence of varying concentrations of GDP. Detection was as described in the legend to Fig. 3. The fitted line corresponds to a value of \(1.12 \times 10^2\) M\(^{-1}\) s\(^{-1}\) for \(k_{-1}\).
To examine possible interaction or communication between the nucleotide and stem-loop binding sites, the experiments of Fig. 9 were repeated in the presence of GDP and GTP. As can be seen from the values for the association and dissociation rate constants given in Table II, there was no detectable influence of the state of the nucleotide binding site of SelB on the kinetics of the interaction.

In complementary experiments, the effect of stem-loop RNA on the interaction of the protein with nucleotides was examined. As shown in Table III, the values for the association and dissociation rate constants for mantGTP and mantGDP obtained in the presence of the fdnG stem-loop at a concentration that is enough to saturate the mRNA binding site are indistinguishable from those obtained in the absence of RNA.

Interaction of the RNA Binding Domain (Domain IVb) with Stem-Loop RNA—To determine to what extent the RNA binding properties of this domain are affected by the rest of the protein, we examined the interaction of the separately expressed domain with the stem-loop structure. Using the fluorescein-labeled fdhF minihelix and the isolated domain in the stopped-flow apparatus, the association kinetics were nearly identical to those seen with full-length SelB ($k_a = 2.58 \times 10^8 \text{M}^{-1} \text{s}^{-1}$).
M\textsuperscript{2}1 s\textsuperscript{2}1) (data not shown). However, the dissociation kinetics were slowed significantly. The rate constant for dissociation was 0.037 s\textsuperscript{2}1 (Fig. 10\textsuperscript{B}), in comparison with 0.28 s\textsuperscript{2}1 for the dissociation from full-length SelB. Thus, the affinity of the isolated fragment (K\textsubscript{d} = 0.14 nM) is increased in comparison with full-length SelB (K\textsubscript{d} = 1.26 nM).

An indication that this difference in the properties of SelB and domain IVb might be of mechanistic importance comes from the second curve shown in Fig. 10A. We have not yet been able to identify a useful direct signal to monitor the interaction of selenocysteyl-tRNASec with SelB, but a clear indication of its binding arises from the observation that in the presence of the charged tRNA, the rate of dissociation of the fdhF minihelix is slowed significantly. In quantitative terms, it is slowed to a rate that is nearly indistinguishable from that of the stem-loop construct from isolated domain IVb, suggesting that whatever the nature of the interactions of domain IVb with the other domains of SelB that lead to a reduction of its affinity for the stem-loop structure, these interactions are removed or relieved when selenocysteyl-tRNA\textsuperscript{Sec} is bound.
FIG. 11. Kinetics of association of fluorescein-labeled fdhF minihelix with SelB in the presence of different concentrations of unlabeled fdhF minihelix. The concentrations of fluorescein-labeled fdhF minihelix (0.136 μM) and SelB (0.128 μM) were constant, whereas the concentration of unlabeled minihelix was varied as indicated in the graph. Fluorescence was detected as described in the legend to Fig. 9A. The fitted lines represent association transients calculated by global analysis of the curves using the program Scientist. They define the values of the rate constants for the association and dissociation reactions as $k_{a1} = 2.39 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ and $k_{1} = 0.28 \text{ s}^{-1}$ for the labeled fdhF minihelix and $k_{a1} = 1.09 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ and $k_{1} = 0.28 \text{ s}^{-1}$ for the unlabeled fdhF minihelix.

TABLE III Rate constants for the interaction of guanosine nucleotides with SelB in the presence of the fdnG stem-loop structure

| Nucleotide | $k_a$ | $k_d$ | $K_d$ |
|------------|-------|-------|-------|
| mantGDP    | $1.60 \times 10^6 \mu M$ | $14.0$ | $8.75$ |
| mantGTP    | $1.71 \times 10^3 \mu M$ | $0.091^a$ | $0.53$ |

$^a$ 3.75 μM fdnG stem-loop was included in all solutions.
$^b$ 1.5 μM fdnG stem-loop was included in all solutions.

DISCUSSION

The results presented show that SelB displays a relatively low affinity for GTP and GDP in binary complexes between the protein and the nucleotides. In contrast to EF-Tu, the GTP affinity is greater (by an order of magnitude) than the GDP affinity. This difference is mainly due to a dramatic difference in the GDP affinities in the two systems, with the GTP affinities being more similar. Although GDP is bound more weakly than GTP, its kinetics of interaction with SelB are considerably faster. Thus, the association rate constant is almost an order of magnitude faster for GDP than for GTP (cf. a factor of 3 for EF-Tu) (22). In terms of affinity, this effect is more than compensated for by the fact that GDP is released 2 orders of magnitude faster than GTP so that GTP binds more strongly than GDP.

The weaker binding of GDP compared with GTP and, in particular, the high dissociation rate constant are consistent with the idea that an exchange factor for SelB is not required since the intrinsic GDP dissociation rate is of the same order of magnitude as the exchange factor-catalyzed dissociation rate with EF-Tu. Somewhat similar properties have been seen for the E. coli signal recognition particle receptor FtsY (23), i.e., weak affinities for GTP and GDP and high exchange rates. However, in this case, GDP is bound more strongly than GTP and exchanges rapidly in comparison with EF-Tu or the Ras family of proteins, but more slowly than GTP.

When the kinetic results obtained in this work are compared with those for other GTPases, the most striking difference observed is the rapid rate of spontaneous GDP dissociation. With the exception of FtsY, all other GTPases involved in signal transduction and regulation that have been characterized in detail with respect to their kinetic properties have slow ($\approx 10^{-8} \text{ s}^{-1}$ for EF-Tu) (22) or very slow ($\approx 10^{-6}$ to $10^{-4} \text{ s}^{-1}$ for Ras and Ras-related proteins) (21, 24) GDP dissociation rates, which are accelerated dramatically by exchange factors. The same is also true for the heterotrimeric G-proteins; and in the case of GTPases involved in signal transduction, it is easy to understand why GDP release must occur in a manner that is dependent on activation or recruitment of an exchange factor, this process being dependent, in general, on primary activation events in the signal transduction pathway. For ribosomal elongation factors, the requirement for an exchange factor is more difficult to rationalize; and in the case described here, SelB appears to function without one, proving that the kinetic properties of the GTPase can be designed so that the principle already known from other elongation factors can work even if the GDP produced concomitantly with peptide bond formation is bound weakly to the factor and dissociates spontaneously, allowing rebinding of GTP and the start of another cycle.

The experiments reported on the kinetics of the interaction of stem-loop constructs with SelB show that this is a high affinity interaction, with a $K_d$ value of $1.3 \text{ nM}$. The association rate constant approaches the diffusion-controlled limit, and dissociation in the absence of tRNA occurs with a half-life of a few seconds. Neither the association nor the dissociation kinetics are affected by the presence or the nature of the guanosine nucleotide at the active site of the GTPase domain. Conversely, as is to be expected based on thermodynamic considerations, the presence of a stem-loop construct at its binding site does not affect the kinetics of the nucleotide interaction with SelB. Thus, there is no coupling between the nucleotide binding site and the RNA binding site, meaning that recruitment of SelB to its binding site on mRNA is not dependent on the state of the nucleotide binding site.

Although we were not able to identify a usable spectroscopic signal for the interaction of selenocysteyl-tRNASec with SelB, there was a clear indication that the rate of dissociation from the stem-loop structure was slower when the charged tRNA was bound, suggesting an interaction between the two binding sites. The fact that a similar slowing down of the dissociation rate from mRNA was seen with the isolated domain IVb suggests that an interaction between the tRNA binding domain and the mRNA binding domain occurs in the absence of tRNA that is weakened or removed when tRNA is bound. Further studies with additional signals will be needed to clarify the nature of the interactions between the two different types of RNA binding sites. The possible functional significance of this effect is that the full affinity of SelB for its binding site on RNA is only realized when selenocysteinylation occurs with SelB and the selenocysteyl-tRNASec is bound. Binding of the charged tRNA thus increases the stability of the SelB-GTP-selenocysteyl-tRNASec-mRNA quaternary complex and thereby favors the conformation of SelB able to interact with the ribosome. This interaction triggers GTP hydrolysis and most probably the release of the mRNA (GTPase sites) (4). As a consequence, there will be an increase in the rate of dissociation of SelB from its mRNA binding site. This is required for the translation of downstream codons since SelB complexed to its SECIS site exerts a considerable kinetic barrier to the processivity of translation (25).

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