A Kinetic Light-Scattering Study of the Binding of Wheat Germ Protein Synthesis Initiation Factor 3 to 40S Ribosomal Subunits and 80S Ribosomes†

Dixie J. Goss* and Donna J. Rounds
Department of Chemistry, Hunter College of the City University of New York, 695 Park Avenue, New York, New York 10021
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ABSTRACT: The rate constants for eucaryotic initiation factor 3 (eIF3) association and dissociation with 40S ribosomal subunits and 80S monosomes have been determined. These rate constants were determined by laser light scattering with unmodified eIF3. The affinity of eIF3 for 40S subunits is about 30-fold greater than for 80S ribosomes. This difference in affinity resides mainly in the association rate constants. Rate constants of 8.8×10^7 and 7.3×10^6 M^{-1} s^{-1} were obtained for eIF3 binding to 40S subunits and 80S ribosomes, respectively. From thermodynamic cycles, the affinity of eIF3–40S subunits for 60S subunits is about 30-fold lower than free 40S subunits for 60S subunits. A calculation shows that under these conditions and assuming simple equilibria, approximately 12% of ribosomal subunits would associate via a reaction of 40S–eIF3 with 60S subunits as opposed to a path where eIF3 dissociates from the 40S subunits prior to association with 60S subunits.

The primary events of initiation of protein synthesis in eucaryotes are the dissociation of the 80S ribosome into subunits and the formation of the preinitiation complex with the 40S subunit, Met-tRNA, and mRNA. While both events are believed to be stimulated by eucaryotic polypeptide chain initiation factor 3 (eIF3),[1] the details of this process remain unclear. Elucidation of the possible translational control mechanisms and the pathway of initiation requires a detailed knowledge of the kinetic parameters for the various reactions. A number of studies have focused on the rates of procaryotic processes. The rate of initiation factor 3 (IF3) binding to ribosomes has been studied by light-scattering and fluorescence techniques (Weil & Hershey, 1981; Wooley & Box, 1979; Chaires et al., 1979; Goss et al., 1980, 1982). Studies of the rates of binding of fMet-tRNA and mRNA to ribosomes have used membrane filtration (Gualerzi et al., 1977; Van der Hofstad et al., 1978) and spectrophotometric techniques (Wintermeyer & Gualerzi, 1983). Recently (Zucker & Hershey, 1986), fluorescence polarization was used to determine the binding affinity of *Escherichia coli* initiation factor 1 (IF1) to 30S subunits and the effects of IF2 and IF3 on the binding affinity.

Less data are available on the rates of eucaryotic initiation factor interactions. Since procaryotic protein synthesis is greater than an order of magnitude faster than eucaryotic protein synthesis, it is important to determine these rates. We have previously reported the rates of *Artemia* ribosomal subunit interactions (Goss & Harrigan, 1986) and the effects of eIF3 on *Artemia*, wheat germ, and rabbit reticulocyte ribosomal subunit interactions (Goss et al., 1988). Earlier studies (Trachsel & Staehelin, 1979; Benne & Hershey, 1978) using ultracentrifugation and membrane filtration have focused on the interaction of eIF3 with 40S ribosomal subunits and the effects of other factors. These methods are unsuited for quantitative kinetic studies.

In this paper, we report the first measurements of an unmodified eucaryotic initiation factor binding. We have investigated the binding interactions of eIF3 with the 80S ribosome and the 40S ribosomal subunit utilizing stopped-flow laser light-scattering techniques. The affinity of eIF3 for 40S subunits is about 30-fold greater than for 80S ribosomes. This difference in affinity resides mainly in the association rate constants.

Materials and Methods

Wheat germ ribosomes were prepared as described by the method of Sperrazza and Spremulli (1983). Ribosomes were stored under liquid nitrogen in buffer consisting of 20 mM HEPES–KOH, pH 7.6, 5 mM $MgCl_2$, 6 mM dithiothreitol, 10% glycerol, and 50 mM KCl. Wheat germ initiation factor 3 was prepared according to Lax et al. (1986). Some of the eIF3 used in this study was the generous gift of Professor J. Ravel. Five different ribosome preparations and four different eIF3 preparations were used in these studies.

Subunit Isolation. Ribosomes (0.5–1.0 A_{260} unit) were incubated 5–10 min and 35 °C in a reaction mixture (0.5 mL) containing 1.0 mM Mg^{2+}. The samples were overlayed on a 12-mL, 10–30% sucrose gradient with a buffer containing 20 mM Tris-HCl (Trizma), pH 7.5, 100 mM KCl, 2 mM dithiothreitol, 0.05 mM EDTA, and 1 mM $MgCl_2$. After centrifugation at 4 °C for 2 h at 32 000 rpm in a Beckman SW40 rotor, 0.25-mL fractions were collected. The absorbance profile at 260 nm was obtained by the use of a Varian 634 spectrophotometer.

Determination of Ribosome and Subunit Concentrations. Ribosomes were assumed to have an absorbance of 0.121 at 260 nm for a 0.001% solution. The molecular weights of the wheat germ 80S ribosome and the 60S and 40S subunits were assumed to be 3.8×10^6, 2.4×10^6, and 1.4×10^6, respectively. The molecular weight of eIF3 was taken to be 660 000 (Behlke et al., 1986).

Kinetic Measurements. Association and dissociation reactions for eIF3 and ribosomes were monitored by 90° light scattering. The scattered light voltage with background subtracted, V_t, given by (Tanford, 1967)

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* Correspondence should be addressed to this author.

[1] Abbreviations: eIF, eucaryotic polypeptide chain initiation factor; IF, procaryotic polypeptide chain initiation factor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid sodium salt; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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$$V_t = a\sum m_i C_i \quad (1)$$

where a is a constant, m_i is the molecular weight of the ith species, and C_i is the concentration in grams per milliliter. If n_i is the number of moles per milliliter, eq 1 then becomes

$$V_t = a\sum m_i^2 n_i \quad (2)$$

V_t/a' is then $1.4^2[40S]_t + 0.66^2[eIF3]_t + 2.06^2[40S–eIF3]_t$; a' is a constant, and $[40S]_t$, $[eIF3]_t$, and $[40S–eIF3]_t$ represent the concentrations of the respective species at time t. Because of the large molecular weight of eIF3, light scattering can be used to monitor these reactions. For the association of eIF3 with 40S subunits or 80S ribosomes, $V_{max} - V_t$ is proportional to the concentration of 40S or 80S ribosomes and eIF3 unreacted. For the dissociation of eIF3 from 40S subunits or 80S ribosomes, the overall voltage change V_{max} is proportional to the initial concentration of eIF3–40S or eIF3–80S complex. At any time t, $V_{max} - V_t$ is proportional to $[eIF3–40S]_t$ or $[eIF3–80S]_t$. The amplitudes of the curves for a reaction going to completion can be calculated from eq 2. For the 40S + eIF3 association, the theoretical amplitude is $2.06^2[40S–eIF3]_\infty - 1.4^2[40S]_0 - 0.66^2[eIF3]_0$. If the reaction goes to completion and $[40S]_0 = [eIF3]_0$, $[40S–eIF3]_\infty = [40S]_0$ and $\Delta V_t/a' = (V_\infty - V_0)/a' = 1.84[40S]_0$.

Kinetic measurements were performed using a Hi-Tech SF-51 stopped-flow device interfaced to a Zenith Z-100 computer with 768K memory and dual disk drives. The light source for the stopped-flow device was a Liconix 4210 NB helium–cadmium laser with either 325- or 441-nm optics. Buffer B consisting of 20 mM HEPES, pH 7.6, 1 mM dithiothreitol, 50 mM KCl, and $MgCl_2$ as indicated was used for kinetic measurements.

Control Assay 1. Isolated 40S and 60S subunits in 1, 2, 4, and 6 mM Mg^{2+} were mixed in the stopped-flow instrument to determine subunit viability and the rate of subunit association.

Control Assay 2. 40S subunits were mixed in the stopped-flow device with buffer B in 2 mM Mg^{2+} to determine any light-scattering changes that occurred upon dilution.

Control Assay 3. 80S ribosomes were mixed alone against buffer B with 2 mM Mg^{2+} to determine any dissociation in the absence of eIF3 that could affect the interpretation of the light-scattering results.

Experiment 1. 40S subunits in buffer B with 2 mM Mg^{2+} were mixed in the stopped-flow instrument against eIF3 in the same buffer to determine association rate constants.

Experiment 2. 40S subunits and eIF3 were incubated together for 20 min in buffer B with 2 mM Mg^{2+}. The samples were mixed in the stopped-flow device against buffer B with equivalent Mg^{2+} concentration. Unequal-driving syringes were used to give a 1:6 dilution. According to Le Chatelier's principle, such a dilution will cause dissociation of some of the eIF3–40S complex. This experiment was used to monitor the dissociation reaction.

Experiment 3. 80S ribosomes were incubated in buffer B with 2 mM Mg^{2+} and mixed against eIF3 in equivalent buffer to monitor the association reaction.

Experiment 4. 80S ribosomes were incubated with eIF3 at 2 mM Mg^{2+}. The samples were flowed against buffer B with equivalent Mg^{2+} concentration. Unequal-driving syringes were used to give a 1:6 dilution. As in experiment 2, this experiment was used to monitor the dissociation of eIF3 from 80S ribosomes.

Determination of Equilibrium Constants. Equilibrium constants were determined both by the ratio of the rate constants ($K_1 = k_1/k_{-1}$) and from the amplitude of the kinetic curves. The amplitude of the kinetic curves is related to the concentration of species as discussed above. From the molecular weights, the expected amplitude for the reaction 40S + eIF3 → 40S–eIF3 is 1.84 times the initial signal with background subtracted. The reaction eIF3 + 80S → eIF3–80S has a theoretical amplitude of 1.34 times the initial signal with background subtracted.

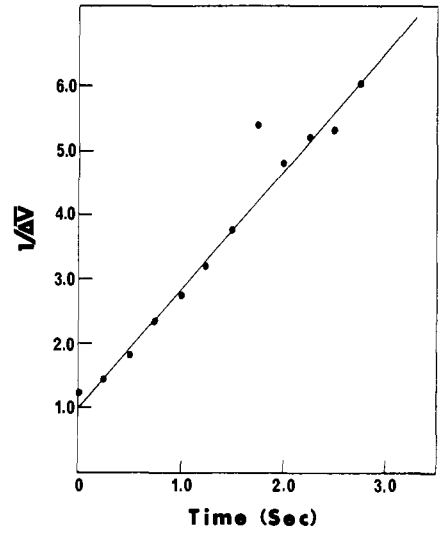

FIGURE 1: Association of wheat germ eIF3 with 40S subunits. The data points represent the average of eight kinetic runs. The ordinate is the reciprocal of the normalized voltage change and is directly related to [40S unreacted]$^{-1}$. The reaction was carried out in buffer B with 2 mM $MgCl_2$. The solid line represents the best fit to the data, and the rate constant is reported in Table I. Ribosome concentration was 0.016 μM; eIF3 concentration was 0.018 μM after mixing. The temperature was 20 °C.

Data Analysis. The data for eIF3 association to either 40S subunits or 80S ribosomes were fit according to a simple bimolecular association: 40S + eIF3 → 40S–eIF3. The data for the dissociation reaction were fit to the reverse of the above reaction. The light-scattering measurements were carried out at 325 or 441 nm. For each sample, a minimum of eight kinetic runs were averaged by computer, thus providing 900 raw data points to generate a curve suitable for data fitting. The minimum of the response function for theoretical models was found by the Fletcher–Powell nonlinear minimization algorithm (Fletcher & Powell, 1963). For each apparent minimum, an estimate of the standard errors in the parameters was obtained by a statistical routine, which gives the variance–covariance matrix of the parameters (Draper & Smith, 1966).

Results and Discussion

Figure 1 shows the second-order reaction of eIF3 binding to 40S ribosomal subunits. The reaction was monophasic, characteristic of a simple bimolecular reaction, and a rate constant of 8.8×10^7 M^{-1} s^{-1} was obtained. For six different ribosome and five different eIF3 preparations studied, the rate constants varied by a maximum of 23%. The greatest variation was seen in the association rate constant which ranged from 7.6×10^7 to 9.3×10^7 M^{-1} s^{-1}. We have previously reported (Goss et al., 1988) static light-scattering data which suggest that eIF3 interacts with 80S ribosomes at low $[Mg^{2+}]$. Figure 2 shows the second-order reaction for 80S ribosomes flowed against eIF3 at 2 mM Mg^{2+}. Control experiments were performed to determine if the ribosomes were in fact 80S particles. One control was to flow the 80S ribosomes against higher Mg^{2+} concentrations and determine if any further association occurred. These experiments showed that no

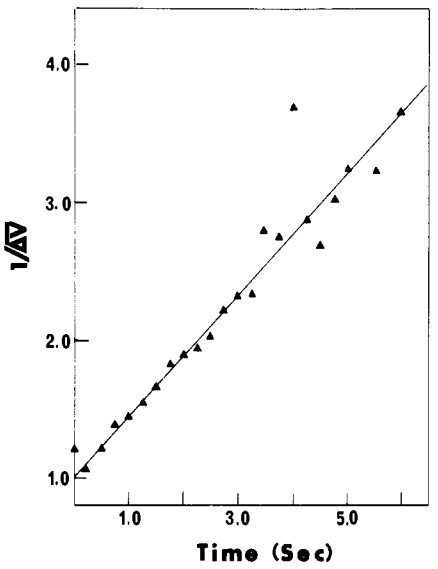

FIGURE 2: Association of wheat germ eIF3 with 80S subunits. The data points represent the average of 30 kinetic runs. The ordinate is the reciprocal of the normalized voltage and is directly proportional to [80S unreacted]$^{-1}$ (see text for details). The reaction was carried out in buffer B with 2 mM $MgCl_2$. The solid line represents the best fit to the data. The rate constant is reported in Table I. Ribosome concentration was 0.054 μM; eIF3 concentration was 0.083 μM after mixing. The temperature was 20 °C.

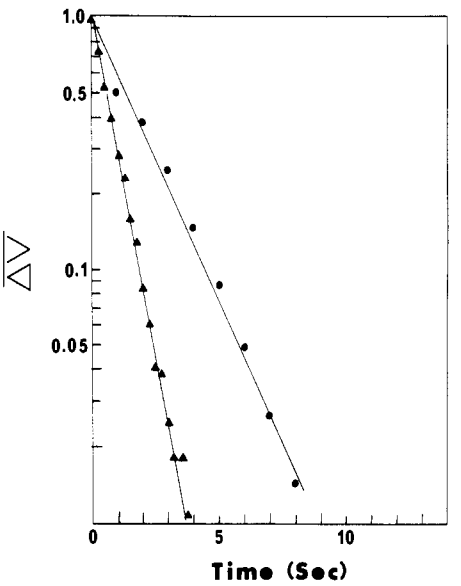

FIGURE 3: Dissociation of eIF3 from 40S subunits and 80S ribosomes. The circles and triangles represent the data for the dissociation of eIF3 from 80S ribosomes and 40S subunits, respectively. Ribosome concentration was 0.16 and 0.22 μM for 40S subunits and 80S ribosomes, respectively. The concentration of eIF3 was 0.22 μM in both cases. The data points represent an average of 12 kinetic runs in each case. The ordinate is the normalized voltage and is directly proportional to the initial concentration of 40S–eIF3 or 80S–eIF3. Buffer B with 2 mM $MgCl_2$ was used. The temperature was 21 °C. The rate constants are reported in Table I.

further association took place; however, it would still be possible to have small amounts of 40S particles present which did not associate. Sucrose gradient analysis indicated at least 95% of the ribosomes were of the 80S form. Static light scattering, taking into account the amplitude of the signal (Goss & Harrigan, 1986), also suggested at least 95% associated 80S ribosomes. The reaction curves were monophasic, suggesting a uniform population. The amplitude of the reaction was too large to be accounted for by the small amount of 40S subunits that may be present. A rate constant of 7.3 $\times$ 10^6 M^{-1} s^{-1} was obtained for the reaction of eIF3 with 80S ribosomes. These rates indicate the association to 40S subunits is about an order of magnitude faster than the association to 80S ribosomes. The 40S–eIF3 association rate constant is also about an order of magnitude greater than the rate constant for procaryotic IF3 association to 30S ribosomes. A rate constant of 8.3 $\times$ 10^6 M^{-1} s^{-1} has been reported for procaryotic IF3–30S association (Goss et al., 1982). The rate of eIF3 binding to 40S subunits is close to the diffusion-controlled rate. This is consistent with the function of eIF3 in mediating ribosomal subunit association. A substantial conformational change of the 40S subunit on this time scale is not very likely; however, a change could occur after binding.

Figure 3 shows the data for eIF3 dissociation from 40S and 80S ribosomes. A rate constant of 0.52 s^{-1} was obtained for the dissociation of 40S–eIF3, and a rate constant of 1.3 s^{-1} was obtained for 80S–eIF3 dissociation. The difference in affinity of eIF3 for 40S and 80S ribosomes is primarily controlled by the association process. The dissociation rate constant for procaryotic IF3–30S has not been directly determined; however, the dissociation rate constant calculated from the reported equilibrium constant of 2.7 $\times$ 10^7 M (Weil et al., 1978) and the association rate of 8.3 $\times$ 10^6 M^{-1} s^{-1} (Goss et al., 1982) is 0.31 s^{-1}, very close to the rate obtained for the eucaryotic factor dissociation.

If one describes the function of eIF3 in terms of simple equilibria, Scheme I can be written.

The four equilibrium constants can be written as ratios of the rate constants where $K_1 = k_1/k_{-1}$ etc. Only three of the equilibrium constants are thermodynamically independent. We have determined equilibrium constants or rate constants for K_1, K_2, and K_4. The K_{eq} values were determined from the amplitude of the curves as described above as well as the ratio of the rate constants. K_3 can then be written as $K_3 = K_4K_1/K_2$. Table I shows the values for these rate constants and the corresponding equilibrium constants. The value of K_3 is about 30-fold lower than the value of K_1. In other words, 40S–eIF3

Scheme I

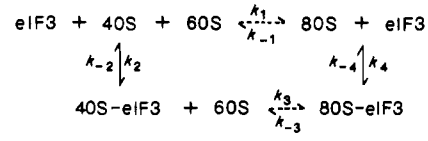

Table I

rate constant	equilibrium constant	comments
k_1 = (4.95 ± 0.09) $\times$ 10^6 M^{-1} s^{-1}	K_1 = 3.3 $\times$ 10^6 M^{-1}	*a, b*
k_{-1} = 0.019 ± 0.002 s^{-1}		
k_2 = (8.8 ± 0.4) $\times$ 10^7 M^{-1} s^{-1}	K_2 = 1.7 $\times$ 10^8 M^{-1} (1.8 $\times$ 10^8 M^{-1})	*b, c*
k_{-2} = 0.52 ± 0.03 s^{-1}		
k_3 = 1.6 $\times$ 10^5 M^{-1} s^{-1}	K_3 = 1.05 $\times$ 10^7 M^{-1}	*d*
k_{-3} = 0.015 s^{-1}		
k_4 = (7.3 ± 0.2) $\times$ 10^6 M^{-1} s^{-1}	K_4 = 5.6 $\times$ 10^6 M^{-1} (5.2 $\times$ 10^6 M^{-1})	*b, e*
k_{-4} = 1.3 ± 0.1 s^{-1}		

[a] Rate constants from Goss et al. (1988). [b] K_{eq} determined from the ratio of the rate constants. [c] The K_{eq} value in parentheses was the average value determined from the amplitude of the association reaction kinetic runs. Three kinetic experiments were used. The concentrations of 40S and eIF3 were approximately equimolar and ranged from 0.016 to 0.091 μM after mixing. K_{eq} values ranged from 1.74 $\times$ 10^8 to 1.83 $\times$ 10^8 M^{-1}. [d] K_{eq} determined from the thermodynamic cycle $K_3 = K_4K_1/K_2$. k_{-3} from Goss et al. (1988). k_3 calculated from K_{eq} and k_{-3}. [e] The K_{eq} value in parentheses was the average value determined from the amplitude of the association reaction. Four kinetic experiments were used. The concentrations of 80S and eIF3 were approximately equimolar and ranged from 0.16 to 0.38 μM. K_{eq} values ranged from 4.9 $\times$ 10^6 to 5.7 $\times$ 10^6 M^{-1}.

has about a 30-fold lower affinity for 60S subunits than free 40S subunits. The dissociation rate of 80S ribosomes to 40S and 60S subunits is unaffected by eIF3. This simple thermodynamic cycle gives an association rate for 40S–eIF3 + 60S → 80S–eIF3 of 1.6×10^5 M^{-1} s^{-1}. If one calculates an initial flow through the two paths of association, about 12% of the 40S subunits would associate via the 40S–eIF3 route as opposed to a prior eIF3 dissociation.

This simple scheme neglects conformational changes in the ribosomes and is probably an oversimplification for detailed analysis. Further studies on the effects of other initiation factors with eIF3 should lead to a more detailed mechanism of the initiation of protein synthesis.

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Multiple Sites and Synergism in the Binding of Inhibitors to Microsomal Aminopeptidase†

Maria DiGregorio, Darryl S. Pickering, and William W.-C. Chan*
Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada L8N 3Z5
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ABSTRACT: The active site of microsomal aminopeptidase has been probed by studying the inhibition of the enzyme in the simultaneous presence of two ligands. The results have been analyzed with the Yonetani–Theorell plot to quantitate the degree of interaction between the two inhibitors. As expected, the enzyme contains a strong binding site for the α-amino group and the hydrophobic side chain of specific substrates. In addition, however, the enzyme can interact with another amine and a second hydrophobic group. Evidence suggests that this extra amine may bind to the zinc in an unprotonated form and that one of the hydrophobic sites is located in the vicinity. Another unexpected finding in this work is a strong synergism between the binding of ammonia and that of zinc ligands such as hydroxamates. This synergism may reflect an induced-fit mechanism that brings the catalytically important zinc atom into the optimal state only in the presence of specific substrates.

The existence of enzymes that specifically cleave the N-terminal residue of peptides has been recognized for some time. [For a review, see Delange and Smith (1971).] These aminopeptidases are widely distributed in nature and may perform a variety of physiological functions.[1] Of particular current interest is the suggestion that an enzyme of this type may participate in the inactivation of neuropeptides (Gros et al., 1985).

In addition to its usefulness as a tool in determining peptide sequence, aminopeptidase has also attracted some attention because of its mechanism (Bryce & Rabin, 1964; Lin & Van Wart, 1982; Makinen et al., 1982; Taylor et al., 1982; Allen et al., 1983). Since the enzyme is known to contain an essential

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[1] In this general introduction, we include for the purpose of background information references to aminopeptidases from many sources, fully recognizing that there are differences in their structural properties and perhaps even in their catalytic mechanism. Subsequent discussions will focus specifically on the microsomal enzyme from pig kidney on which our work is based.

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