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The Dimer of the β Subunit of *Escherichia coli* DNA Polymerase III Holoenzyme Is Dissociated into Monomers upon Binding Magnesium(II)†

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ABSTRACT: The β subunit of *Escherichia coli* DNA polymerase III holoenzyme binds Mg^{2+}. Reacting β with fluoresceinmaleimide (FM) resulted in one label per β monomer with full retention of activity. Titration of FM–β with Mg^{2+} resulted in a saturable 11% fluorescence enhancement. Analysis indicated that there was one noncooperative magnesium binding site per β monomer with a dissociation constant of 1.7 mM. Saturable fluorescence enhancement was also observed when titration was with Ca^{2+} or spermidine(3+) but not with the monovalent cations Na^+ and K^+. The Mg^{2+}-induced fluorescence enhancement was specific for FM–β and was not observed with FM–glutathione, dimethoxystilbenemaleimide–β, or pyrenylmaleimide–β. Gel filtration studies indicated that the β dimer–monomer dissociation occurred at physiologically significant β concentrations and that the presence of 10 mM Mg^{2+} shifted the dimer–monomer equilibrium to favor monomers. Both the gel-filtered dimers and the gel-filtered monomers were active in the replication assay. These and other results suggested that the fluorescence increase which accompanies β dissociation is due to a relief from homoquenching of FM when the β dimer dissociates into monomers.

DNA polymerase III holoenzyme is the multisubunit enzyme that is responsible for the majority of replicative DNA synthesis in *Escherichia coli* [for reviews, see Kornberg (1982) and McHenry (1985, 1988)]. The β subunit can be chromatographically separated from holoenzyme,[1] to yield pol III*, a subassembly of the remaining subunits (Wickner & Kornberg, 1973; McHenry & Kornberg, 1977).

β is the *dnaN* gene product (Burgers et al., 1981). From the DNA sequence (Ohmori et al., 1984) and the amino-terminal sequence of β (Johanson et al., 1986), it is known that β has 366 amino acid residues for a molecular mass of 40.6 kDa. While all other holoenzyme subunits are present at only 10–20 copies per cell (Kornberg, 1980), there are about 90–100 β molecules per *E. coli* cell. This value was calculated from β purification data (Johanson & McHenry, 1980): 12 900 β units/g of cell paste in the first fraction and a final specific activity of 12.6×10^6 units/mg. For this calculation, it was assumed that there are 10^{12} *E. coli* cells/g of cell paste. If the volume of an *E. coli* cell is estimated to be 4 fL (~0.8 × 0.8 × 2.0 μm) (DeRobertis & DeRobertis, 1980), then the concentration of β in the cell is about 40 nM monomers.

The ability of β to increase the processivity of holoenzyme subassemblies has become the best understood enzymatic process of DNA polymerase III. DNA polymerase III holoenzyme can replicate an entire 5000-nucleotide G4 circle without dissociating (Fay et al., 1981). β has a significant role in this processivity since pol III*, which lacks β, adds only 200 nucleotides to a primer before it dissociates from the DNA (Fay et al., 1982). β is also required in the formation of initiation complex, the step preceding elongation in which holoenzyme binds to oligonucleotide-primed single-stranded DNA coated with single-stranded DNA binding protein.

There appear to be two pathways to initiation complex formation, involving either the presence or absence of ATP. The complex formed in the presence of ATP is stable; it remains a functional complex even after gel filtration (Wickner & Kornberg, 1973; Wickner, 1976; Burgers & Kornberg, 1982a,b; Johanson & McHenry, 1980, 1982). When a deoxynucleoside triphosphate mixture is added to this complex, highly processive replication occurs. Studies with anti-β IgG (Johanson & McHenry, 1980, 1982) have shown that β becomes immersed within the complex such that it is resistant to the antibody's inhibitory action and remains with the replicative complex during elongation. The stability of the complex is thought to be related to the hydrolysis of ATP (Burgers & Kornberg, 1982b; Johanson & McHenry, 1984; Oberfelder & McHenry, 1987). Once formed, it continues to exhibit ATPase activity (Wickner & Kornberg, 1973; Burgers & Kornberg, 1982a,b; Johanson & McHenry, 1984). However, the full role of the DNA-dependent ATPase has not been elaborated.

Excess β is required to form initiation complex in the absence of ATP. Even though this complex is not as stable to

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[1] Abbreviations: DMSM, 2,5-dimethoxystilbene-4-maleimide; FM, fluorescein-5-maleimide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; holoenzyme, DNA polymerase III holoenzyme; PM, *N*-1-pyrenylmaleimide; pol III*, DNA polymerase III holoenzyme subassembly lacking the β subunit.

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gel filtration (Crute et al., 1983), a nondisruptive technique has shown that this initiation complex is nearly as stable as that formed with ATP (Kwon-Shin et al., 1987). When a deoxynucleoside triphosphate mixture is added to this complex, highly processive replication occurs without the lag observed in the presence of ATP. The synthesis of long DNA products by a preformed initiation complex had similar stability whether formed in the presence of ATP or excess β. In fact, excess β will also increase the processivity of other holoenzyme subassemblies, proving that at least part of β's contribution is through interaction with the polymerase core subunits to produce the essential complex necessary for efficient elongation (LaDuca et al., 1986). There is also genetic evidence that the β subunit and the polymerase subunit interact (Kuwabara & Uchida, 1981).

Most binding studies with β and pol III* have measured the strength of this interaction by indirect functional methods, such as determining the increase in processivity; only a few studies have used equilibrium methods. Holoenzyme requires both ATP and β in order to be efficiently reconstituted from pol III*. The dissociation constant K_D for β in this reaction is 1 nM, whereas in the absence of ATP it is greater than 5 nM (Lasken & Kornberg, 1987). The concentrations of β necessary to form functional initiation complexes are of similar magnitude. The concentration of β for half-maximum stimulation of long product synthesis is 1 nM (Lasken & Kornberg, 1987), while in the absence of ATP it is closer to 90 nM (Kwon-Shin et al., 1987). Pol III* binds ATP with a K_D of greater than 5 μM. Adding β to this subassembly decreases the K_D to 600 nM (Burgers & Kornberg, 1982a). Therefore, β modulates the ATP affinity of pol III*.

To further characterize the many binding interactions necessary for holoenzyme activity, we have fluorescently labeled the β subunit. The availability of an efficient β overproducer has aided obtaining the quantities required for this type of analysis (Johanson et al., 1986). Since fluorescence is sensitive to a wide variety of molecular processes, it should be possible to monitor assembly of the holoenzyme through the eyes of β. We have found that FM reacts with one cysteine per β monomer with full retention of β activity. Several polyvalent cations, such as Mg^{2+}, Ca^{2+}, and spermidine(3+), were found to specifically bind to native β and FM–β in the absence of pol III* or DNA. Polyvalent cation binding caused the dissociation of β dimers into monomers.

Experimental Procedures

Proteins and Assays. *E. coli* DNA polymerase III* (1.3 × 10^6 units/mg) was prepared by the method of Fay et al. (1982). β subunit (7.9 × 10^6 units/mg; ϵ_{280} = 17 900 M^{-1} cm^{-1}) was prepared and quantitated by the method of Johanson et al. (1986).

β activity was measured by the reconstitution of holoenzyme from a mixture of pol III* and β. The holoenzyme assay was performed essentially as described (Johanson & McHenry, 1984). The enzyme dilution buffer was changed to 50 mM HEPES (pH 7.5), 20% glycerol, and 10 mM dithiothreitol, in order to more closely reflect the conditions in the fluorescence experiments. In assays with native β, the β activity obtained with this buffer was about 70% as great as that obtained with the standard buffer. However, this difference was inconsequential since it was only necessary to know the activity of the labeled β's relative to that of the native β. One unit of holoenzyme was defined as 1 pmol of (total) deoxynucleoside monophosphate incorporated per minute at 30 °C on an M13Goril DNA template with priming by *dnaG* primase in situ.

Fluorescence Labeling Reaction. The fluorescent reagents were purchased from Molecular Probes (Eugene, OR). Maleimides react specifically with the sulfhydryl amino acid side chains of proteins at neutral pH. The final reaction conditions were 130 μM β and fivefold molar excess of fluorescent label in 54 μL of 50 mM K_2HPO_4 (pH 7.5)–8% dimethyl sulfoxide. The reaction was allowed to proceed for 2 h in the dark at room temperature. Upon completion of the reaction excess glutathione was added; the mixture was applied to either a Sephadex G-25 (150 mesh, 15 × 250 mm) or Sephacryl S-200 (15 × 210 mm) gel filtration column and then eluted with 50 mM HEPES (pH 7.5) buffer in order to separate β from the low molecular weight reactants. The reaction of β with FM resulted in one label per β monomer and did not significantly alter its active site as determined in the replication assay. Two preparations of FM–β have been used, and they had 1.04 and 0.94 label/monomer and were respectively 99 and 96% active. There were no discernible fluorescence differences between the preparations. Similar values were achieved with the labels DMSM (0.67 label/monomer and 90% active) and PM (0.94 label/monomer and 96% active).

Glutathione, γ-L-glutamyl-L-cysteinylglycine, was labeled with FM to serve as a fluorescence control compound. To a 2 μM solution of FM in 50 mM HEPES (pH 7.5) buffer, a fivefold molar excess of glutathione was added. The reaction was considered complete when the fluorescence intensity of the label had reached a maximum and doubled.

The concentration of protein in the labeled β was determined by the Coomassie Blue (Bio-Rad Laboratories, Richmond, CA) protein assay, based on the dye-binding procedure of Bradford (1976). The protein standard in these assays was native β which had been accurately quantitated with its molar extinction coefficient (Johanson et al., 1986). The concentration of fluorophore in the labeled β was determined with the extinction coefficients for the dyes given by Haugland (1985). The fluorophore absorbance wavelength maxima and their molar extinction coefficients were as follows: DMSM, 344 nm, 2.5 × 10^4 M^{-1} cm^{-1}; FM, 496 nm, 7.5 × 10^4 M^{-1} cm^{-1}; PM, 344 nm, 3.75 × 10^4 M^{-1} cm^{-1}. The labeled β's were stored at −80 °C, and when subsequently thawed, they exhibited full fluorescence. In contrast, if stored at 4 °C, the fluorescence intensity decreased about 10% in 1 week. Only the results from freshly thawed samples are reported in this paper.

Fluorescence Measurements. The fluorescence measurements were made on a SLM-Aminco 4800 spectrofluorometer (SLM Instruments, Urbana, IL) controlled by an IBM AT computer. The sample compartment was equipped with temperature regulation and a stirring assembly. The excitation and emission bandwidths were respectively 1 and 4 nm to minimize photobleaching. All samples were corrected for the wavelength dependence of exciting light intensity through the use of a quantum counter, rhodamine B (Lakowicz, 1983), in the reference channel.

Additions of salts were made from concentrated stock solutions: 200 mM $MgCl_2$; 200 mM $CaCl_2$; 100 mM $MnCl_2$; 4 M NaCl; 2 M KCl; 1 M Na_2SO_4; 1 M KI; 10 mM $Co(NH_3)_6Cl_3$; 10 mM spermidine trihydrochloride. The concentration of $MgCl_2$ stock was quantitated by Galbraith Laboratories, Inc. (Knoxville, TN). The salts were in 50 mM HEPES (pH 7.5) buffer except $MnCl_2$ which was in distilled, deionized water. All intensity measurements have been corrected for dilution and photobleaching. The cobalt salt required an additional correction because of its significant absorption at the exciting and emitting wavelengths of FM. This

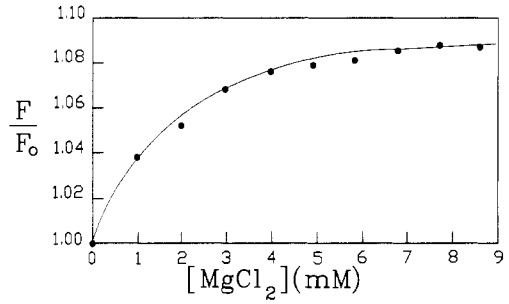

FIGURE 1: FM–β fluorescence enhancement by $MgCl_2$. The fluorescence of FM–β was monitored as indicated under Experimental Procedures with an excitation wavelength of 496 nm and an emission wavelength of 518 nm. Aliquots of a 200 mM $MgCl_2$ stock solution were added to 100 nM FM–β in 50 mM HEPES (pH 7.5) at 20 °C, and the fluorescence intensity was corrected for dilution and photobleaching.

inner-filter effect was corrected with the equation from Lakowicz (1983): $F_{cor} \approx F_{obsd}$ antilog $[(A_{ex} + A_{em})/2]$. The molar extinction coefficients were 152 M^{-1} cm^{-1} at 496 nm and 76 M^{-1} cm^{-1} at 518 nm.

The titrations with those metal ions that caused fluorescence enhancement were analyzed with a nonlinear regression program (Duggleby, 1981) on an Apple IIe personal computer. The data were fit to the equation $y = y_{max}S/(K_D + S)$, where y is percent enhancement, S is free metal ion concentration, y_{max} is maximum fluorescence enhancement, and K_D is the metal ion–protein dissociation constant. Given values for y and S, the program generates best-fit values of y_{max} and K_D along with their standard errors. Titrations with those compounds which quenched fluorescence were analyzed according to the modified Stern–Volmer equation of collisional quenchers: $x = x_{max}Q/(K_{sv} + Q)$, where x is percent quenching, Q is quencher concentration, x_{max} is maximum percent quenching, and K_{sv} is the Stern–Volmer quenching constant.

RESULTS

$MgCl_2$ Titration of FM–β. Studies of the interaction of β with other replication components require a sensitive, specific probe. β was labeled with the sulfhydryl-specific fluorescent reagent FM. The fluorescence of FM–β was saturably increased while titrating with $MgCl_2$ (Figure 1). The effect was fully reversible by EGTA (data not shown). A linear regression fit of these data indicated 10.6 ± 0.3% maximum enhancement and a dissociation constant, K_D, of 1.7 ± 0.2 mM (Table I). Hill and Scatchard analyses indicated 1.02 independent, noncooperative Mg^{2+} binding sites per FM–β monomer (data not shown). Thus each β dimer has two independent, noncooperative Mg^{2+} binding sites.

Several experiments proved that the Mg^{2+}-induced fluorescence enhancement was specific to β and its FM label. When FM–glutathione was titrated with Mg^{2+}, no fluorescence enhancement was observed (Table I), indicating that this effect was specific to the protein and not the fluorophore. When PM–β or DMSM–β was titrated with Mg^{2+}, no fluorescence enhancement was observed (data not shown). Thus, fluorescence enhancement was a property of FM attached to β.

Ion Specificity of FM–β Fluorescence Enhancement. Several other salts were tested for their effect on FM–β and FM–glutathione. The fluorescence of FM–β was not effected by up to 500 mM NaCl, KCl, or Na_2SO_4 (data not shown), indicating that neither anions nor monovalent cations can cause the effect. Calcium(II) and spermidine(3+) induced a saturable fluorescence enhancement in FM–β but not in FM–glutathione (Table I). The concentration of Ca^{2+} which caused a half-maximum effect was similar to that for Mg^{2+}, K_D = 2.0 mM, indicating a lack of binding site preference between these two divalent cations. It took less of the trivalent cation, spermidine(3+), to cause the same effect, K_D = 330 μM. Scatchard and Hill analyses that indicated 1.0 independent, noncooperative spermidine(3+) binding site per β monomer. However, the two cations did bind to the same site since spermidine(3+) saturation precluded any further effect by Mg^{2+} (data not shown).

Table I: Ion Specificity of FM–β Fluorescence Effects

	FM–β		FM–glutathione	
salt	effect	K_{50}	effect	K_{50}
$MgCl_2$	11% enhance	1.7 mM[a]	none	
$CaCl_2$	10% enhance	2.0 mM[a]	none	
$MnCl_2$	100% quench	91 mM[b]	100% quench	100 mM[b]
spermidine trihydrochloride	15% enhance	330 μM[a]	none	
$Co(NH_3)_6Cl_3$	76% quench	57 μM[b]	80% quench	420 μM[b]
KI	100% quench	320 mM[b]	100% quench	260 mM[b]

[a] K_{50} is equal to the dissociation constant, K_D, and was determined from binding plots as indicated under Experimental Procedures. K_D is the metal ion–protein dissociation constant. [b] K_{50} is equal to the Stern–Volmer constant, K_{SV}, and was determined from the Stern–Volmer equation as indicated under Experimental Procedures. K_{SV} is the concentration of quencher which quenches half the total fluorescence.

In contrast, two of the cations, Mn^{2+} and $Co(NH_3)_6^{3+}$, quenched the fluorescence of both FM–β and FM–glutathione. Manganese(II) is an electron scavenger capable of efficient collisional quenching of the fluorescence of tryptophan derivatives (Steiner & Kirby, 1969). Since Mn^{2+} completely quenched the fluorescence of both FM–β and FM–glutathione, the quenching appeared to be a specific interaction between this metal ion and the fluorophore. The trivalent cation, $Co(NH_3)_6^{3+}$, quenched about 80% of the fluorescence of both labeled compounds, which indicated that β did not have a role in this phenomenon. However, the quenching constant for FM–β was sevenfold less than that for FM–glutathione, implying that β was binding to $Co(NH_3)_6^{3+}$ and thereby increasing its ability to quench FM. That is, there could be a negatively charged domain on β near the attached FM which provides a binding site for $Co(NH_3)_6^{3+}$. This binding site may or may not be distinct from the Mg^{2+} binding site.

Quenching of FM–β and FM–glutathione by I^- was also studied since its quenching properties have been well documented. Iodide is a collisional quencher (Kaska, 1952; Lehrer, 1971) and was capable of quenching both the labeled protein and the model compound (Table I). The quenching constant was slightly higher for FM–β than for FM–glutathione, demonstrating that the label may be positioned in either a protected or a negatively charged domain. The polyvalent cation poly(dA), at a concentration of 10 μg/mL in the presence or absence of 10 mM $MgCl_2$, had no significant effect on the fluorescence of FM–β (data not shown). Neither was there an effect from adding 250 μM of the ribonucleoside triphosphates separately or as a mixture.

The molecular basis for the Mg^{2+}-induced fluorescence enhancement was investigated. Since the enhancement was specific for FM-labeled β, a likely explanation was that Mg^{2+} binding leads to β dimer dissociation; when the FM labels on adjacent halves become separated, the relief from homoquenching causes an increase in fluorescence from these labels. Proof of this was obtained from gel filtration studies.

Gel Filtration of β. The elution behavior of β from a S-200 gel filtration column was studied in order to confirm that Mg^{2+}

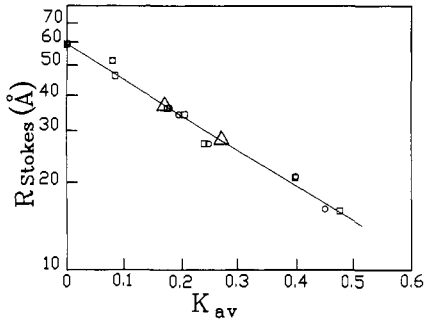

FIGURE 2: Radius of β varies depending on its eluting concentration. When β, in 50 mM HEPES (pH 7.5), 80 mM NaCl, 0.1 mM EDTA, and 0.02% azide, was eluted at a concentration of 1.31 μM, its apparent radius was 37.1 Å (left △); at 21 nM, it was 29.4 Å (right △). The Sephacryl S-200 superfine (Pharmacia) siliconized column (15 × 210 mm) was calibrated with standards. The column was equilibrated at room temperature with either 50 mM HEPES (pH 7.5), 80 mM NaCl, 0.1 mM EDTA, and 0.02% azide (○) or 50 mM HEPES (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, and 0.02% azide (□). A 400-μL sample of protein was applied to the column, 400-μL fractions were collected, and the elution positions were assayed by A_{280} for the calibration proteins and holoenzyme reconstitution for β. The standard curve was obtained by plotting the log of the Stokes radius of each calibration protein versus K_{av}, the fraction of gel volume available for protein diffusion: $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the sample's peak fraction, V_0 is the void volume, and V_t is the total column volume. The Stokes radii for the calibration proteins were calculated with the diffusion constants, D, compiled by Smith (1970) and the equation $R_{Stokes} = kT/6\pi\eta D$, where k is the Boltzmann constant, T is temperature in kelvin, and η is viscosity in Poise. The standards used and their Stokes radii were as follows: horse spleen apoferritin, 59.4 Å (used to determine the void volume); bovine liver catalase, 52.3 Å; rabbit muscle aldolase, 46.3 Å; bovine serum albumin, 35.6 Å; horse liver alcohol dehydrogenase, 34.5 Å; hen egg albumin, 27.5 Å; bovine pancreas chymotrypsinogen, 20.9 Å; horse heart cytochrome c, 16.5 Å. K_{av} was determined for each eluting β, and from the standard curve, R_{Stokes} was obtained.

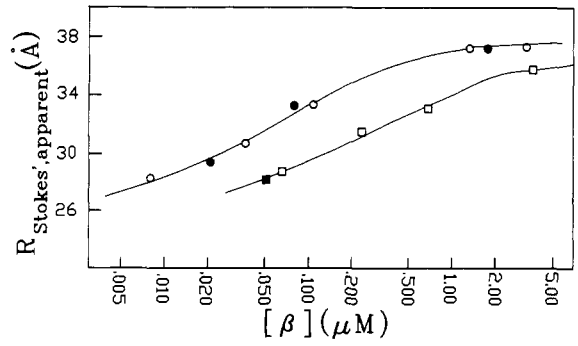

FIGURE 3: Apparent Stokes radius, $R_{Stokes,app}$, of β versus eluting concentration. Native β (○, □) or FM–β (●, ■) is loaded at various concentrations (400-μL total loading volume), and the elution concentrations and radii were determined as described in the legend of Figure 2. The column was equilibrated with either 50 mM HEPES (pH 7.5), 80 mM NaCl, 0.1 mM EDTA, and 0.02% azide (○, ●) or 50 mM HEPES (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, and 0.02% azide (□, ■).

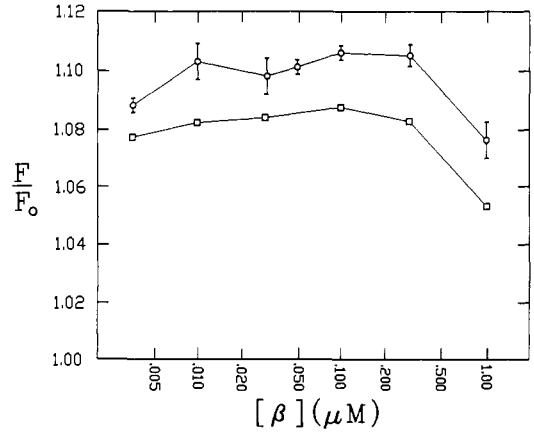

FIGURE 4: Fluorescence enhancement of FM–β caused by 10 mM Mg^{2+} (□) and by saturating Mg^{2+} (○) as a function of β concentration. The experiment was carried out essentially as described under Figure 1. The saturating fluorescence was extrapolated from titration data as indicated under Experimental Procedures.

was enhancing the fluorescence of FM–β by dissociating the dimers into monomers. The column was equilibrated at room temperature with either of two buffers at the same ionic strength: 50 mM HEPES (pH 7.5), 80 mM NaCl, 0.1 mM EDTA, and 0.02% azide; or 50 mM HEPES (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, and 0.02% azide. The presence of the Mg^{2+} did not influence the elution positions of the standard proteins (Figure 2). Various concentrations of β were loaded onto the column, either in the presence or in the absence of 10 mM Mg^{2+}, and it was observed that the elution position of β varied as a function of eluted β concentration. Since the column had been calibrated with standards, the apparent Stokes radii of eluted β's could be obtained given their elution positions (Figure 2). β varied from a dimer with Stokes radius of about 37 Å at the high elution concentrations to a monomer of about 27 Å at the low elution concentrations (Figure 3). In the presence of 10 mM Mg^{2+} the radius of β is smaller at every intermediate elution concentration (Figure 3).

At β concentrations of 30 nM to 1 μM, a 30-fold range, 10 mM Mg^{2+} decreases the average radius of β by 3 Å (Figure 3). The Mg^{2+} effect occurred for both native β and FM–β. This is another confirmation that Mg^{2+} is interacting with β and not simply with a site created when β was labeled with a fluorophore. In addition, FM–β appears to be identical with β in its hydrodynamic properties since they elute from the column with similar radii.

The dissociation constant for the β dimer cannot be obtained directly from these gel filtration data since such a determination requires equilibrium conditions. During gel filtration, the concentration of protein is constantly changing, and the midpoints from these curves (Figure 3) will only give K_D's relevant to the conditions during gel filtration. Significant dilution and band broadening occur during the 40-min interval that β is on the gel filtration column. For example, when 400 μL of 16.3 μM β is loaded onto the column in the 80 mM NaCl buffer, the peak elutes at a concentration of 1.3 μM with a 3.2-mL width at half-maximum and an apparent Stokes radius of 37.1 Å (left △, Figure 2). In addition, the recovery of eluted β was also somewhat concentration dependent with 60% recovery at the higher elution concentrations down to 30% at the lower concentrations. Thus Figure 3 does not provide an equilibrium value for the dimer–monomer dissociation constant of β, but it does indicate a relative sixfold shift in the K_D caused by 10 mM Mg^{2+}.

FM–β Concentration Dependence of Fluorescence Enhancement Effect. The fluorescence enhancement caused by 10 mM Mg^{2+} (Figure 4) was a function of FM–β concentration. There is a plateau of ~8.3% enhancement at the intermediate FM–β concentrations from 10 to 300 nM, a 30-fold range. The fluorescence enhancement effect decreases at β concentrations greater than 300 nM and also begins to decrease at less than about 5 nM. At higher β concentrations fewer of the dimers should be able to dissociate, due to mass action, and the maximum fluorescence enhancement will decrease. Likewise, at lower β concentrations more of the protein is in the monomeric form, and again, the maximum fluorescence enhancement will decrease. More detailed analysis of

the Mg^{2+} titrations also indicated that the maximum fluorescence enhancement was decreasing at high FM–β concentration (Figure 4).

Discussion

The fluorophore FM reacted with β to label one cysteine in a 2-h period and did not alter its activity as determined by the replication assay. This maleimide label was more specific than *N*-ethylmaleimide which rapidly derivatized one cysteine, followed by slower derivatization of the others over a 2-h period (Johanson et al., 1986). Specific site labeling by fluorophores, that is, "fast thiols", has been observed in a wide variety of proteins [for selected references, see Haugland (1985)].

6-(Iodoacetamido)fluorescein reacted with β to give more than one label per β with total loss of activity (unpublished results). These results are similar to those observed with iodoacetamide (Johanson et al., 1986), in which sulfhydryl modification of β by iodoacetamide leads to loss of all activity.

While testing the various replication assay components for their effect on FM–β, it was observed that titration with $MgCl_2$ saturably increased the fluorescence. At 100 nM FM–β, the maximum enhancement was 10.6%, the K_D for Mg^{2+} was 1.7 mM (Table I), and Hill and Scatchard analyses indicated 1.02 independent, noncooperative Mg^{2+} binding sites per β monomer. Enhancement was not a trivial characteristic of the FM fluorophore since the fluorescence control compound, FM–glutathione, was not effected by Mg^{2+}. It also was not due to β having a fluorescent label since neither PM–β nor DMSM–β was effected by Mg^{2+}. In addition, Mg^{2+} decreases the apparent Stokes radius of β to the same extent whether it has a fluorescent label or not. Thus, fluorescence enhancement was specific to FM-labeled β.

FM is capable of homoquenching, a feature that is absent from PM or DMSM. Homoquenching occurs for those fluorescent molecules that have significantly overlapping fluorescence excitation and emission spectra. Thus, if these homomolecules are close enough to one another, their fluorescence can be quenched through Förster energy transfer. This phenomenon suggested an explanation for the fluorescence increase when Mg^{2+} binds to FM–β dimer: the homoquenching of FM's on adjacent halves is somehow relieved. One of the ways this might be achieved would be for the dimer to dissociate to monomers. In fact, Mg^{2+} was observed to favor the formation of β monomers as determined by gel filtration.

All other factors remaining constant, various concentrations of β were loaded on a gel filtration column, and the elution concentrations were plotted against their apparent Stokes radii. The size of β ranges from a dimer with a 37-Å radius at high eluting concentration to a monomer with a 27-Å radius at low eluting concentrations. β eluted as a single peak at every radius and concentration indicating that β was in rapid equilibrium between monomers and dimers on this time scale (data not shown). Thus the intermediate sizes of β represent the average size of a population of β monomers and dimers. If β had not been in rapid equilibrium during gel filtration, it would have eluted as two peaks, monomers and dimers, whose relative peak sizes would be somehow related to β's dissociation constant.

When half of the β dimer has dissociated, it can be calculated to have an apparent radius of 30.1 Å. From the gel filtration data in Figure 3, this size would correspond to $K_{D,\text{gel filtration}}$ for β dimer–monomer dissociation of 26 nM in 80 mM NaCl and 150 nM in 50 mM NaCl–10 mM $MgCl_2$. However, these $K_{D,\text{gel filtration}}$'s are not analogous to equilibrium K_D's, since the concentration of β is being continuously diluted during filtration. In order to determine the actual K_D for β dimer dissociation, the gel filtration data have to be extrapolated to the equilibrium conditions of no dilution or zero eluting time. Gel filtration can only accurately determine the apparent radii of β and indicate that 10 mM Mg^{2+} shifts the β dimer dissociation constant by sixfold to favor monomers.

In conjunction with the gel filtration data, another type of experiment was used to establish the equilibrium K_D of β. If the dissociation of dimers corresponded exactly with the fluorescence enhancement effect, it should be possible to relate the two phenomena. In the gel filtration experiments, the apparent size of β is decreased about 3 Å by 10 mM Mg^{2+} over the range from 30 nM to 1 μM β (a 30-fold range). Since the complete change in radius from dimers to monomers is about 10 Å, this represents approximately 30% dissociation of the dimers. In the fluorescence experiments there is also a plateau over a 30-fold concentration range of 8.3% fluorescence enhancement. At very high concentrations, mass action prevents dissociation of the dimers despite the addition of 10 mM Mg^{2+} and, thus, prevents fluorescence enhancement. At very low concentrations, all of the FM–β is in the form of monomers and, thus, cannot undergo the dissociation necessary for fluorescence enhancement.

The fluorescence experiment represents the equilibrium measurement of the same phenomenon observed by gel filtration. The midpoint of the fluorescence enhancement plateau, 60 nM, should thus correspond to the midpoint concentration of the Mg^{2+}-induced 3-Å radius decrease from gel filtration, 250 nM. That is, the gel filtration experiment would correspond to equilibrium conditions if its midpoint were shifted from 250 to 60 nM. If the abscissa of Figure 3 is shifted accordingly, the gel filtration curves reveal K_D's of 6.6 nM in the 80 mM NaCl buffer and 35 nM in the 50 mM NaCl–10 mM $MgCl_2$ buffer.

Given that 10 mM Mg^{2+} dissociates 30% of the β dimers and causes an 8.3% fluorescence enhancement in FM–β, it should be possible to establish a distance between FM's on adjacent dimer halves. The efficiency of energy transfer is defined as $E = (1 - F_{DA}/F_{DD})/\chi$, where F_{DA} is the fluorescence of the donor in the presence of acceptor, F_{DD} is the fluorescence of donor in the absence of acceptor, and χ is the fraction of donors capable of participating in energy transfer. In the present case F_{DA} is 1.000, F_{DD} is 1.083, and χ is 0.3 since only 30% of the dimers are dissociating. This relation is not strictly applicable to homoquenching however, since there is the dual effect of increasing the fluorescence of both the donor and the acceptor; if homoquenching were accounted for, the distance calculated below would be slightly greater. The measured efficiency of energy transfer can be related to the distance R between the donor and acceptor molecules by the equation (Förster, 1948; Lakowicz, 1983) $R = R_0(E^{-1} - 1)^{1/6}$, where R_0 is the characteristic distance at which the efficiency of nonradiative transfer, E, is 50%. Given an R_0 of 50 Å for fluorescein, the distance between FM's on adjacent halves of the β dimer is calculated to be 60 Å. This value is only an approximation, and future experiments will determine this distance more accurately using nonidentical donor/acceptor pairs.

The results from this study provide further insight into the role of β in the DNA polymerase III holoenzyme. β dimer binds Mg^{2+} and dissociates to monomers in a physiologically significant concentration range; the concentration of β in the *E. coli* cell is approximately 40 nM. Whether the monomer and dimer have different functional roles in the holoenzyme has yet to be determined. However, since it was not known that β dissociates to monomers, previous kinetic studies with β have been interpreted as though it was a dimer under all

conditions. For example, Lasken and Kornberg (1987) found that the K_D for β binding to pol III* in the presence of saturating ATP was 1 nM β. Our results indicate that under these conditions β should be completely dissociated to monomers. Conversely, Kwon-Shin et al. (1987) found that 90 nM β, in the absence of ATP, could cause half-maximum stimulation of long product synthesis with pol III*. Under these conditions our results indicate that β is present in solution as mostly a dimer. It is possible that the dimeric state of β might somehow be involved in the pathways leading to these two types of initiation complexes. However it is not yet established how rapidly the dimer dissociates to monomers or whether the β dimer reconstitutes once it is bound to pol III*.

The results presented here also indicate the utility of fluorescence in studying the many binding interactions that occur during replication. This is the first holoenzyme subunit to be studied with this technique, and it has already led to new ideas concerning dissociation of β dimers. As overproducers are made for the other holoenzyme subunits, these methods will be applied to them as well in an attempt to understand their individual roles in replication. Through the use of various fluorescent dyes and Förster energy transfer on the many subunits, nucleotides, primed ssDNA, and associated replication proteins, it should be possible to map out the arrangement of subunits within the holoenzyme and within the replisome.

Registry No. Mg, 7439-95-4; Ca, 7440-70-2; DNA polymerase III, 37217-33-7; spermidine, 124-20-9.

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