Monoclonal Antibodies as Probes of the Topological Arrangement of the α Subunits of *Escherichia coli* RNA Polymerase†

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Received July 19, 1988; Revised Manuscript Received December 13, 1988

ABSTRACT: Three monoclonal anti-α antibodies were used to study the properties of the α subunit of *Escherichia coli* RNA polymerase. None of the monoclonal antibodies inhibited the d(A-T)$_n$-directed synthesis of r(A-U)$_n$. Reassembly of the RNA polymerase core was blocked by mAb 129C4 or mAb 126C6 while no effect was observed with mAb 124D1. The conversion of premature to mature core was partially inhibited by mAb 129C4 and almost totally inhibited by mAb 126C6. The data suggest that during the course of core assembly at least one of the α subunits undergoes conformational changes. The increase in affinity of mAb 126C6 for assembled α compared with free α also implies that α undergoes conformational changes during RNA polymerase assembly. Double antibody binding studies showed that the epitopes for mAb 124D1 and mAb 129C4 are available on only one of the α subunits in RNA polymerase. It would appear that the relevant domain on one of the α subunits in RNA polymerase is well exposed whereas this domain on the second α subunit is shielded by interaction with regions of the large β and β' subunits. The α domain in which the epitope for mAb 126C6 resides is not impeded by subunit interactions in the RNA polymerase. The data obtained also suggest that in the holoenzyme the σ subunit may be positioned close to one of the α subunits, probably to the more exposed α. The $\alpha\beta$ complex is the minimal stable subassembly since one of the α subunits dissociates from the $\alpha_2\beta$ complex following binding of any of the monoclonal antibodies studied. These studies suggest that the in vitro assembly of the polymerase core may proceed via the formation of the more stable $\alpha\beta$ complex followed by addition of the second α subunit.

The DNA-dependent *Escherichia coli* RNA polymerase is a complex multisubunit enzyme which can be isolated as a catalytically competent core enzyme ($\alpha_2\beta\beta'$) or as a promoter-selective holoenzyme ($\alpha_2\beta\beta'\sigma$). The function of each of the subunits is only incompletely understood. The σ subunit is involved in initiation specificity, and at least part of the active site is present in the β subunit; the role of the α and β' subunits remains to be defined (von Hippel et al., 1984; Burgess et al., 1987). The structural organization of the *E. coli* RNA polymerase has been studied by electron microscopy (Lubin, 1969; Kiselev & Stel'maschuk, 1981), small-angle neutron scattering (Stockel et al., 1980a,b), and X-ray scattering (Meisenberger et al., 1980a,b) as well as by cross-linking with bifunctional reagents (Coggins et al., 1977; Hillel & Wu, 1977) and by partial proteolysis (Lill & Hartmann, 1975; Lowe & Malcolm, 1976; Fisher & Blumenthal, 1980). Subunit-specific polyclonal antibodies have been used to investigate the accessibility and function of the subunits in RNA polymerase (Stender, 1980, 1981) and to study the mode of subunit interaction (Tichelaar et al., 1983).

The several approaches have provided information regarding the dimensions of RNA polymerase and the gross shape of the individual subunits and the enzyme and have suggested possible modes of subunit interactions. Thus far, the regions involved in subunit–subunit interactions and possible changes in subunit conformation attendant to enzyme assembly remain undefined. The development of hybridoma technology (Kohler & Milstein, 1975) has allowed the preparation of site-specific monoclonal antibodies. This paper describes the use of monoclonal antibodies directed against the α subunit of the *E. coli* RNA polymerase to study the conformational changes in α that occur during enzyme assembly and the topological arrangement of these subunits in the enzyme.

Materials and Methods

Materials. Reagents were obtained as follows: radioimmunoassay-grade bovine serum albumin and 4-methylumbelliferyl β-D-galactopyranoside, Sigma Chemical Co.; goat anti-mouse IgG–β-galactosidase, Hyclone; Immunoprecipitin, Bethesda Research Laboratories; d(A-T)$_n$ and ribonucleoside triphosphates, Pharmacia; [^{3}H]UTP, ICN; [^{125}I]iodine, New England Nuclear; urea, Schwarz/Mann; acrylamide, Serva; Scintisol, Isolab.

Preparation of RNA Polymerase and Monoclonal Antibodies. RNA polymerase was purified from *E. coli* K12 by a modification of the procedure of Burgess and Jendrisak (1975). Holoenzyme and core enzyme were resolved by chromatography on denatured calf thymus DNA–agarose (Lowe et al., 1979). Protein concentration was determined by using the following extinction coefficients; core polymerase, $E^{1\%}_{280nm}$ = 5.8; holoenzyme, $E^{1\%}_{280nm}$ = 6.7. Subunit-specific monoclonal antibodies (DeFalco et al., 1983; Rockwell et al., 1985) were prepared as indicated in Rockwell et al. (1985). The immunoglobulin isotype of the anti-α monoclonal antibodies was determined by using the mouse immunoglobulin subtype identification kit purchased from Boehringer Mannheim Corp. Immunoglobulin concentration was determined by using the extinction coefficient $E^{1\%}_{280nm}$ = 14.0 (Ey et al., 1987).

Separation of RNA Polymerase Subunits. α subunit was separated from β and β' subunits by gel filtration on Sephacryl SF-400 in 10 mM potassium phosphate (pH 7.2), 6 M guanidine hydrochloride, 1 mM dithiothreitol, 0.1 mM EDTA,

† This work was supported by a research grant from the National Institutes of Health (GM19673).

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0006-2960/89/0428-3299$01.50/0

and 5% glycerol. The separated subunits were stored in the above buffer at −20 °C. For the binding studies, α subunit was prepared by chromatography of the urea-denatured core enzyme on Bio-Rex 70 (Lowe & Malcolm, 1976). The purified α subunit was dialyzed against phosphate-buffered saline (PBS)[1] containing 0.1 mM dithiothreitol and stored at −20 °C.

Preparation of $\alpha_2\beta$ Subassembly. $\alpha_2\beta$ subassembly prepared from isolated α and β subunits was obtained by chromatography of urea-denatured core enzyme on either Affi-Gel Blue (Wu et al., 1977) or Bio-Rex 70 (Lowe & Malcolm, 1976). Fractions containing α plus β subunits were pooled and dialyzed against storage buffer containing 20 mM potassium phosphate (pH 7.5), 150 mM NaCl, 60% glycerol, 1 mM dithiothreitol, and 0.1 mM EDTA and stored at −20 °C.

Reconstitution of Core Enzyme. The conditions for reconstitution were essentially as described by Palm et al. (1975). One hundred picomoles of α and 50 pmol each of β and β′ subunits (obtained by gel filtration and stored in the presence of 6 M guanidine hydrochloride) were combined, and the total volume of the mixture was adjusted to 250 μL with reconstitution buffer (50 mM Tris-HCl, pH 8, 0.2 M KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, and 20% glycerol) to obtain the optimal core enzyme concentration of 0.2 mg/mL. Alternatively, core enzyme was dissociated by dialysis against 10 mM potassium phosphate (pH 7.0), 6 M guanidine hydrochloride, 0.1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol for 2 h at 4 °C after which the protein concentration was adjusted to 0.2 mg/mL. Reconstitution mixtures in the presence or absence of the indicated monoclonal antibody were dialyzed against reconstitution buffer at 4 °C overnight and then incubated for 30–40 min at 30 °C. Protein concentration after reconstitution was determined by the method of Schaffner and Weissmann (1973). To determine the activity of the reconstituted core enzyme, 25-μL aliquots of the reconstituted mixtures were used. The activity of the reconstituted core enzyme was assayed by $d(A\text{-}T)_n$-directed synthesis of $r(U\text{-}A)_n$. α was dialyzed against PBS containing 0.1 mM dithiothreitol before running reconstitution assays with mAb 124D1. The activity of reconstituted core enzyme was not affected whether α had been stored in the presence of 6 M guanidine hydrochloride or in PBS.

Assays of $d(A\text{-}T)_n$-Directed $r(A\text{-}U)_n$ Synthesis. Monoclonal antibody–polymerase complexes were formed by incubation for the time and temperature indicated in the legends. After addition of 30 nmol of $d(A\text{-}T)_n$, the mixture was incubated for 10 min at 37 °C. Synthesis of $r(A\text{-}U)_n$ was carried out in a reaction mixture (90 μL) which contained 40 mM Tris-HCl (pH 8.0), 13 mM potassium phosphate (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 10 mM mercaptoethylamine, 0.1–0.25 mM dithiothreitol, 400 nmol of ATP, 100 nmol of [^{3}H]UTP (44 000 cpm/nmol), and 5.5% glycerol. After incubation at 37 °C for the time indicated, the $r(A\text{-}U)_n$ was precipitated with 5% trichloroacetic acid, collected on glass fiber filters (Whatman GFC), and counted in Scintisol.

Preparation of ^{125}I-Labeled Monoclonal Antibodies. Twenty micrograms of monoclonal antibody was labeled with 1 mCi of sodium [^{125}I]iodide (specific activity 17 Ci/mg of iodine) by the chloramine T procedure (Hunter, 1967). The specific activity of the ^{125}I-labeled monoclonal antibody was determined by TCA precipitation.

Double Antibody Binding Assays. A modification of the method of Pestka et al. (1983) was used to assay for binding of the mAbs to free and polymerase-associated α. Costar 96-well EIA polystyrene plates were coated with antibody by incubation overnight at 4 °C. Each well received 500 ng of the indicated monoclonal antibody in 100 μL of PBS, 10 mM potassium phosphate (pH 7.4), and 150 mM NaCl. Remaining protein binding sites were blocked by incubation for 60 min at 37 °C with 200 μL per well of PBT: PBS containing 2 mg/mL bovine serum albumin, 0.05% Tween 80, and 0.04% NaN_3. The plates were then washed twice with PBT. Binding of RNA polymerase or the indicated subunit to the immobilized monoclonal antibody was carried out by incubation for 1 h at 37 °C or for 16 h at 4 °C in a solution (100 μL) containing 365 ng of α or 700 ng of $\alpha_2\beta$ or 1 μg of core or 1 μg of holoenzyme in PBS + 2 mg/mL bovine serum albumin. After the plates were washed 3 times with PBT, 100 μL of a solution containing a saturating amount (determined from Scatchard plots) of the indicated ^{125}I-mAb in PBS + 2 mg/mL bovine serum albumin was added. After incubation for 90 min at 37 °C or for 16 h at 4 °C, the plates were washed with PBT. ^{125}I-Labeled monoclonal antibody bound was determined after incubation for 30 min with 140 μL of 1 M NaOH. After transfer to 10 × 75 mm tubes, radioactivity was determined in an LKB Autogamma counter.

When *Staphylococcus aureus* cells (Immunoprecipitin) containing formalin-fixed protein A were used, the binding assays were performed as follows: 1 mg of *S. aureus* cell suspension (binding capacity 12 μg of IgG/mg of cell suspension) was incubated with 15 μg of the indicated monoclonal antibody in 100 μL of PBS–BSA for 15 min at room temperature. To saturate unoccupied sites on the *S. aureus* cells, bovine IgG (20 μg) was then added and incubated for an additional 10 min at room temperature. Unbound antibody was removed by centrifugation of the suspension at 15 000 rpm for 2 min, and the *S. aureus*–monoclonal antibody complex was washed 2 times with 1 mL of PBS–BSA. The ^{125}I-mAb 129C4 (10 pmol, 1.5×10^6 cpm/pmol) was incubated for 30 min at 37 °C with 1 μg of $\alpha_2\beta$, 2.4 μg of core enzyme, or 3 μg of holoenzyme in PBS–BSA (final volume of 250 μL for $\alpha_2\beta$, 300 μL for core or holoenzyme). After addition of 50 μL of the indicated ^{125}I-mAb 129C4–antigen complex to 10 μL of the *S. aureus*–monoclonal antibody suspension, the mixture was incubated for 30 min at 37 °C. Unbound ^{125}I-labeled monoclonal antibody was removed by washing 2 times with 1 mL of washing buffer followed by centrifugation at 15 000 rpm for 2 min. After transfer to 10 × 75 mm tubes, radioactivity was determined in an LKB Autogamma counter.

Monoclonal Antibody Binding Constants. Dissociation constants (K_d) for mAb 129C4 and mAb 124D1 and for α, core, and holoenzyme were calculated from the Scatchard plots obtained with ^{125}I-labeled monoclonal antibodies in double antibody binding assays on polystyrene plates using the procedure described above. K_d values for mAb 126C6 and α, core, and holoenzyme, as well as for mAb 129C4 and mAb 124D1 and α, were determined by the method of Friguet et al. (1985) with 4-methylumbelliferyl β-D-galactopyranoside as a fluorescent substrate for goat anti-mouse IgG–β-galactosidase. The plates (MicroFluoro "B" flat-bottom plates, Dynatech Corp.) were coated with core enzyme overnight at 4 °C. Concentrations of monoclonal antibodies were 2 nM for mAb 126C6 and 1 nM for mAb 129C4 and mAb 124D1; antigen concentrations varied from 1 to 500 nM. Antigens were incubated with monoclonal antibodies overnight at 4 °C. One hundred-microliter aliquots were removed from these mixtures for

[1] Abbreviations: BSA, bovine serum albumin; PBS, phosphate-buffered saline; mAb, monoclonal antibody; SDS, sodium dodecyl sulfate; CRP, cAMP receptor protein.

Table I: Properties of Anti-α Monoclonal Antibodies[a]

mAb	Ig class	effect of mAb on reaction directed by d(A-T)$_n$[b]
129C4	IgG2a, κ	92
124D1	IgG2b, κ	90
126C6	IgG2b, κ	95

[a] RNA polymerase holoenzyme (2 pmol) was preincubated with the indicated monoclonal antibody for 30 min at 37 °C. The molar ratio of antibody to α was 10 to 1 for the d(A-T)$_n$-directed reaction. The RNA polymerase–monoclonal antibody complex was assayed as described under Materials and Methods. [b] Residual activity in the presence of monoclonal antibody is expressed as the percent of the control value for incorporation of [^{3}H]UMP in 20 min at 37 °C which was 5 nmol.

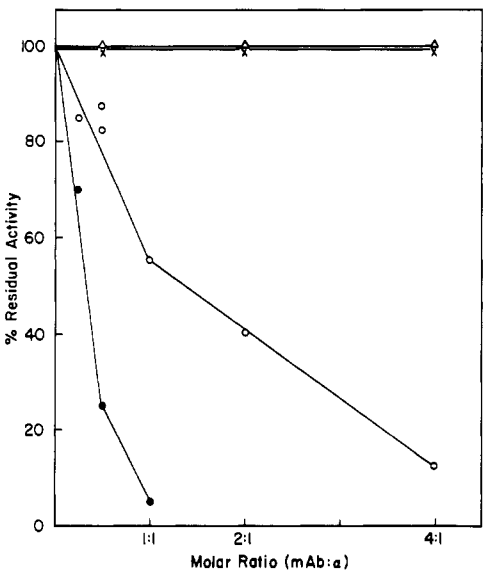

FIGURE 1: Effect of increasing concentration of anti-α monoclonal antibodies on reconstitution of core enzyme. Core enzyme was reconstituted from α and β + β′ subunits separated by gel filtration in the presence of 6 M guanidine hydrochloride. Reconstitution mixtures contained 100 pmol of α and 50 pmol each of β and β′ and the indicated antibody. Reconstitution mixtures were dialyzed against reconstitution buffer overnight at 4 °C followed by incubation for 30 min at 37 °C. Aliquots (25 μL) of reconstituted core enzyme were removed for determination of d(A-T)$_n$-directed synthesis of r(A-U)$_n$. The control value for incorporation of [^{3}H]UMP was 1.6 nmol. mAb 129C4 (●); mAb 126C6 (○); mAb 124D1 (△); bovine IgG (×).

binding to the plate-immobilized core enzyme; the reaction was carried out for 1 h at 37 °C. The fluorescence of the product was determined with a Dynatech MicroFluor Reader.

Results

Shown in Table I are properties of the monoclonal antibodies raised against purified α subunit used in this study. The monoclonal immunoglobulins contain G2a or G2b heavy chains and κ light chains, α specificity of the monoclonal antibodies was verified by immunoblotting using RNA polymerase holoenzyme resolved by SDS–polyacrylamide gel electrophoresis and by solid-phase ELISA using purified α, β, and β′ subunits (data not shown). None of the three monoclonal antibodies inhibit the d(A-T)$_n$-directed synthesis of r(A-U)$_n$. mAb 126C6 strongly inhibits cAMP–CRP-dependent initiation with *lac* P$^+$ and to a lesser extent inhibits initiation with the *lac* UV5 promoter (Riftina et al., 1987); mAb 129C4 and mAb 124D1 are without effect. Reactions involving a functional promoter are more sensitive to inhibition by mAb 126C6 than are reactions directed by d(A-T)$_n$.

The lack of effect on the d(A-T)$_n$-directed synthesis of r(A-U)$_n$ can be used to assay for possible effects of the antibodies on the recovery of active polymerase following reconstitution from subunit mixtures. The data presented in Figure 1 show that mAb 124D1 and bovine IgG have no effect on reconstitution. When reconstitution is carried out in the presence of mAb 129C4 or mAb 126C6, recovery of active polymerase is inhibited. A complete inhibition of reconstitution ensues in the presence of a 4 to 1 ratio of mAb 129C4 to α. At a mAb 126C6 to α ratio of 4 to 1, approximately 10% of the core polymerase activity is recovered.

Table II: Effect of Anti-α Monoclonal Antibodies on Reconstitution of RNA Polymerase Core Enzyme[a]

antibody	effect of mAb when incubated with [b] subunits	premature core	mature core
mAb 129C4	1 (4:1)	62 (16:1)	115 (16:1)
mAb 124D1	100 (10:1)	ND	ND
mAb 126C6	11 (4:1)	8 (13:1)	90 (13:1)

[a] Core enzyme was denatured by incubation in buffer containing 6 M guanidine hydrochloride for 2 h at 4 °C. The reconstitution mixtures contained 100 pmol of α subunit and 50 pmol each of β and β′ subunits, and antibody was added at the indicated stage of reconstitution. Reconstitution to form the premature core was carried out by dialysis for 16 h at 4 °C against reconstitution buffer. Incubation of the antibody with the premature or mature core enzyme was for 3 h on ice followed by incubation for 40 min at 30 °C. Aliquots (25 μL) containing 4 pmol of reconstituted core enzyme were assayed for d(A-T)$_n$-directed synthesis or r(A-U)$_n$ as described under Materials and Methods. ND indicates that the effect of the antibody was not determined. The ratio of antibody to α used is given in parentheses. [b] Residual activity in the presence of monoclonal antibody is expressed as the percent of the control value for incorporation of [^{3}H]UMP: 100% was 1.6 nmol in 10 min at 37 °C.

Table III: Effect of Urea Treatment on the Binding of Anti-α Monoclonal Antibodies to Immobilized α and RNA Polymerase[a]

immobilized antigen	[urea] (M)	^{125}I-mAb bound (fmol) 129C4	124D1	126C6
α subunit	0	150	175	85
	4	160	170	105
core enzyme	0	55	58	100
	4	95	87	50
holoenzyme	0	55	33	75
	4	77	58	39

[a] The indicated antigen (0.5 μg/60 μL of PBS) was adsorbed onto wells of polystyrene plates overnight at 4 °C; remaining protein binding sites were blocked with PBS + 2 mg/mL bovine serum albumin. Where indicated, 100 μL of 4 M urea in PBS was added and incubated for 1 h at 37 °C. The plates were washed 3 times with PBT. Immobilized antigens were incubated for 1 h at 37 °C with 3.4 pmol of the indicated ^{125}I-labeled monoclonal antibody in 100 μL of PBS + 2 mg/mL bovine serum albumin. After the plate were washed 3 times with PBT, binding of the ^{125}I-labeled monoclonal antibody was determined. The specific radioactivity was as follows: mAb 129C4, 217 cpm/fmol; mAb 124D1, 155 cpm/fmol; mAb 126C6, 155 cpm/fmol.

Ishihama (1981) proposed that the assembly of the RNA polymerase core proceeds in a sequential fashion; the last step involves the temperature-dependent conversion of a premature core to the active core enzyme. The data presented in Table II indicate that incubation of the subunit mixture with mAb 129C4 completely blocks reconstitution of active core enzyme while mAb 124D1 is without apparent effect. When mAb 129C4 is incubated at 0 °C with premature core, only an incomplete inhibition of the conversion to the mature core enzyme occurs. In contrast, mAb 126C6 inhibits reconstitution when incubated with either the subunit mixture or the premature core. The data reflect some critical changes that involve the α subunit(s) initially during assembly into premature core enzyme and subsequently during the temperature-dependent conversion of the premature core enzyme to the active form.

Table IV: Dissociation Constants for Binding of the Anti-α Monoclonal Antibodies to Free and RNA Polymerase Associated α Subunit[a]

antibody	K_D (nM)		
	α	core	holoenzyme
mAb 126C6	0.28 ± 0.008[b]	0.16 ± 0.005[b]	0.064 ± 0.006[b]
mAb 129C4	3.11 ± 0.06[c]	2.86 ± 0.023[c]	2.75 ± 0.18[c]
mAb 124D1	2.20 ± 0.042[c]	3.33 ± 0.037[c]	2.90 ± 0.093[c]

[a] Conditions used are described under Materials and Methods. [b] The method of Friguet et al. (1985) was used to determine the K_d. [c] The K_d was determined from Scatchard plots using ^{125}I-labeled monoclonal antibodies; the concentration of the ^{125}I-labeled monoclonal antibody was varied from 0.25 to 12 nM. For ^{125}I-mAb 129C4, the immobilized antibody was mAb 124D1; for ^{125}I-mAb 124D1, the immobilized antibody was mAb 129C4.

The data presented in Table III indicate that the anti-α monoclonal antibodies bind to their respective epitopes in free α and RNA polymerase immobilized on the surface of polystyrene microtiter wells. Inherent in such solid-phase assays are the difficulties in determining the amount of the antigenic protein adsorbed to the polystyrene surface and knowing to what extent adsorption to the polystyrene affects the native conformation of the proteins (Friguet et al., 1984). Both of the monoclonal antibodies shown to be noninhibitors of RNA polymerase activity show relatively greater binding to the free α subunit compared to α assembled in RNA polymerase core enzyme or holoenzyme. In contrast, the inhibitory mAb 126C6 binds equally well to free α and to α present in RNA polymerase. Incubation of the immobilized core enzyme with 4 M urea results in an approximately 2-fold increase in the amount of ^{125}I-mAb 124D1 and ^{125}I-mAb 129C4 bound. This is not due to a possible effect on the conformation of α since the prior incubation of the immobilized α with 4 M urea does not increase binding by ^{125}I-mAb 124D1 and ^{125}I-mAb 129C4. mAb 126C6 behaves differently from the other two monoclonal antibodies in that urea treatment of immobilized α increases binding by about 25% while urea treatment of immobilized core enzyme decreases binding of ^{125}I-mAb 126C6 by about 50%. The data suggest that particular regions of α may be conformationally altered and/or sterically blocked when α is assembled in RNA polymerase.

The affinities of mAb 129C4, mAb 124D1, and mAb 126C6 for their respective epitopes on the free and RNA polymerase associated α subunit are determined by using the indirect ELISA assay devised by Friguet et al. (1985) or by direct binding with ^{125}I-labeled monoclonal antibodies. The data for mAb 129C4 and mAb 124D1 indicate that the affinity of these antibodies for their respective epitopes in free α is similar to or slightly lower than that for polymerase-associated α (Table IV). In contrast, the data obtained with mAb 126C6 indicate that this antibody shows a higher affinity for α when it is part of the polymerase core or holoenzyme structure. The competition assay shown in Figure 2 also demonstrates the greater affinity of mAb 126C6 for polymerase-associated α. The results indicate that conformational changes in α resulting in an increased affinity for mAb 126C6 occur during the course of RNA polymerase assembly.

Depending on the topological arrangement of the subunits within the core enzyme, a particular epitope may be accessible on one, both, or neither of the α subunits. The assay developed by Pestka et al. (1983) provides a method for determining whether one or both of the α epitopes in RNA polymerase are accessible to a particular monoclonal antibody. In this assay, an unlabeled monoclonal antibody is adsorbed onto the polystyrene surface of the wells of microtiter plates. Subsequently α, $\alpha_2\beta$, core enzyme, or holoenzyme is incubated with the immobilized monoclonal antibody, and finally the immune complexes which form are tested for binding with a ^{125}I-labeled monoclonal antibody. The data presented in Table V show that when the α subunit is used there is an obvious difference between homologous and heterologous ^{125}I-labeled monoclonal antibody binding. The data are consistent with the predominant species being the α monomer since the homologous pairs of immobilized monoclonal antibody and soluble ^{125}I-labeled monoclonal antibody show only low α binding activity. The lack of interference for the binding of the labeled antibody to α bound to a heterologous antibody indicates that each monoclonal antibody binds to an independent antigenic domain on the α subunit.

When assayed with each of the homologous antibody pairs, binding to an $\alpha_2\beta$ preparation (Lowe & Malcolm, 1976) is very low (Table VI). When the immobilized anti-β mAb 210E8–$\alpha_2\beta$ complex is used, only ^{125}I-mAb 126C6 is bound.

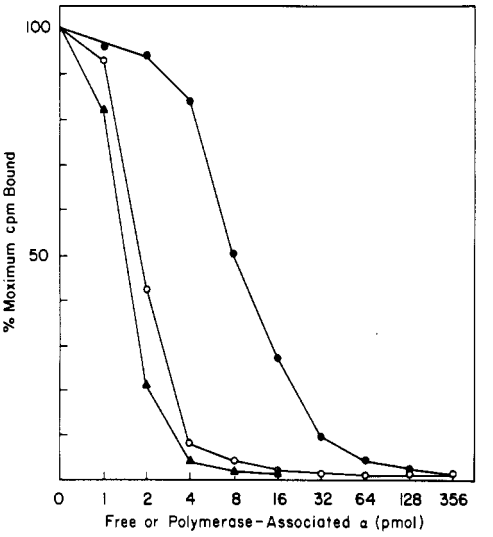

FIGURE 2: Competitive inhibition of mAb 126C6 binding to ^{125}I-α by free and RNA polymerase associated α. Wells of a Costar 96-well EIA plate were coated by overnight incubation at 4 °C with 0.5 μg of mAb 126C in 100 μL of PBS. Remaining protein binding sites were blocked by incubation with PBS + 2 mg/mL bovine serum albumin. A fixed concentration of ^{125}I-α (5.1 ng, 69 000 cpm) was added to tubes containing serially diluted α (●), core enzyme (○), or holoenzyme (▲). The mixtures (in 150 μL of PBS + 2 mg/mL bovine serum albumin) were then transferred to the wells containing the immobilized mAb 126C6 and incubated for 2 h at 37 °C. After the wells were washed with PBS + 2 mg/mL BSA, the amount of ^{125}I-α bound was determined as described under Materials and Methods.

Table V: Binding of ^{125}I-Labeled Anti-α Monoclonal Antibodies to Plate-Immobilized Monoclonal Antibody–α Complexes[a]

immobilized antibody	^{125}I-mAb bound (fmol)		
	129C4	124D1	126C6
mAb 129C4	2	36	45
mAb 124D1	50	7	56
mAb 126C6	50	40	5

[a] The indicated antibody (0.5 μg/60 μL of PBS) was adsorbed onto wells of polystyrene plates overnight at 4 °C; remaining protein binding sites were blocked with PBS + 2 mg/mL bovine serum albumin. α (10 pmol in 100 μL of PBS + 2 mg/mL bovine serum albumin) was incubated with the immobilized monoclonal antibody for 1 h at 37 °C. After plates were washed 3 times with PBT, 1 pmol of the indicated ^{125}I-labeled monoclonal antibody in 100 μL of PBS + 2 mg/mL bovine serum albumin was added and incubated for 90 min at 37 °C. After plates were washed 3 times with PBT, binding of the ^{125}I-labeled monoclonal antibody was determined. α and ^{125}I-labeled antibodies were added at saturating concentrations. The specific radioactivity was the following: mAb 129C4, 1311 cpm/fmol; mAb 124D1, 1124 cpm/fmol; mAb 126C6, 100 cpm/fmol.

Table VI: Binding of ^{125}I-Labeled Monoclonal Antibodies to Plate-Immobilized Monoclonal Antibody–Polymerase and $\alpha_2\beta$ Complexes[a]

	^{125}I-mAb bound (fmol)								
	$\alpha_2\beta$			core enzyme			holoenzyme		
immobilized mAb	129C4	124D1	126C6	129C4	124D1	126C6	129C4	124D1	126C6
anti-α									
mAb 129C4	2	163	198	1	110	187	1	29	73
mAb 124D1	182	5	137	105	1	137	30	0	77
mAb 126C6	180	133	7	3	7	59	2	4	25
anti-β									
mAb 210E8	5	7	68	1	1	185	1	0	173

[a] The indicated antibody (0.5 μg/100 μL of PBS) was adsorbed onto wells of polystyrene plates overnight at 4 °C. $\alpha_2\beta$ (0.7 μg), core enzyme (1 μg), or holoenzyme (1 μg) was incubated with the immobilized monoclonal antibody for 16 h at 4 °C. After plates were washed, 1 pmol of the indicated ^{125}I-labeled monoclonal antibody was added and incubated for 16 h at 4 °C. For details, see Materials and Methods. The specific radioactivity was as follows: mAb 129C4, 1170 cpm/fmol; mAb 124D1, 300 cpm/fmol; mAb 126C6, 249 cpm/fmol.

The observation that only one of the anti-α monoclonal antibodies is able to bind to the immune complex formed between mAb 210E8 and the $\alpha_2\beta$ complex while no binding is observed when the homologous anti-α monoclonal antibody pairs are used indicates that a stable $\alpha_2\beta$ is not present. It would appear that in the $\alpha_2\beta$ subassembly one of the α subunits is weakly bound and that it may have dissociated from the subassembly following binding of the monoclonal antibody. The extent of binding with the immobilized mAb 129C4–core plus ^{125}I-mAb 129C4 and immobilized mAb 124D1–core plus ^{125}I-mAb 124D1 is very low compared to the results obtained when each of the immobilized immune complexes is incubated with the heterologous ^{125}I-labeled monoclonal antibodies. In contrast, the immobilized mAb 126C6–core immune complex shows only weak binding with ^{125}I-mAb 124D1 or ^{125}I-mAb 129C4 and relatively strong binding with the homologous ^{125}I-mAb 126C6. The data indicate that the epitope specific for mAb 126C6 is available on each of the α subunits in RNA polymerase while the epitopes for mAb 124D1 and mAb 129C4 are accessible for binding on only one of the α subunits in RNA polymerase. The impaired binding of mAb 129C4 and mAb 124D1 to the mAb 126C6–polymerase complex may be a consequence of steric hindrance resulting from the particular orientation of the enzyme with respect to the plate surface.

Core or holoenzyme bound to the plate-fixed mAb 126C6 does not permit the binding of either mAb 124D1 or mAb 129C4 to its epitope on the available α subunit. Since each of the epitopes is present in separate antigenic domains in free α, the observed interference is not a function of steric blockage by the immobilized mAb 126C6. It is possible that polymerase bound to the plate-immobilized mAb 126C6 does not allow access of the soluble mAb 124D1 or mAb 129C4 to its site due to the proximity of the polystyrene surface. Binding of the monoclonal antibody to the protein A present on the surface of *S. aureus* cells could allow for a greater degree of accessibility for binding to epitopes present on RNA polymerase. The binding of ^{125}I-mAb 129C4–core enzyme to the protein A bound mAb 126C6 (Table VII) contrasts with the lack of binding of ^{125}I-mAb 129C4 to the polystyrene plate bound mAb 126C6–core enzyme. Binding of ^{125}I-mAb 129C4 complexed with αβ or core enzyme to the protein A bound mAb 210E8 is also evident. The polystyrene plate and protein A assays indicate that the epitope for mAb 129C4 is available on only one of the α subunits of RNA polymerase.

Table VII: Binding of ^{125}I-Labeled mAb 129C4 Complexed with Polymerase and $\alpha_2\beta$ to Protein A–Monoclonal Antibody Complexes[a]

	^{125}I-mAb 129C4 bound (fmol)		
immobilized mAb	$\alpha_2\beta$	core enzyme	holoenzyme
anti-α			
mAb 129C4	30	0	0
mAb 124D1	563	393	251
mAb 126C6	610	418	226
anti-β			
mAb 210E8	96	72	14

[a] ^{125}I-mAb 129C4 (10 pmol, 1500 cpm/fmol) was incubated with $\alpha_2\beta$ (1.1 μg), core enzyme (2.4 μg), or holoenzyme (3 μg) for 30 min at 37 °C. The indicated monoclonal antibody adsorbed to protein A on *S. aureus* cells was mixed with 50 μL of the preformed immune complex containing ^{125}I-mAb 129C4 and incubated for 30 min at 37 °C. Bound ^{125}I-mAb 129C4 was determined after washing with PBT. For details, see Materials and Methods.

Discussion

Reconstitution of the *E. coli* RNA polymerase core enzyme from its constituent subunits has been extensively studied (Ishihama & Ito, 1972; Ito & Ishihama, 1973; Fukuda & Ishihama, 1974; Harding & Beychok, 1974; Yarbrough & Hurwitz, 1974; Palm et al., 1975; Saitoh & Ishihama, 1976; Ishihama, 1981). The sequence proposed for assembly of RNA polymerase core enzyme (Ishihama, 1981) is as follows:

$$\alpha + \alpha \rightarrow \alpha_2 + \beta \rightarrow \alpha_2\beta + \beta' \rightarrow \alpha_2\beta\beta'$$

Reassembly at 4 °C results in an enzymatically inactive premature core enzyme; conversion of the premature core to the catalytically active core enzyme requires incubation at 37 °C. The physical and enzymatic properties of the active form of the reconstituted polymerase closely resemble those of the native RNA polymerase (Harding & Beychok, 1974; Ishihama et al., 1979). Analysis of the absorbance spectra, far- and near-UV CD spectra, and tritium exchange rates of the premature and reconstituted active polymerase indicates that although most of the secondary structure of the premature core is similar to that of the active enzyme there are minor but significant differences. Studies using sensitivity to trypsin digestion, chemical cross-linking, sedimentation velocity, and elution profiles from phosphocellulose and DEAE-Sephadex indicate that the subunits in the premature core are weakly associated (Ishihama et al., 1979). The results of glycerol gradient centrifugation suggested that the premature core is in rapid equilibrium with the $\alpha_2\beta$ subassembly and β′ subunit (Ishihama et al., 1979).

Only one of the α subunits of RNA polymerase is bound by mAb 124D1. The affinity of mAb 124D1 for its epitope on free α and the polymerase-associated α subunit is similar. While these properties are also seen for mAb 129C4, incubation of polymerase subunits with mAb 124D1 does not inhibit reconstitution. This lack of inhibition may result from a conformational change in one of the α subunits affected during the reassembly process. The proposed conformational change in the affected α would take place during assembly of the premature core since incubation of the α subunits with mAb 124D1 prior to addition of β and β′ results in an 80% recovery of polymerase activity (data not shown). The an-

tibody binding data indicate that the minimal stable complex is $\alpha\beta$ and that reassembly of core enzyme in the presence of the mAb 124D1 may proceed as follows:

$$2[\text{mAb 124D1}-\alpha] + \beta \rightarrow \text{mAb 124D1}-\alpha\beta + \text{mAb 124D1}-\alpha$$

$$\text{mAb 124D1}-\alpha\beta + \text{mAb 124D1}-\alpha + \beta' \rightarrow \text{mAb 124D1}-\alpha\alpha\beta\beta' + \text{mAb 124D1}$$

Concerted interactions between mAb 124D1-α, mAb 124D1-$\alpha\beta$, and β' may cause the dissociation of mAb 124D1 from one of the α subunits. Subsequently, the α domain in which the epitope for mAb 124D1 resides may be blocked by interaction with the other polymerase subunits. The inferred conformational change must take place in the affected domain of only one of the polymerase α subunits since the affinity of mAb 124D1 for free α and the available α subunit in the core enzyme is similar. The postulated pathway for core assembly (Saitoh & Ishihama, 1976) has been questioned by Coggins et al. (1977), who studied RNA polymerase subunit proximity by chemical cross-linking; the major products were β-β', α-β, and α-β'; no α-α species were found. Incubation of core enzyme with 2 M urea resulted in the release of $\alpha\beta$ and $\alpha\beta'$ complexes (Ishihama, 1972). Using the monoclonal antibodies, we were unable to demonstrate the presence of a stable $\alpha_2\beta$; however, the antibody binding data clearly indicate the existence of the $\alpha\beta$ complex. A destabilizing effect of the monoclonal antibodies on subunit interactions within subassemblies cannot be ruled out. It is possible that in an $\alpha_2\beta$ subassembly one of the α subunits is weakly associated with the more stable $\alpha\beta$ complex and is released on binding of the monoclonal antibody.

Incubation of mAb 129C4 with the subunit mixture results in an almost complete inhibition of reconstitution. Incubation of the premature core enzyme with mAb 129C4 results in only a partial inhibition of the temperature-dependent conversion to the active core enzyme. A model in which $\alpha_2\beta$ and β' are in rapid equilibrium with the premature core (Ishihama et al., 1979) is not consonant with the data obtained using mAb 129C4. If $\alpha_2\beta$ were formed, it would be bound by mAb 129C4 with its consequent dissociation into mAb 129C4-$\alpha\beta$ and mAb 129C4 resulting in the inhibition of polymerase assembly. It is possible that the premature core is composed of a mixture of intermediates of varying stability to dissociation into subassemblies. Alternatively, during reconstitution, one of the α subunits may undergo conformational changes that result in only partial accessibility of the mAb 129C4 epitope. During the course of the transition to the active enzyme, the epitope in the premature core enzyme for mAb 129C4 present on one of the α subunits becomes totally inaccessible. The affinity of mAb 129C4 for free α and the available α present in RNA polymerase is similar, suggesting that the available mAb 129C4 epitope in polymerase has the same conformation as that present in the free α subunit.

Inhibition of d(A-T)$_n$-directed activity is not observed when mAb 126C6 is incubated with the reconstituted mature core enzyme. However, about 90% of the recoverable enzyme activity is lost when mAb 126C6 is incubated with either the subunit mixture or the premature core enzyme. In native RNA polymerase, the mAb 126C6 epitope is available on each of the α subunits. Inhibition of the maturation of the premature core enzyme by mAb 126C6 may be due to antibody-induced conformational changes in α. In the premature core enzyme, the α subunits may be less conformationally stabilized by interactions with neighboring subunits than in the mature or native enzyme. The observed increase in the affinity of mAb 126C6 for α present in RNA polymerase also implies that the α domain in which the epitope for mAb 126C6 residues undergoes conformational changes during enzyme assembly.

It would appear that there are at least local differences in the conformation of each of the polymerase-associated α subunits. This is clearly seen in the increased affinity of mAb 126C6 for α present in polymerase. The relative affinity of mAb 126C6 for α is $\alpha_2\beta\beta'\sigma > \alpha_2\beta\beta' > \alpha$, and its epitope is exposed on both of the polymerase α subunits. In contrast, the domains in which the epitopes for mAb 129C4 and mAb 124D1 reside are exposed in only one of the polymerase α subunits. There is evidence indicating that the α subunits in RNA polymerase are not equivalent. Following infection of *E. coli* with phage T4, initially only one of the α subunits is ADP-ribosylated (Rohrer et al., 1975), indicating that the α subunits are differently arranged in RNA polymerase. Treatment of RNA polymerase with 0.1 mM *p*-(chloromercuri)benzoate results in the release of one of the α subunits (Ishihama, 1972, 1981).

The unequal exposure of the α subunits in RNA polymerase is also indicated by the data obtained with the monoclonal antibodies. It would appear that one of the α subunits is relatively exposed while the other α is positioned with more of its surface shielded by the larger β and β' subunits. This is consistent with the observations of Stender (1980) regarding the low accessibility of core enzyme to polyclonal anti-α antibodies. Tichelaar et al. (1983) showed by immunoelectron microscopy that almost half of the α surface is covered by the β and β' subunits. The proposal that the α subunits differ in their exposure may also explain the low extent of binding of mAb 129C4 and mAb 124D1 to core or holoenzyme bound to mAb 126C6 adsorbed to the polystyrene surface (Table VI). The α subunit which is more shielded by the polymerase β and β' subunits may bind poorly or not at all to the plate-fixed mAb 126C6. Binding of polymerase to the plate-fixed mAb 126C6 would presumably occur through its epitope on the more exposed α subunit. It is assumed that the available epitopes for mAb 129C4 and mAb 124D1 are also located on the α subunit bound by the immobilized mAb 126C6.

Results obtained by using small-angle neutron scattering (Stockel et al., 1980a,b) or circular dichroism (Levine et al., 1980) did not reveal any major differences between the core enzyme and the core component of the holoenzyme. Chemical cross-linking of the holoenzyme indicated that σ interacts with β and β' and with at least one of the α subunits (Hillel & Wu, 1977). Only minimal differences in the profile of core and holoenzyme were discerned by electron microscopy (Tichelaar et al., 1983). Analysis of tryptic cleavage of RNA polymerase provided evidence for association between the β and σ subunits (Fisher & Blumenthal, 1980). On the basis of studies with subunit-specific antibodies, Tichelaar et al. (1983) proposed that the σ subunit resides at the concave side of the core where it interacts with the α dimer. The reduced binding of the anti-α monoclonal antibodies to the holoenzyme may be attributable to a combination of a steric effect of the σ subunit and a particular orientation of the antibody-bound polymerase with respect to the polystyrene plate surface. This is consistent with the data indicating that there is no significant difference in the affinity of mAb 129C4 and mAb 124D1 for core and holoenzyme whereas the affinity for mAb 126C6 for holoenzyme is greater than for core enzyme. The steric effect of the σ subunit is only seen in the double anti-α antibody assays. The σ subunit does not impede binding of mAb 126C6 to its epitope on each of the α subunits when there is no steric hindrance imposed by the plate surface. The lower binding

of mAb 129C4–holoenzyme relative to that for the mAb 129C4–core to the *S. aureus* protein A–mAb 124D1 (or mAb 126C6) complex suggests that when two anti-α monoclonal antibodies bind to the same subunit (presumably the more exposed α) the steric effect of σ remains.

ACKNOWLEDGMENTS

We thank Christopher Roman for excellent technical assistance.

Registry No. RNA polymerase, 9014-24-8.

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