Dimeric Association of *Escherichia coli* RNA Polymerase α Subunits, Studied by Cleavage of Single-Cysteine α Subunits Conjugated to Iron−(*S*)-1-[*p*-(Bromoacetamido)benzyl]ethylenediaminetetraacetate†

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ABSTRACT: Proximity relationships between the two associated monomers of the *Escherichia coli* RNA polymerase α subunit were studied using a set of four mutant α subunits, each with a single Cys residue at one of the naturally occurring positions (54, 131, 176, and 269). These mutant α subunits were conjugated with the cutting reagent iron−(*S*)-1-[*p*-(bromoacetamido)benzyl]ethylenediaminetetraacetate (Fe−BABE), and the peptide backbone was cleaved at locations near the modified Cys. Analysis of the cleavage sites identified segments within ≈12 Å of the conjugation site. These results show that, for *inter*molecular cutting, segments of the subunit assembly domain (N-terminal domain) of one subunit and the linker region between N- and C-terminal domains of the other subunit are near each other, and the N-terminal domains of both subunits are in close proximity to one another. Intramolecular cutting however, was observed only within an individual N- or C-terminal domain.

The *Escherichia coli* RNA polymerase core enzyme is composed of two α, one β, and one β' subunit. This complex enzyme is assembled in vivo and in vitro in the sequence $2\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta'$ (*1*). Dimerization of the α subunit is the first step of core enzyme assembly; β and β' subunits cannot bind together without the α subunits. The α_2 dimer is the first stable intermediate on the assembly pathway. Each α subunit contains two independently folded domains, the amino-terminal domain (NTD) and the carboxy-terminal domain (CTD) (*2*, *3*). Amino acid residues 20−235 of the α NTD are involved in the formation of active enzyme molecules in vivo (*4*, *5*) and in vitro (*6*−*8*). On the other hand, the 94-residue CTD contains the contact sites for transcription factors (*9*−*11*) and the DNA upstream (UP) element, which has transcription enhancer activity (*12*). Recently the role of each α subunit in making contact with the *rrnB*P1 UP element was studied by DNA cleavage analysis with Fe−BABE[1] (*13*). This study showed that the two α subunits bind to the UP element, but only one α subunit appears to bind to the promoter-distal region of the UP element.

Although much information has been gained about polymerase function, relatively little is known about how the subunits contact each other (*14*, *15*). For example, low-resolution (≈25 Å) X-ray structures of both core and holo RNA polymerase are available (*16*), but these do not permit identification of separate subunits or other molecular details. Neither a high-resolution crystal structure nor an NMR structure of a single intact RNA polymerase subunit has been determined, though an NMR structure is available for the α CTD (*17*), and a crystal structure is available for a large fragment of the σ subunit (*18*).

Chemical approaches such as cross-linking can help fill this void by showing which proteins in a multisubunit complex are adjacent to each other (*19*). More recently, site-directed cleavage techniques have provided precise information on the identities of proximal residues in protein complexes (*20*−*22*). Establishing the proximity of a single site on one subunit to one or more sites on another adds considerably to our understanding of the overall architecture of a large macromolecular complex. As part of a program to map contacts between RNA polymerase subunits we have examined the α_2 dimer, the simplest stable substructure on the assembly pathway.

The bifunctional chelating agent Fe−BABE consists of EDTA−Fe and a reactive group that covalently conjugates

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[1] Abbreviations: BABE, (*S*)-1-[*p*-(bromoacetamido)benzyl]ethylenediaminetetraacetic acid; biotin-HPDP, *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide; CPM, *N*-[4-(7-diethylamino-4-methylcoumarin-3-yl)phenyl]maleimide; Fe−BABE, iron chelate of (*S*)-1-[*p*-(bromoacetamido)benzyl]ethylenediaminetetraacetic acid (also denotes the chelate moiety conjugated to cysteine); HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-morpholinopropanesulfonic acid.

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Table 1: Fe−BABE Cleavage Sites of α_2 Dimer[a]

protein	MW	terminus	aa residues	band intensity	inter
$C^{54}\alpha$	31 965	C	(47 ± 5)−329	s	
	30 999	C	(54 ± 5)−329	w	
	26 393	C	(92 ± 7)−329	s	
	26 830	N	1−(239 ± 4)	s	*
	23 301	C	(123 ± 4)−329	m/w	
	20 581	C	(145 ± 4)−329	s/m	
	19 919	C	(152 ± 4)−329	s/m	
$C^{131}\alpha$	26 890	C	(93 ± 7)−329	w	
	23 960	C	(121 ± 4)−329	s	
	22 049	C	(134 ± 4)−329	m	
	20 516	C	(146 ± 4)−329	m/w	
	19 405	C	(157 ± 4)−329	m/w	
$C^{176}\alpha$	32 386	C	(42 ± 1)−329	s	*
	31 781	C	(47 ± 5)−329	s	*
	20 593	C	(151 ± 4)−329	m	
	19 240	C	(163 ± 4)−329	m	
$C^{269}\alpha$	33 106	N	1−(294 ± 2)	s	
	30 403	N	1−(269 ± 2)	s	
	29 530	N	1−(265 ± 2)	m	

[a] MW is the size of each fragment. Terminus shows whether the fragment contains an original C- or N-terminus. aa residues are the residue numbers determined for each fragments. The number after the ± sign is the standard deviation. Band intensity is divided into (s) strong, (m) medium, or (w) weak. Inter with an asterisk denotes intermolecular cleavage. Only the most clearly indicated intermolecular bands were included.

to the free sulfhydryl groups of cysteine residues in proteins. After addition of ascorbate and peroxide, Fe−BABE cleaves polypeptide chains in proximity to the chelate, apparently independent of the amino acid side chains involved (*23*, *24*). This reagent is useful to study protein topography, and—because it also cleaves nucleic acid backbones—to study contact sites between proteins and DNA (*13*, *25*). This methodology can be applied even to complex biological systems such as heme-containing membrane proteins (*20*).

The wild-type α subunit contains four cysteine residues, at amino acid positions 54, 131, 176, and 269. In our study four different single-Cys α subunits, each of which contains a cysteine residue at only one of these positions, were constructed and used as probes. These mutant α subunits were conjugated with the cutting reagent Fe−BABE, and the peptide backbones in proximity to the tethered Fe−BABE were cleaved. The polypeptide fragments were separated by SDS−PAGE and blotted. The cleavage sites were analyzed by comparing the fragment sizes with molecular weight (MW) markers, which were prepared from two series of cloned truncated α subunits.

Since the α subunit exists as a dimer in solution, it is necessary to distinguish between *inter*subunit cleavage and cleavage within each α monomer. To accomplish this, "heterodimers" were prepared in which one monomer was tagged with biotin at Cys-176 and the other with Fe−BABE at one of the four Cys positions. All the fragments that could be identified contained residue 176 (Table 1), so this allowed fragments from α subunits that did not contain Fe−BABE—and therefore were produced by intersubunit cleavage—to be identified by staining with streptavidin−alkaline phosphatase conjugate (streptavidin−AP) (Figure 1).

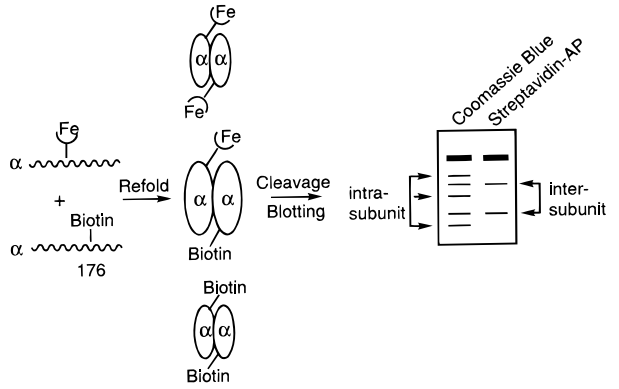

FIGURE 1: Fe−BABE/biotin α "heterodimers" were prepared with one subunit containing Fe−BABE and the other containing biotin. Three types of dimers can be formed: Fe−BABE homodimer, biotin homodimer, and Fe−BABE/biotin heterodimer. The cleavage reaction was performed on the mixture. Only the intersubunit cleavage fragments from heterodimers could be visualized with streptavidin−AP, while both intra- and intersubunit cleavage fragments could be visualized with Coomassie blue staining. The fragments from Fe−BABE homodimer could not be visualized with streptavidin−AP. The biotin homodimer could not produce any fragments.

MATERIALS AND METHODS

Materials. The fluorescent dye CPM was purchased from Molecular Probes Inc. Ascorbic acid (vitamin C, microselect grade) was purchased from Fluka and hydrogen peroxide (Ultrex grade) was from J. T. Baker. The biotin-HPDP reagent and streptavidin−AP were purchased from Pierce. Broad-range protein markers for SDS−PAGE were from New England Biolabs. All other reagents were obtained from commercial sources and used without further purification. Pure water (18 MΩ cm^{-1}) was used throughout. All lab ware was either purchased as metal-free or acid-washed (*26*).

Buffers. Wash buffer consisted of 10 mM Tris-HCl (pH 8.0 at 4 °C), 1 mM EDTA, and 150 mM NaCl. Buffer A consisted of 20 mM MOPS (pH 8.0), 0.1 mM EDTA, 10 mM $MgCl_2$, and 0.2 M KCl. Storage buffer consisted of 20 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 10 mM $MgCl_2$, 0.2 M KCl, and 50% glycerol. Reconstitution buffer consisted of 50 mM Tris-HCl (pH 7.8 at 4 °C), 0.1 mM EDTA, 10 mM $MgCl_2$, 0.3 M KCl, and 20% glycerol. Cleavage buffer consisted of 10 mM MOPS (pH 7.9), 1.0 mM EDTA, 10 mM $MgCl_2$, and 120 mM NaCl. Buffer B consisted of 50 mM Tris-HCl (pH 7.8 at 4 °C), 0.1 mM EDTA, 10 mM $MgCl_2$, and 0.15 M KCl. Sample application buffer (5 × SAB) consisted of 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% glycerol, 25 mM EDTA, and 0.0025% (w/v) bromophenol blue.

Protein Preparation. pGEMA54C, pGEMA131C, pGEMA176C, and pGEMA269C are overexpression plasmids of mutant α subunits, which contain a single cysteine residue at position 54, 131, 176, or 269, respectively (*13*, *27*). Each plasmid was transformed into strain BL21(λDE3). The transformed cells were grown in LB medium containing 200 μg/mL ampicillin at 37 °C until $OD_{600} = 0.4$. Expression was induced by adding isopropyl thiogalactoside (0.4 mM final) and incubating at 37 °C for 2−3 h. The cells were harvested, washed with 50 mL of wash buffer, and stored at −70 °C. The purification of each subunit was performed as described for the wild-type α preparation (*28*).

Conjugation with Fe−BABE under Folded Conditions. Each mutant α subunit (27 μM final) was dialyzed into buffer A and mixed with Fe−BABE (270 μM final) (in dimethyl

sulfoxide) at pH 8 (*29*). The final dimethyl sulfoxide concentration was adjusted to <5% with buffer A. The mixture was incubated at 37 °C for 1 h and the excess Fe−BABE was dialyzed away against storage buffer (4 °C, overnight).

Conjugation with Fe−BABE under Unfolded Conditions. Each mutant α subunit (27 μM final) was unfolded by dialysis at 4 °C into buffer A containing 6 M urea and mixed with Fe−BABE (270 μM final) at pH 8 and at <5% dimethyl sulfoxide. The mixture was incubated at 37 °C for 1 h. The excess Fe−BABE was removed and the α subunits were refolded by dialyzing against reconstitution buffer (4 °C, overnight).

Biotinylation of Mutant α Subunit. The free cysteine residue of C^{176}α was biotinylated using biotin-HPDP, a disulfide biotinylation reagent that reacts with the free thiol group of cysteine. The mutant α subunit was unfolded by dialysis into buffer A containing 6 M urea. A fresh solution of biotin-HPDP (4 mM) was prepared in dimethylformamide. The conjugation was carried out by incubating the C^{176}α (27 μM final) and biotin-HPDP (270 μM final) for 90 min at room temperature. The excess biotin-HPDP was dialyzed away against buffer A. The conjugation yield was monitored by measuring the absorbance of pyridine-2-thione product at 343 nm.

Exchange of Fe−BABE-Conjugated Subunit and Biotinylated Subunit. A chosen Fe−BABE-conjugated mutant α subunit (α−Fe−BABE) and the C^{176}α-biotinylated subunit were unfolded in buffer A containing 6 M urea and mixed in a 1:1 ratio. The mixture was dialyzed against reconstitution buffer at 4 °C overnight to refold the proteins. This was carried out for each of the four α−Fe−BABE conjugates.

Circular Dichroism Spectroscopy. The protein samples were prepared in pure water at 2.5 μM, and the CD from 185 to 260 nm was measured in a 1 mm thick cell using a Jasco J-600 spectropolarimeter.

Assay of Free Sulfhydryl Groups. The presence of free Cys sulfhydryl groups before and after conjugation was determined fluorometrically by the CPM test (*29, 30*).

Amino Acid Analysis. The four mutant α subunits (27 μM) were unfolded by dialysis into buffer A containing 6 M urea and then conjugated with BABE by mixing with BABE (270 μM) and incubating for 1 h at 37 °C. The excess BABE was dialyzed away against buffer B. To convert cysteine into cysteic acid, samples were oxidized with performic acid prior to standard 24 h HCl hydrolysis (*31*). Both conjugated and unconjugated α samples were analyzed using a Beckman 6300 amino acid analyzer.

Mass Spectrometry. A Hewlett-Packard G2030A MALDI/TOF mass spectrometer that employs a nitrogen laser (337 nm) was used. Samples were prepared by vacuum evaporation using sinapinic acid as matrix. Spectra were generated in positive ion mode.

Cleavage Reaction. The protein was dialyzed into cleavage buffer containing 10% glycerol. In general, the cleavage buffer was a 10−50 mM HEPES or MOPS buffer. The protein solution in cleavage buffer also contained a final concentration of 1 mM EDTA to scavenge adventitious metals that might cleave the peptide chains and a final concentration of 10% glycerol to scavenge free radicals. Simple salts are compatible, but phosphate and imidazole inhibit cleavage. The pH of the buffer was usually between 7 and 8; however, the reaction is fairly insensitive to pH. Ascorbate was derived from ascorbic acid by titrating to pH 7 with NaOH. Both stock solutions of ascorbate and hydrogen peroxide (H_2O_2) were prepared fresh in 1 mM aqueous EDTA prior to use. The proteins were titrated with different concentrations of ascorbate and H_2O_2 in order to determine the optimal concentrations: 1 mM ascorbate/H_2O_2 for 5 μM α−Fe−BABE. After addition of ascorbate and H_2O_2 to the protein solution at room temperature, the reaction was quenched within 15 s by adding 0.25 volume of 5× SAB. The reaction then was quickly frozen in liquid N_2 and stored at −70 °C.

The cleaved fragments were separated by SDS−PAGE and blotted. Coomassie blue staining was used to visualize all protein fragments. An affinity-purified rabbit antibody specific for the C-terminal end of α was used to identify the cleavage fragments containing the original C-termini (*32*). Streptavidin−AP was used to visualize the biotinylated fragments.

Preparation of MW Markers. Two sets of MW markers were prepared: an N-terminal truncation set and a C-terminal truncation set. pETMAΔ*N*20, 30, 40, 60, 100, 120, and 160 are overexpression plasmids for a series of α subunits truncated at the N-terminus (*7*). pGEMAD235 and 256 are overexpression plasmids for a series of α subunits truncated at the C-terminus (*6*).

Oxidizing MW Markers (Control Experiment). αΔN30, αΔN60, αΔN120, αΔN160, α1−235, and α1−256 in cleavage buffer were titrated with Fe−EDTA, ascorbate, and H_2O_2. The reaction was incubated at 37 °C for 1 min and quenched by adding 0.25 volume of 5× SAB (*32*), and the effect of this treatment on protein migration distance during SDS−PAGE was determined.

Determination of Cleavage Fragment Sizes. The conventional way to determine the mass of an unknown protein by comparison with markers on SDS−PAGE involves a plot of log MW versus migration distance (R_m). However, sequence-dependent variations in R_m degrade the accuracy of the results, and markers made from a nested set of fragments of the original protein should improve accuracy (*15*). Several mathematical analyses were compared using the nested set of eight cloned α-fragment markers truncated at the N-terminus. To obtain estimates of accuracy, each of the eight standards was treated in turn as an unknown, whose molecular weight was to be estimated by comparison to the other seven. To explore gel to gel reproducibility (precision), eight different 12% slab gels were compared. An example is given in Supporting Information, Table 1S. To summarize, both the conventional log MW vs R_m plot (Supporting Information, Table 1SA) and a point-to-point plot of MW vs R_m (Supporting Information, Table 1SB) allow reproducible estimates of molecular weights of "unknown" fragments, with a precision comparable to the 110 Da mass of an average residue (average standard deviation for the eight proteins 78 and 100 Da, respectively). However, the conventional log MW vs R_m plot is inferior to the point-to-point plot of MW vs R_m in the *accuracy* of its predictions (average error for the eight proteins 773 vs 374 Da, respectively). It is clear that—due to the systematic variations in R_m—MW assignments can be highly accurate when there are fragments with closely similar masses in the set of

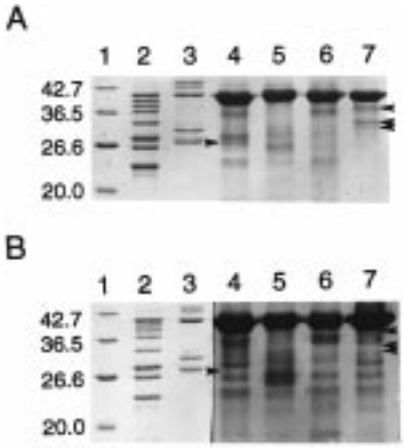

FIGURE 2: Fe−BABE-conjugated α subunits were cleaved with 1 mM ascorbate and H_2O_2. (A) The blot was stained with Coomassie blue. (B) The blot was stained with antibody specific for the α C-terminus (*32*). Lane 1, broad-range protein markers; lane 2, N-terminal truncated α MW markers (wt-α, αΔN20, αΔN30, αΔN40, αΔN60, αΔN100, αΔN120, and αΔN160); lane 3, C-terminal truncated α MW markers (wt-α, α1−256, and α1−235); lane 4, cleavage reaction of C^{54}α; lane 5, cleavage reaction of C^{131}α; lane 6, cleavage reaction of C^{176}α; lane 7, cleavage reaction of C^{269}α.

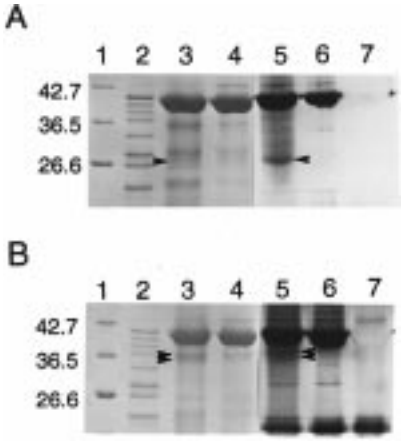

FIGURE 3: Cleavage fragments of Fe−BABE/biotin α_2 heterodimer were stained with Coomassie blue (lanes 1−4) and streptavidin-AP (lanes 5−7). (A) C^{54}α; (B) C^{176}α. Lane 1, broad-range protein markers; lane 2, N-terminal truncated α MW markers (wt-α, αΔN20, αΔN30, αΔN40, αΔN60, αΔN100, αΔN120, and αΔN160); lanes 3 and 7, cleavage reaction performed with 4 mM ascorbate/H_2O_2 on Fe−BABE-conjugated α without biotin; lanes 4 and 5, cleavage reaction performed with 4 mM ascorbate/H_2O_2 on Fe−BABE/biotin α; lane 6, no cleavage reaction performed on Fe−BABE/biotin α. Lanes 3 and 4 confirm that the same cleavage products were observed for Fe−BABE-conjugated α and Fe−BABE/biotin α subunit. Lane 7 confirms no cross-reactivity on Fe−BABE-conjugated α cleavage fragments with streptavidin−AP.

standards, but they can be inaccurate when the markers do not have similar masses. Cleavage fragment sizes were analyzed using a point-to-point plot of MW vs R_m.

RESULTS

The proper folding of the four mutant α subunits was confirmed by transcription activity assay (*28*) and circular dichroism (CD). The CD spectra of all four mutant α subunits, measured at 185−260 nm, were practically identical with that of wild-type α (data not shown).

The Fe−BABE conjugation reaction was performed on native protein dimers first. The conjugation yield determined by measuring residual thiol with CPM was very low (less than 10%) for C^{54}α, C^{131}α, and C^{269}α, but C^{176}α gave a 55% conjugation yield. CPM reacts with 100% ± 5% of the cysteine on each folded α subunit (1 mol of Cys/mol of protein). Fe−BABE conjugation was tried on each unfolded protein monomer next. Each α mutant was unfolded with 6 M urea, conjugated with Fe−BABE, and then refolded. The CPM test showed much higher conjugation yields for all four subunits (65−70%). The proper folding of each Fe−BABE-conjugated mutant α subunit was also confirmed by CD (data not shown). The conjugation yield indicated by the CPM test was in reasonable agreement with the loss of Cys shown by amino acid analysis, and the mass spectrum showed no doubly conjugated protein molecules.

Fragments from cleavage reactions were separated on SDS−14%, 12%, 10%, and 8% polyacrylamide gels followed by blotting and visualization with either Coomassie blue staining or immunostaining specific for the α C-terminus. Each mutant α subunit showed a different cleavage pattern (Figure 2); the cleaved fragment bands were not present in controls (Supporting Information, Figure 1S). By comparing Coomassie blue staining (Figure 2A) and α C-terminal antibody staining (Figure 2B), it is clear that some bands are not visualized by antibody staining. These fragments do not contain the original C-terminus; rather, they have the original N-terminus (as confirmed by Edman sequencing) and a new C-terminus. A protein fragment near α1−235 (Figure 2, lane 3) for C^{54}α (Figure 2, lane 4, arrow) and all three dark fragment bands for C^{269}α (Figure 2, lane 7, arrows) were not observed by antibody staining.

The fragment sizes were determined by using two sets of cloned, truncated α MW markers (Supporting Information, Figures 2S and 3S). When they contained the original C-terminus, Fe−BABE cleavage sites were assigned by using the nested set of N-terminal truncated α MW markers. The fragments containing the original N-terminus were analyzed with a set of three C-terminal truncated α fragments as MW markers. As a control experiment, it was determined that the truncated α subunits migrate identically on SDS−PAGE even when they have been oxidized in the presence of Fe−EDTA, ascorbate, and H_2O_2. No change of migration distance was observed on any of the truncated α markers: αΔN30, αΔN60, αΔN120, αΔN160, α1−235, and α1−256 (αΔN160 is shown in Supporting Information, Figure 4S).

Due to the formation of the α_2 dimer, the experiments in Figure 2 cannot distinguish whether a given cleavage event occurred intra- or intermolecularly. To solve this problem, Fe−BABE/biotin α "heterodimers" were prepared with Fe−BABE on one subunit and biotin on the other (Figure 1). When a heterodimer is cleaved, the fragments containing biotin can be visualized with streptavidin−AP on a blot. One fragment cut by Fe−BABE on C^{54}α and two fragments cut by Fe−BABE on C^{176}α (just below the uncut band) were visible with streptavidin−AP staining (Figure 3, lane 5, arrows). These three bands were not present in uncut proteins (Figure 3, lane 6). Thus, these three bands are identified as intersubunit products. Lanes 3 and 4 in Figure 3 confirm that both Fe−BABE homodimer and Fe−BABE/biotin heterodimer show the same cleaved fragments. The heterodimer cleavage of C^{131}α and C^{269}α showed no inter-

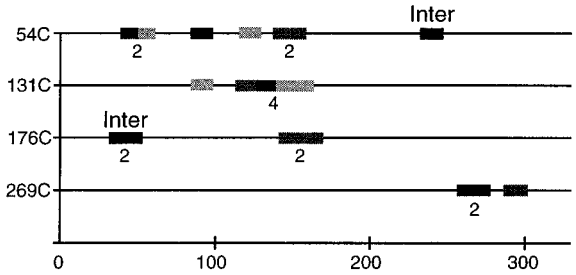

FIGURE 4: Summary of α subunit cleavage sites. The number below each box indicates the number of cutting sites in that region. The cutting sites marked Inter above the box are intersubunit cleavages. Darkness of box corresponds to the strength of cutting, determined from band intensity on SDS−PAGE; dark = strong, light = weak.

subunit products (Supporting Information, Figure 5S). Cleavage site analysis and inter/intrasubunit assignments are summarized in Table 1.

DISCUSSION

The great improvement of the Fe−BABE conjugation yield in $C^{54}\alpha$, $C^{131}\alpha$, and $C^{269}\alpha$ under unfolded conditions suggests that cysteine residues at positions 54, 131, and 269 are located in hydrophobic regions because the hydrophobic reagent CPM reacted with high yield under native conditions, while the hydrophilic molecule Fe−BABE did not. This may indicate that cysteine residue 176 is located in a hydrophilic region within the folded structure of the protein. CD spectra indicated that the conjugates refolded correctly after removal of urea.

The determination of cleavage sites has been investigated by several methods. Automated Edman degradation on the cleaved fragments containing new N-termini was not successful. Blocking of N-termini during separation and analysis is possible (*33*−*35*). Alternatively, this resistance of the α fragments to Edman sequencing may result from an oxidative, rather than hydrolytic, cleavage pathway. Although Fe−BABE is capable of hydrolytically cleaving a peptide (*36*), an oxidative cleavage pathway has also been proposed for a tethered Fe chelate (*37*). Whether peptide cleavage occurs by oxidative, hydrolytic, or both pathways may depend strongly on the orientation of chelate and peptide backbone. The physical reach of the tethered chelate is ≈12 Å; hydrolysis requires nucleophilic attack perpendicular to the peptide plane (*36*), while cleavage by a diffusible species such as hydroxyl radical is less restricted with respect to orientation and distance.

As summarized in Table 1 and Figure 4, three intersubunit cleavage sites and many intrasubunit cleavage sites have been observed. Several intrasubunit cleavages occurred within the amino acid residues 47−176, but none were observed in the 177−264 region. This suggests that residues 47−176 lie within a highly folded structure, since Fe−BABE at Cys-54 can reach amino acid residues from near 47 through 152, producing six cleavage sites, and Fe−BABE at Cys-131 can reach residues from near 93 through 157, producing five cleavage sites. The set of 19 proximity relations represented in Table 1 is not sufficient to allow us to propose a structure but provides constraints for structures proposed on other grounds.

Interestingly, all cleavage sites of $C^{269}\alpha$ determined in this study are intramolecular ones. These cleavage sites are

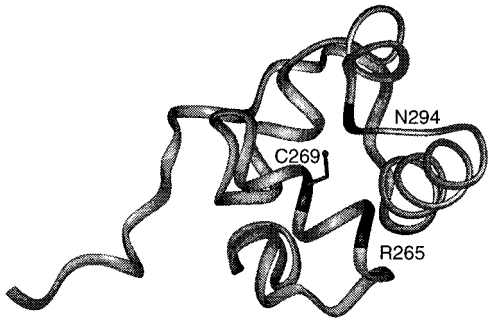

FIGURE 5: Intrasubunit cleavage sites of $C^{269}\alpha$ marked on the α CTD NMR structure (*17*).

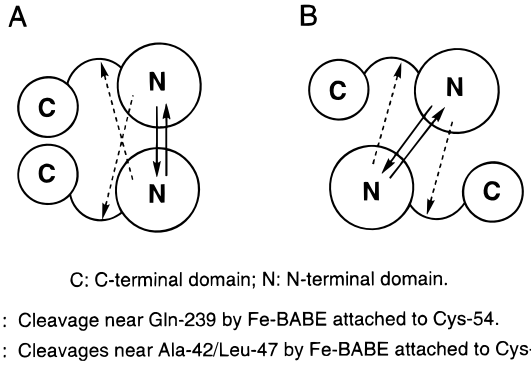

FIGURE 6: Intersubunit cleavages of the α_2 dimer. The α_2 dimer may have either (A) a parallel dimerization domain, similar to catabolite gene activation protein (CAP or CRP) (*39*), or (B) an antiparallel dimerization domain, similar to *trp* repressor (*40*).

plausible when compared to the NMR structure of the α CTD (residues 249−326), which also contains Cys-269 (*17*). In the α CTD, the distances from Cys-269 sulfur to the backbones of Arg-265, Cys-269, and Asn-294 are 3−6 Å (Figure 5), which are within the reach of the Fe−BABE reagent. This is in good agreement with the study by Rana and Meares (*36*), in which the human carbonic anhydrase I peptide chain was cleaved hydrolytically at Leu-189 by Cys-212 modified with Fe−BABE.

On the basis of the NMR structure of α CTD, Glu-273 is also close enough to be cleaved by Cys-269. However, this was not observed. One explanation could be that charge repulsion between the negative side chain of Glu-273 and the negatively charged Fe−BABE residue forces Fe−BABE more toward positive Arg-265 and neutral Asn-294, both of which were cleaved. The limited number of products observed suggests cleavage by an activated metal complex rather than by the small, neutral, diffusible hydroxyl radical.

The α CTD is already known to form its own folded structure (*17*). The limited-proteolysis (*2*, *3*) and NMR analysis (*38*) of the intact α subunit showed that at least 13 residues between Asp-236 and Glu-248 constitute a flexible linker between the NTD and CTD. In our experiments, intersubunit cleavage by Fe−BABE attached to Cys-54 was observed at the N-terminal edge of this flexible linker region, near Gln-239. This shows close proximity between the NTD of one subunit and the N-terminal edge of the linker region of the other. Two additional intersubunit cleavages near Ala-42 and Leu-47 by Fe−BABE attached to Cys-176 indicate that the middle of the NTD of one subunit is close to the N-terminal segment of the other (Figure 6).

It will be interesting to determine any changes in the α cleavage pattern after assembly of the core enzyme. We are currently extending this work to map interactions of α with the other RNA polymerase subunits.

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SUPPORTING INFORMATION AVAILABLE

Table 1S, showing our error analysis for molecular weights of α fragments by SDS−PAGE, and Figures 1S−5S, showing various control experiments (7 pages). Ordering information is given on any current masthead page.

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