Mapping Protein−Protein Interactions with a Library of Tethered Cutting Reagents: The Binding Site of σ^{70} on *Escherichia coli* RNA Polymerase†

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Received December 22, 1998; Revised Manuscript Received February 16, 1999

ABSTRACT: Surface-exposed lysine amino groups and other reactive nucleophiles of the σ^{70} protein were conjugated with the cutting reagent iron (*S*)-1-[*p*-(bromoacetamido)benzyl]ethylenediaminetetraacetate (FeBABE) via 2-iminothiolane (2IT) with low efficiency. The result is a library of σ^{70} conjugates, with an average of 1−2 cutting reagents tethered to any of a variety of sites (lysine, cysteine, etc.) on the surface of the protein. Model calculations indicate that the conjugates in this library should be capable of cutting nearby sites on the backbone of almost any protein or nucleic acid to which σ^{70} binds. Since cutting occurs only when the protein is bound, the cleaved sites indicate proximity; since only proximal sites are cleaved, interpretation of the results is straightforward. We used this library to map the periphery of the binding site on the core enzyme ($\alpha_2\beta\beta'$) of *Escherichia coli* RNA polymerase. The β subunit was cut primarily within its conserved regions C, D, Rif I, and G; additional sites were also cut between A and B and near conserved regions E and H. The cut sites within the β' subunit were intensely clustered between residues 250−450, which include its conserved regions C and D, along with two additional cut sites in conserved regions A and G. No cut sites on the α subunit were observed. These results recapitulate and extend those obtained using FeBABE conjugates of seven strategically placed single-Cys σ^{70} mutants [Owens, J. T., Miyake, R., Murakami, K., Chmura, A. J., Fujita, N., Ishihama, A., and Meares, C. F. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 6021−6026]. This technique provides a straightforward, general approach to mapping protein interactions without mutagenesis.

Many questions in molecular biology involve the binding of a protein, such as a transcription factor, to a macromolecular assembly. It is important to identify and characterize the sites involved in these interactions. There are a variety of techniques that can be used to study the interactions of macromolecules, each providing a unique perspective.

X-ray crystallography has been used to determine the structure of a fragment of the *Escherichia coli* σ^{70} protein (*1*), and NMR has been used to structurally characterize the C-terminal and N-terminal domains of the α subunit of RNAP[1] (*2*, *3*). To date, these techniques are of limited value for studying an intact multisubunit complex such as the 450 kDa *E. coli* RNAP holoenzyme ($\alpha_2\beta\beta'\sigma^{70}$).

Genetic approaches used to study interactions between proteins include such diverse approaches as site-directed mutagenesis and the yeast two-hybrid system (*4*, *5*). Deletions and point mutations in the σ subunit have been shown to decrease binding to the RNAP core enzyme ($\alpha_2\beta\beta'$) (*6*−*8*). Amino acid substitutions in the β and β' subunits of RNAP affect the stability of the transcription complex (*9*). Techniques such as energy transfer, footprinting, and cross-linking are also useful in studying macromolecular contacts (*10*−*15*).

FeEDTA footprinting has been used to investigate protein−protein interactions by comparing the solvent-exposed sites of one protein in the presence and absence of another (*16*, *17*). FeEDTA footprinting, however, cannot distinguish between actual binding sites and sites blocked due to conformational changes. Moreover, unless the binding between the two proteins is strong, a footprint is difficult to detect.

Attaching a tethered cleavage reagent, such as FeBABE, has been used to identify intra- or intermolecular sites proximal to the reagent (*18*−*22*) without interference from conformational changes or weak binding. The FeBABE conjugates of seven σ^{70} mutants with strategically placed single cysteines have proven to be powerful tools for mapping the contact sites of σ^{70} on core RNA polymerase (*23*). The selection of each site for cysteine substitution was based on experimental data from the crystal structure of a σ^{70} fragment, as well as many mutation and deletion studies (see ref *1* and references therein). Without such a foundation, it would be difficult to choose effective locations to place a probe.

The basis of our new technique is to take advantage of the many reactive nucleophiles that are abundant on the surface of almost any protein, such as the ϵ-amino group of lysine. The concept is similar to one described by Hanai and Wang (*24*) in which surface-exposed lysine residues underwent a two-step modification as a way of protein footprinting.

† This work was supported by Research Grant GM25909 to C.F.M. from the National Institutes of Health.

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[1] Abbreviations: 2IT, 2-iminothiolane; BABE, (*S*)-1-[*p*-(bromoacetamido)benzyl]ethylenediaminetetraacetate; BSA, bovine serum albumin; CITC, (*S*)-1-[*p*-(isothiocyanato)benzyl]ethylenediaminetetraacetate; CNBr, cyanogen bromide; EDTA, ethylenediaminetetraacetate; FeCITC, iron chelate of CITC; FeBABE, iron chelate of BABE; GaBABE, gallium chelate of BABE; MOPS, 3-morpholinopropanesulfonate; NTCB, 2-nitro-5-(thiocyano)benzoic acid; RNAP, RNA polymerase.

10.1021/bi983016z CCC: $18.00
Published on Web 03/19/1999

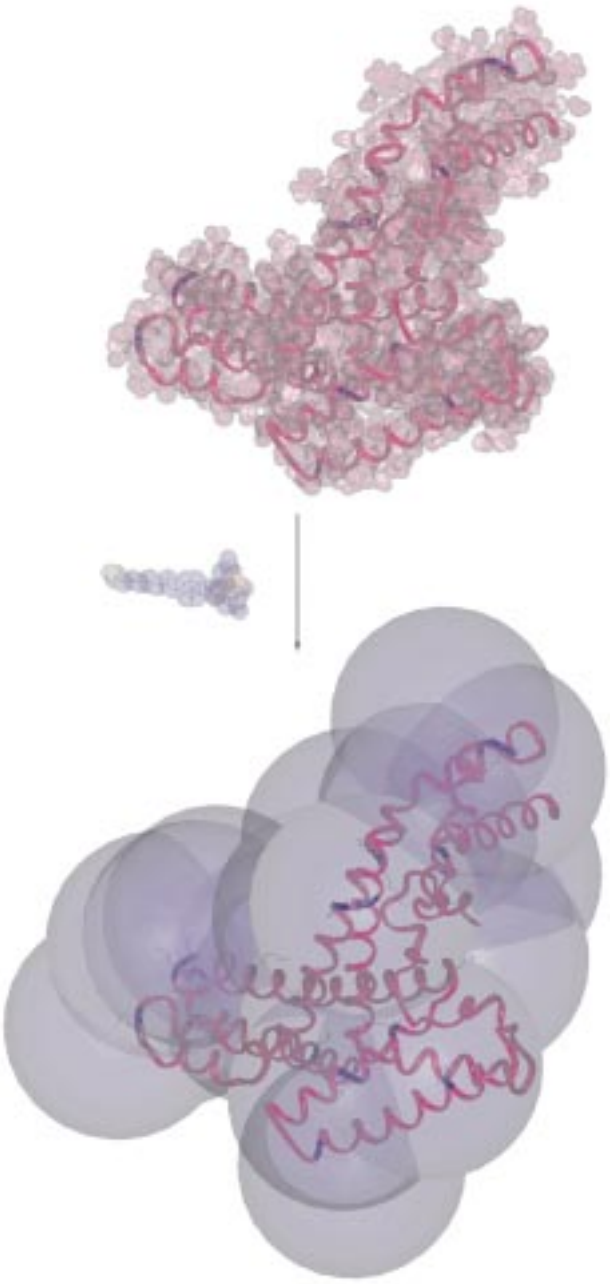

FIGURE 1: Structure of the σ^{70} fragment (*1*) shown as a space-filling model (top) and in ribbon form with 18 Å spheres centered on the lysine ϵ-amino groups (the length of the 2IT−FeBABE chelate is 18 Å) (bottom). This illustrates how the library of cutters is able to cover the surface of σ^{70}. 2IT−FeBABE is shown to scale (center) for comparison. The Fe atom is colored red and the ϵ N is in green.

Our strategy is to attach cutters at many possible sites with a low efficiency, so that a particular protein molecule is not likely to have more than one or two cutters. The result is a collection of molecules with a cutter at a different place on each molecule, yielding a library of tethered chelate conjugates. Reagents are chosen so that the reach of each cutter is sufficient to extend beyond the nearest neighboring lysine, providing almost complete surveillance of the protein surface. Figure 1 shows a space-filling model of σ^{70} demonstrating the ability of 2IT−FeBABE to cover the surface of the protein.

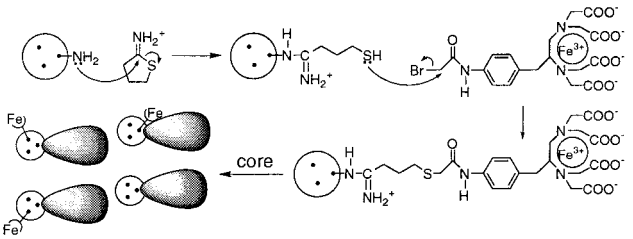

FIGURE 2: General scheme for creating and using a library of σ^{70} conjugates. Surface-exposed reactive sites on σ^{70} are depicted as black dots. σ^{70} is incubated with a 2-fold excess of FeBABE over 2IT. FeBABE is added first and in excess to prevent disulfide aggregates from forming. Attack by a Lys ϵ-amino group on a protein opens the five-membered ring of 2IT, yielding a thiol. Nucleophilic attack by the thiol on FeBABE yields the desired protein conjugate. Excess reagents are removed by gel filtration. When the library is incubated with core enzyme (shaded oval), some conjugates will have a chelate close enough to cleave at the periphery of the binding site. Other chelates will be farther away and will not generate any cleavage products, nor will *unbound* protein be cleaved.

The basic scheme for preparing and using a 2IT−FeBABE conjugate library is shown in Figure 2. The reagent 2IT (Traut's reagent) is commonly used to cross-link proteins to other molecules by way of lysine side chains. Studies with peptides show that 2IT is not very selective for labeling the N-terminus over the lysine side chain, even though there is a considerable difference in pK_a (*25*).

If we use a reagent such as 2IT that conjugates to a particular residue such as lysine, and if all such residues on the macromolecule are equally and independently reactive, we can describe the mixture of conjugation products using the binomial formula. We can define a statistical weight or *conversion ratio s* as the ratio of conjugated residues (Lys−hapten) to unconjugated residues (Lys):

$$\frac{\text{Lys}-\text{hapten}}{\text{Lys}} \equiv s \tag{1}$$

Clearly, *s* is a number that increases as the degree of conjugation increases. If our target contains only a single reactive residue, we need go no farther. But suppose each macromolecule of interest P contains *n* reactive residues. Note that there will be *n* possible species having one hapten h at one of the *n* sites but $n(n-1)/2$ possible species having two haptens distributed among *n* sites and so forth. This can be accounted for by the appropriate binomial coefficients. The ratio of singly conjugated to unconjugated P is

$$\frac{[\text{Ph}]}{[\text{P}]} = sn \tag{2}$$

because there are *n* sites to attach the hapten, all with conversion ratio *s*. The appropriate formula for the ratio of doubly conjugated to unconjugated P is

$$\frac{[\text{Ph}_2]}{[\text{P}]} = s^2 n(n-1)/2 \tag{3}$$

because there are $n - 1$ targets for the second hapten and we cannot distinguish the first from the second.

In general, for a macromolecule conjugated to i haptens

$$\frac{[\mathrm{Ph}_i]}{[\mathrm{P}]} = s^i \frac{n!}{i!(n-i)!} \tag{4}$$

If we wish to know the fraction f of the preparation that contains r haptens, the appropriate formula is

$$f = \frac{[\mathrm{Ph}_r]}{P_{\mathrm{tot}}} = \frac{\dfrac{[\mathrm{Ph}_r]}{[\mathrm{P}]}}{\displaystyle\sum_i \frac{[\mathrm{Ph}_i]}{[\mathrm{P}]}} = \frac{\dfrac{n!}{r!(n-r)!}s^r}{\displaystyle\sum_{i=0}^{n} \frac{n!}{i!(n-i)!}s^i} \tag{5}$$

Also, we can multiply by r and sum the numerator to find the average number ν of haptens per protein:

$$\nu = \frac{\displaystyle\sum_{r=0}^{n} \frac{n!}{r!(n-r)!}rs^r}{\displaystyle\sum_{i=0}^{n} \frac{n!}{i!(n-i)!}s^i} \tag{6}$$

If we consider σ^{70}, which has 34 lysines plus one N-terminal amino group, we may choose $n = 35$. If the average number of haptens on σ^{70} is $\nu = 1$, it turns out that the conversion ratio $s = 0.0294$. In that case the fraction of σ^{70} molecules containing exactly 0 haptens is 0.36; 1 hapten, 0.37; 2 haptens, 0.19; 3 haptens, 0.06; etc. (see examples in Supporting Information, Figure 1S).

In this paper, we describe a method for preparing a library of tethered chelate conjugates that, collectively, cover the surface of the protein and thus any potential points of contact. We also demonstrate the specific cleavage of a bound protein and its ability to map protein−protein interactions. The interaction of the σ^{70} protein with the core subunits of *E. coli* RNA polymerase has been mapped previously by protein footprinting *(17)* and single-Cys σ^{70}−FeBABE conjugates (*23*), forming the basis for a critical analysis of this new approach.

EXPERIMENTAL PROCEDURES

Materials. The σ^{70} subunit and *E. coli* RNA polymerase were purified and tested for transcriptional activity as described (*26*, *27*). The anti-chelate monoclonal antibody CHA255 was obtained from Dr. David Goodwin (*28*). FeBABE was prepared according to Greiner et al. (*29*). CITC was prepared according to Meares et al. (*30*). 2-Iminothiolane was purchased from Sigma. Ascorbic acid (vitamin C, microselect grade) was purchased from Fluka, and hydrogen peroxide (Ultrex grade) was purchased from J. T. Baker. Pure water (18 MΩ cm) was used throughout.

σ^{70}−2IT−FeBABE Conjugation. Conjugation conditions are essentially as in McCall et al. (*31*) with minor modifications. The σ^{70} protein was transferred from storage buffer into conjugation buffer (10 mM MOPS, pH 8, 100 mM NaCl, 0.1 mM EDTA, 5% glycerol) using a gel filtration spin column (*32*). FeBABE (23.4 mM) in DMSO was added to a final concentration of 1.4 mM, immediately followed by freshly prepared 2IT (final concentration 0.7 mM). The reaction was incubated at 37 °C for 1 h. Excess reagents were removed by gel filtration spin column equilibrated in 2× cleavage buffer (1×: 10 mM MOPS, pH 8, 120 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA). An equal volume of glycerol was added, and the σ^{70} conjugate library was stored at −70 °C. For control experiments, BSA was conjugated in the same manner. The concentration of the resulting conjugates was determined by a dye-binding assay (Bio-Rad).

σ^{70}−FeCITC Conjugation. CITC was loaded with iron prior to the protein conjugation reaction. The acidic aqueous solution containing 35 mM CITC was carefully adjusted to pH 4−5 with triethylamine. An equimolar amount of freshly prepared ferrous chloride was added to aqueous CITC, pH 4−5. The mixture was vortex mixed and allowed to sit at room temperature for 30 min. The FeCITC was analyzed by thin-layer chromatography (TLC) and determined to be 93% complexed with Fe by ^{57}Co competition (*33*). For FeCITC conjugation, σ^{70} was transferred into 50 mM tetramethylammonium phosphate, pH 10, 100 mM NaCl, 1 mM EDTA, and 5% glycerol, to a final σ^{70} concentration of 22 μM. FeCITC was added to a final concentration of 67 μM, and the reaction was allowed to incubate at room temperature for 1 h. The resulting conjugate was prepared for storage as above.

σ^{70}−2IT−GaBABE Conjugation. To measure loss of transcriptional activity due only to conjugation, without complications from oxidative damage by iron, a σ^{70}−2IT−GaBABE library was also prepared (gallium is the same size as iron but does not facilitate the oxidative chemistry that iron does). An aqueous solution, pH 4−5, containing 27 mM BABE was adjusted to approximately pH 1 with 1 M HCl. An equimolar amount of freshly prepared gallium nitrate was added to aqueous BABE, and the mixture was vortex mixed and then allowed to sit at room temperature for 30 min. The GaBABE was analyzed by thin-layer chromatography (TLC) and determined to be 96% complexed with Ga by ^{57}Co competition (*33*). The σ^{70}−2IT−GaBABE conjugate was prepared at the same time and in the same exact manner as σ^{70}−2IT−FeBABE.

Conjugation Yield. The conjugation yields of σ^{70}−2IT−FeBABE, σ^{70}−2IT−GaBABE, and σ^{70}−FeCITC were estimated by comparison to a set of standards. The σ^{70} conjugate libraries were separated by SDS−PAGE alongside five serially diluted standards of 496C−σ^{70}−FeBABE [91% conjugation yield determined by fluorometric assay (*29*) for thiols]. The σ^{70} conjugate libraries were loaded on the gel in triplicate to obtain a standard deviation. The western blot of this gel was developed using the anti-chelate antibody CHA255 (*28*), followed by rabbit anti-mouse IgG(H+L)−alkaline phosphatase, and a quantitative fluorescent substrate (Vistra ECF substrate, Amersham). The blots were imaged using a Fluor-S MultiImager with the Quantity One software package (Bio-Rad).

2IT−FeBABE Conjugation Distribution. The amounts of 2IT and FeBABE in the conjugation reaction were increased to obtain libraries with increasing conjugation yields below and above 1 chelate per σ^{70} molecule. During the conjugations, σ^{70} was held constant at 25 μM and FeBABE was always a 2-fold excess over 2IT. A conjugation reaction was performed at each of the following FeBABE concentrations: 25 μM, 50 μM, 100 μM, 200 μM, 400 μM, 800 μM, and 1.6 mM.

To demonstrate that the conjugation occurred at lysine side chains as well as at the amino terminus, the conjugate library was subjected to CNBr (*34*) and Lys-C (Promega) digests. The digests were separated by SDS−PAGE, electrophoretically blotted, and separately visualized with the anti-chelate antibody (CHA255) and with an antibody developed against the C-terminus of the σ^{70} subunit. The anti-chelate antibody will detect any fragment of σ^{70} which contains FeBABE. The antibody developed against the C-terminus does not detect fragments with the original N-terminus. The two stains were carefully compared to find bands common to both. Bands that test positive for both the C-terminus and the chelate confirm that sites other than the N-terminus are conjugated.

Activity Assay of Conjugate Library. The single round runoff transcription assay (*35*) was done in triplicate immediately after conjugation. Briefly, template DNA (185 bp *Bam*HI−*Hin*dIII fragment (*36*) of pLAC12] was incubated with reconstituted RNA polymerase (prepared in a 1 to 1 ratio of σ^{70} to core) for 15 min at 37 °C to allow open complex formation. A solution containing the NTPs and heparin was added to initiate RNA synthesis. After ethanol precipitation, labeled transcripts were separated by an 8% polyacrylamide−8 M urea gel, visualized by film autoradiography, and analyzed by densitometry.

Affinity Cleavage and Assignment of Cut Sites. A mixture of the core enzyme and the σ^{70}−2IT−FeBABE conjugate library in a 1:1 molar ratio was incubated in cleavage buffer for 30 min at room temperature to allow complex formation. The final concentration of glycerol in the cleavage reaction was kept at 10% to scavenge diffusible hydroxyl radicals. A 40 mM stock solution of ascorbate (titrated to approximately pH 7 with NaOH) was prepared in advance and stored in aliquots at −70 °C. A 40 mM stock solution of hydrogen peroxide was prepared just before use. Both of these 10× stock solutions also contain 10 mM EDTA to sequester adventitious transition metal impurities. After complex formation, ascorbate and hydrogen peroxide were added to final concentrations of 4 mM with vortex mixing after each addition. The reaction was allowed to proceed for 1 min, followed by the addition of 5× sample application buffer [62.5 mM Tris·HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.02% (w/v) bromophenol blue, 25 mM EDTA]. The sample was immediately frozen in liquid nitrogen and stored at −70 °C. The samples were separated by SDS−PAGE, electrophoretically blotted onto poly(vinylidene difluoride) (PVDF) membrane, and visualized with affinity-purified antibodies against the termini of the core enzyme subunits as described by Greiner et al. (*17*). The sizes of the fragments were determined by comparison to markers created by NTCB and CNBr digests of the core enzyme as described by Owens et al. (*23*).

Competition with Unconjugated σ^{70}. Unconjugated σ^{70} was used to compete with σ^{70}−2IT−FeBABE for binding to the core enzyme. Six samples were prepared by mixing 1 μM core with 1 μM σ^{70}−2IT−FeBABE and unconjugated σ^{70} in the following ratios of σ^{70} to σ^{70}−2IT−FeBABE: 1:4, 1:2, 1:1, 2:1, and 4:1. The samples were incubated for 15 min, then treated with ascorbate and hydrogen peroxide for affinity cleavage, and analyzed as described above.

RESULTS

2IT−FeBABE Library. Changing any of the following can vary the conjugation yield of a 2IT−FeBABE library: the pH of the conjugation buffer, the conjugation reaction time, or the concentrations of 2IT and FeBABE. A titration of increasing amounts of 2IT and FeBABE was used to demonstrate the minimal conjugation yield needed to produce clearly visible product bands (Supporting Information, Figure 2SA). As the conjugation yield increases, the intensity of the cleavage products increases, but no new products are seen. For 2IT−FeBABE, 2IT−GaBABE, and FeCITC labeling a conjugation yield, ν, of approximately 1−2 chelates per protein molecule was used for subsequent experiments.

It is important to demonstrate that the reach of the 2IT−FeBABE cutter is sufficient to cover the periphery of σ^{70}. For comparison, FeCITC was attached directly to primary amines via the isothiocyanate group, without a 2IT linker; the result is a cutter with a shorter reach than 2IT−FeBABE. Cleavage with σ^{70}−FeCITC is identical to that obtained from σ^{70}−2IT−FeBABE, indicating the reach of the cutter is long enough to cover all nearby sites on the core enzyme (Supporting Information, Figure 3S).

Chemical and proteolytic digests of the σ^{70}−2IT−FeBABE library showed bands that tested positive for both the C-terminus and the chelate, confirming that sites other than the N-terminus were conjugated under these conditions (data not shown). A σ^{70}−2IT−FeBABE library (and a σ^{70}−FeCITC library) prepared at pH 10 rather than pH 8 gives the same cleavage pattern as seen in Figure 3 (except that the intensities of the product bands increase due to an increased conjugation yield).

The σ^{70}−2IT−GaBABE and σ^{70}−2IT−FeBABE conjugate libraries prepared for the transcription assay were determined to have conjugation yields of 1.9 ± 0.1 and 1.6 ± 0.2 chelates per σ^{70} by anti-chelate antibody staining and comparison to standards (Supporting Information, Figure 4S). The σ^{70}−2IT−GaBABE tested for the loss of activity due to the physical presence of the chelate; the σ^{70}−2IT−FeBABE tested for additional loss due to oxidative damage caused by iron. The transcriptional activity for a 1:1 molar ratio of unconjugated σ^{70} with core RNA polymerase was assigned to 100% activity. Only a single band representing the transcript was detected (Supporting Information, Figure 5S). σ^{70}−2IT−GaBABE, determined to have 1.9 ± 0.1 chelates per σ^{70}, was 62 ± 4% active, demonstrating a modest loss of activity due to conjugation of the chelate. The activity of σ^{70}−2IT−FeBABE, determined to have 1.6 ± 0.2 chelates per σ^{70}, was 20 ± 3%, demonstrating that oxidative damage by iron can further affect activity.

2IT−FeBABE Cleavage. The cleavage pattern of σ^{70}−2IT−FeBABE is shown in Figure 3, alongside single cysteine mutant 396C−σ^{70}−FeBABE and the FeEDTA footprint (*17*). Control experiments to demonstrate the chemistry for cleaving the core included treating the following with ascorbate and peroxide: (1) core alone, (2) core with unconjugated σ^{70}, (3) core with a library of BSA conjugates, and (4) core with a library of σ^{70} conjugates. Only experiment 4 (lane 4 of Figure 3) showed the cleavage pattern. Experiment 3 (lane 3 of Figure 3) demonstrates that the cleavage pattern is only generated when the cutting protein binds to the core and not through random interactions. FeEDTA footprinting reactions

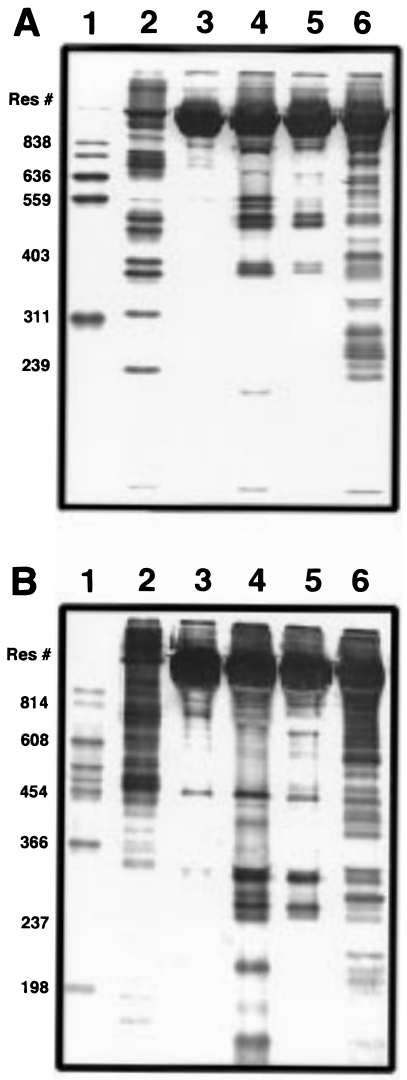

FIGURE 3: Immunostained western blots of 10% SDS−PAGE detecting either (A) β or (B) β' using antibodies against the N-termini of the respective subunit. No cleavage was observed for the α subunit (data not shown). Panels A and B: lane 1, NTCB chemical digest; lane 2, CNBr chemical digest; lane 3, BSA−2IT−FeBABE + core enzyme (unbound control); lane 4, σ^{70}−2IT−FeBABE + core enzyme; lane 5, 396C−σ^{70}−FeBABE + core enzyme; lane 6, untethered FeEDTA cleavage of the core enzyme. The residue numbers (cut-site locations) for some of the NTCB and CNBr marker fragments are indicated on the left.

show the pattern of a nonspecific, unbound cutter. A competition experiment in which native σ^{70} was added to the σ^{70}−2IT−FeBABE holoenzyme resulted in diminished cleavage yield (Supporting Information, Figure 2SB). When BSA was added in the same manner, there was no effect on the cleavage yield (data not shown).

DISCUSSION

2IT−FeBABE Library. Thirty-four of the 613 amino acid residues in σ^{70} are lysine. The side chain ϵ-amino groups of lysine residues tend to have similar chemical reactivity, presumably because of their occurrence on the surface of proteins. Lysine side chains are widely distributed on protein surfaces. From the crystal structure of the σ^{70} fragment, the average distance from a lysine ϵ-amino group to its nearest neighbor lysine ϵ-amino group is 12 ± 4 Å. A very similar result is found for the Fab fragment of antibody CHA255 (*37*), suggesting this nearest neighbor distribution is likely to be similar for most proteins.

If we conjugate 2IT−FeBABE to lysine side chains that are distributed on average 12 Å apart, the 12 Å reach of FeBABE plus the 6 Å extension due to 2IT means that, on average, the cutter will reach past the next nearest lysine side chain (Figure 1). Thus, a collection of macromolecules bearing a library of lysine-tethered cutters will be likely to have sufficient members to cut almost any site on a target macromolecule. For cutting caused by diffusible species such as hydroxyl radicals, the reach of each tethered reagent will be even greater. In our experiments, the distance a diffusible hydroxyl radical can travel was limited by the presence of 10% glycerol, a hydroxyl radical scavenger. The flexibility of the lysine side chain itself also allows for a longer reach. The similar results we observed for FeCITC (11 Å length) and 2IT−FeBABE (18 Å length) demonstrate that the reach is far enough in practice to cover all sites of interest.

Our experimental data suggest a random distribution of chelates, rather than conjugation at one reactive site. As the conjugation yield increases, no new cleavage products are seen, and an increase in conjugation leads to a proportional increase in the intensity of the cleavage products. If the conjugation had occurred preferentially at the amino terminus, for example, one would expect that libraries with conjugation yields greater than 1 chelate per σ^{70} would result in additional cut sites due to conjugated lysines. This is not the case, since a library with a conjugation yield of 5 shows the same (although more intense) cut sites as one with 0.6 chelates per σ^{70} (Supporting Information, Figure 2SA). We infer that the same set of reactive groups on the surface of σ^{70} is conjugated. When the pH of the conjugation reaction is increased to favor further deprotonation of lysine ϵ-amino groups, the cleavage pattern does not change. In addition, digests of the conjugates visualized with a C-terminal antibody and an anti-chelate antibody qualitatively show that conjugation not only was occurring at the N-terminus (pK_a ∼7.5) but was distributed among lysine side chains (pK_a ∼ 9.5) as well.

In Figure 3, we see that there is a large amount of uncut protein remaining after the cleavage reaction. By comparing the band intensities, we determined that approximately 10% of the RNAP molecules are being cleaved while 90% remain uncut. Based on the binomial distribution (eq 5), when 90% of all the molecules remain uncut, 9.5% of the molecules have been cut once and only 0.5% of all the molecules have been cut more than once. Thus, we can conclude that single-cut conditions prevail. The same mathematics described here can be applied to DNA footprinting (*38*).

2IT−FeBABE Conjugation Yield and Cleavage Specificity. A titration of increasing amounts of 2IT and FeBABE was used to determine optimal conjugation reaction conditions. The conjugation should be high enough for easy detection of all possible cuts, but low enough to maintain biological activity. In our studies, a conjugation yield of approximately 1−2 chelates per protein molecule gave clearly visible cleavage products and maintained 62% biological activity in the absence of iron. If some molecules can no longer bind after conjugation, they should not interfere with the experiment because the conjugate must bind in order to cleave. BSA was chosen as a control to demonstrate specificity because it is approximately the same size as σ^{70} but does

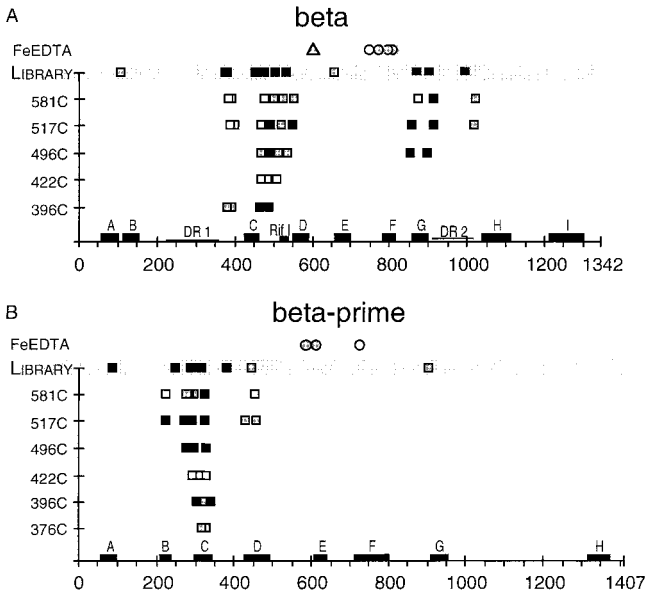

FIGURE 4: Summary of 2IT−FeBABE cleavage data in Figure 3. The sequence and conserved regions of β and β' are indicated along the horizontal axes. Library refers to the σ^{70}−2IT−FeBABE library. 376C through 581C represent cleavage patterns of FeBABE conjugates of the single-Cys mutants described by Owens et al. (*23*). FeEDTA is a summary of the results reported by Greiner et al. (*17*), which depicts only the few cut sites which are involved in the footprint rather than the entire cleavage patterns (example shown in lane 6 of Figure 3). Sites that are no longer sensitive to untethered footprinting reagents in the presence of σ^{70} are designated as circles. The triangle indicates a new cut site near residue 604 on β, which represents a conformational change. Commercial molecular weight standards were used in the assignment of FeEDTA footprinting results; these assignments are less accurate than the tethered chelate mapping results which were assigned using CNBr and NTCB fragments of the actual subunits. The shading of the symbols corresponds to the intensity of the fragment band: the darker the shading, the higher the cutting yield.

not bind core RNA polymerase. A BSA−2IT−FeBABE library with the same conjugation yield did not cleave the core enzyme, indicating that the cleavage products of the σ^{70}−2IT−FeBABE library are produced only after binding. The addition of unconjugated σ^{70}, which can compete for the core binding site, diminished the σ^{70}−2IT−FeBABE cleavage intensity of the core enzyme. In contrast, adding BSA had no effect on the σ^{70}−2IT−FeBABE cleavage intensity of the core enzyme, demonstrating that the presence of a nonbinding protein does not change the intensity of the cutting.

β and β' Subunit Contact Sites. Specific criteria were set for a critical comparison of these data to those of Owens et al. (*23*). All cut sites reported must be reproducible and clearly visible. Cut sites reported here were indicated by the presence of a band in the cut lane that was not in the uncut lane, rather than by intensity differences. The intensities of the cut site bands were carefully compared for consistency. For accuracy, all cut sites were assigned on the basis of the migration of known fragments produced from chemical digests of the same protein (rather than commercial standards).

The σ^{70}−2IT−FeBABE cleavage pattern essentially summarizes that obtained from seven single-Cys mutant σ^{70}−FeBABE conjugates (Figure 4). Both techniques show regions of β subunit contact clustered within the rifampicin-binding cluster I and near β conserved regions C, D, G, and H. On the β' subunit, contact sites are clustered in and around β' conserved regions B, C, and D. In addition, σ^{70}−2IT−FeBABE cleavage reveals some contact sites not observed previously (*23*). Conserved regions A, B, and E on β and conserved regions A and G, as well as a site between regions C and D, on β' were also found to be close to σ^{70}. These observations, that the σ^{70}−2IT−FeBABE library not only reproduces all the cut sites previously observed with seven different single-Cys mutants but also reveals more sites, are consistent with the expectation that 2IT−FeBABE conjugation occurs at a large number of sites on σ^{70}.

Comparison to FeEDTA Footprinting. Protein footprinting with FeEDTA cuts surface-exposed residues, generating a large number of fragments. However, only a few of those cuts will be involved in the footprint. For example, only 4 cut sites out of 39 on the β subunit are affected by the binding of σ^{70}. In contrast, the library approach generates relatively few products: 10 cut sites in the β subunit, all due to proximity. In practice, the differences in the cleavage patterns of unbound FeEDTA on the core enzyme and holoenzyme frequently involve changes in intensity, rather than the complete disappearance of a fragment. These subtle differences are often difficult to detect without a densitometer. The library cutting results are easy to identify, since all fragments are absent in the control experiments.

There are differences between protein footprinting with untethered FeEDTA and both types of tethered FeBABE experiments. Sites identified on β and β' by FeEDTA protein footprinting are different from those cut by tethered FeBABE (Figure 4). Interpretation of the FeEDTA footprint requires comparing the cleavage pattern of the surface-exposed residues on the RNA polymerase $\alpha_2\beta\beta'$ core enzyme to the $\alpha_2\beta\beta'\sigma^{70}$ holoenzyme. Presumably, σ^{70} will sterically protect its binding surface from the cleavage reagents, creating a "footprint" in the cleavage pattern. However, FeEDTA footprinting can also detect conformational changes (*11*, *17*). A clear example is a new site on β which becomes sensitive to FeEDTA after σ^{70} binds. When a protein undergoes a conformational change, some sites might also become protected, creating a false footprint.

It is important to note that FeEDTA footprinting should elucidate regions that are within the interface between σ^{70} and the core enzyme, whereas cutting with tethered chelates—either by the σ^{70}−2IT−FeBABE library or by single-Cys FeBABE conjugates—should occur at the periphery of the binding surface. Thus, the experiments with untethered FeEDTA should provide information complementary to that obtained with tethered cutters. One common result among all these experiments is that none identify any sites on α that are close to σ^{70}.

The potential strength of the library approach is in mapping the overall binding surface in a straightforward way. Currently, we are using this to determine regions of σ^{70} that are at the periphery of the binding surface with the core. We envision that the 2IT−FeBABE library can be easily extended to other systems for mapping protein−protein interactions.

ACKNOWLEDGMENT

We thank Dr. Akira Ishihama for the σ^{70} overexpression plasmid, Reiko Miyake and Dr. Jeffrey Owens for the single

cysteine σ^{70}−FeBABE conjugates, Dr. Sydney Kustu for helpful discussions, and Janice Wu for technical assistance.

SUPPORTING INFORMATION AVAILABLE

Five figures showing the distribution of conjugated σ^{70} species at various conjugation yields, titration of 2IT and FeBABE during the σ^{70} conjugation reaction and competition of σ^{70}−2IT−FeBABE with wild-type σ^{70}, a comparison of σ^{70}−2IT−FeBABE and σ^{70}−FeCITC cleavage of the core, quantification of chelates of σ^{70}−2IT−GaBABE and σ^{70}−2IT−FeBABE used for the transcription assay, and separation of the transcript by 8% polyacrylamide−8 M urea gel electrophoresis. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI983016Z