Use of Aryl Azide Cross-Linkers To Investigate Protein–Protein Interactions: An Optimization of Important Conditions as Applied to *Escherichia coli* RNA Polymerase and Localization of a σ^{70}–α Cross-Link to the C-Terminal Region of α†

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ABSTRACT: In an effort to better understand protein–protein photoaffinity cross-linking using aryl azides, we have tested a number of factors influencing the cross-linking of the σ^{70} subunit of *Escherichia coli* RNA polymerase to core RNA polymerase. These factors include the effect of the incubations necessary for the derivatization of the protein on enzyme activity, the effect of overhead lighting on azide stability, the effect of reducing agents on azide stability, aggregation of the derivatized protein, and a comparison of two types of aryl azide cross-linkers, *N*-[(5-azido-2-nitrobenzoyl)oxy]succinimide (ANB-NOS) and (*N*-hydroxysuccinimidyl)-4-azidosalicylic acid (NHS-ASA). We found that derivatization proceeds effectively in a buffer similar to the buffer used during protein purification, that overderivatization can cause protein aggregation, that room lighting does not appreciably destroy aryl azides, and that 0.1 mM DTT is a better choice of reducing agent than 5 mM 2-mercaptoethanol. The cross-link products were separated by SDS gel electrophoresis and identified on Western blots by cross-reactivity with monoclonal antibodies to the individual subunits of RNA polymerase. In agreement with previous work (Coggins *et al.*, 1977; Hillel & Wu, 1977), it was possible to cross-link σ^{70} to all three of the subunits of RNA polymerase. With a combination of gel analysis, chemical cleavage, and immunodetection, it is possible to demonstrate that σ^{70} cross-links to the α subunit between residues 209 and 329.

A practical technique for the study of multisubunit enzymes has been the use of chemical cross-linkers. With these compounds, it has been possible to determine the subunits that are adjacent to each other [see Coggins *et al.* (1977) and Hillel and Wu (1977) for examples]. For all the knowledge gained by these methods, they suffer from two shortcomings. The first is the limited reactivity of the functional groups of the cross-linkers. Because they often react with only one amino acid side chain, it is possible to miss generating a cross-link between two adjacent subunits if the distance between reactive side chains is not less than or equal to that of the length of the cross-linker. The second disadvantage is the complexity of the cross-link products generated. Both functional groups react simultaneously, generating all possible cross-link pairs and even more complicated products. Although this is fine when studying the enzyme as a whole, it adds unnecessary complications when studying the interactions of a specific subunit.

Since these initial RNA polymerase cross-linking studies, heterobifunctional photoaffinity cross-linkers in general, and aryl azides specifically, have simplified the study of protein–protein interactions in multisubunit proteins [see Fleet *et al.* (1969) and Lewis *et al.* (1977) for some examples]. Using these reagents, it is now possible to activate the functional groups of the cross-linker in a stepwise manner instead of simultaneously. A purified subunit can be reacted with just one of the functional groups, producing a protein that has been derivatized with a photoactive cross-linker. This derivatized protein can be incubated with the other subunits and then the second functional group of the cross-linker photoactivated. This limits the cross-link products to only those that have the derivatized protein as a component. Such a method was used to determine that the ω protein interacts primarily with the β' subunit and to a lesser extent with the α subunit of *Escherichia coli* RNA polymerase (Gentry & Burgess, 1993).

E. coli RNA polymerase is the enzyme that is responsible for RNA synthesis. The catalytic function can be reconstituted *in vitro* from three purified polypeptide chains to form a core enzyme with the subunit structure $\alpha_2\beta\beta'$ (Burgess, 1969; Heil & Zillig, 1970). Promoter specificity is conferred by a fourth polypeptide belonging to a class of proteins called σ factors. The main σ factor is designated σ^{70} because its molecular mass is 70 kDa (Burton *et al.*, 1981; Gross *et al.*, 1992).

In the past, cross-linking agents have been used in an attempt to learn more about the organization of the subunits in this enzyme. Using the periodate-cleavable diimido ester, *N*,*N*′-bis(2-carboximidoethyl)tartaramide dimethyl ester, it was shown that the binary cross-link products σ^{70}–β,[1] σ^{70}–β', and β–β' could be formed (Coggins *et al.*, 1977). Using cross-

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[1] Abbreviations: ANB-NOS, *N*-[(5-azido-2-nitrobenzoyl)oxy]succinimide; NHS-ASA, (*N*-hydroxysuccinimidyl)-4-azidosalicylic acid; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline (137 mM NaCl, 2.68 mM KCl, 8.06 mM Na_2HPO_4, and 1.47 mM KH_2PO_4); Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin; ANB-Tris, *N*-(5-azido-2-nitrobenzoyl)aminotris(hydroxymethyl)methane; σ^{70}-ANB, σ^{70} derivatized with ANB-NOS; σ^{70}–α, σ^{70} cross-linked to the α subunit; σ^{70}–β, σ^{70} cross-linked to the β subunit; σ^{70}–β', σ^{70} cross-linked to the β' subunit; HSAB, *N*-hydroxysuccinimidyl-4-azidobenzoate; mAb, monoclonal antibody; 2-ME, 2-mercaptoethanol.

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linking compounds with specificity toward other amino acid side chains, it was later shown to be possible to generate the remaining binary cross-link products, σ^{70}–α, α–β, α–β', and α–α (Hillel & Wu, 1977).

We feel these experiments make *E. coli* RNA polymerase a good enzyme to use to better understand the aryl azide cross-linkers that have become increasingly popular for studying protein–protein interactions. We have used *N*-[(5-azido-2-nitrobenzoyl)oxy]succinimide (ANB-NOS) to modify the primary amines of σ^{70}. Activation of the azido group with UV light was then used to covalently bind σ^{70} to core RNA polymerase. The resultant complexes were separated by SDS–PAGE and transferred to nitrocellulose in the preparation for identification by monoclonal antibodies.

Using this system, we were able to find a number of important factors that influence the efficiency of cross-linking using aryl azide cross-linkers attached at the lysine residues of proteins. We feel it will be helpful for those working with a similar system to be aware of these factors. We were also able to subject the resultant σ^{70}–α cross-link to hydroxylamine cleavage. This method cleaves proteins between asparagine–glycine pairs. There are no such sites on σ^{70}, and α has only one at residues 208 and 209. In this way, it was possible to determine that σ^{70} cross-links to the C-terminal region of α.

EXPERIMENTAL PROCEDURES

Chemical Reagents. All reagents used were purchased from commercial sources. Cross-linking reagents ANB-NOS, which places a 5-azido-2-nitrobenzoyl group on primary amines, NHS-ASA, which places a 4-azido-2-hydroxybenzoyl group on primary amines, and HSAB, which puts a 4-azidobenzoyl group on primary amines, were purchased from Pierce.

Protein Purification. Core RNA polymerase was purified from an overproducing strain (Bedwell & Nomura, 1986) according to the procedure of Burgess and Jendrisak (1975). σ^{70} subunit was purified according to the method of Gribskov and Burgess (1983).

Immunological Reagents. σ^{70} monoclonal antibody (mAb) 1H6 has been described previously (Strickland *et al.*, 1988), as have β mAb NT63 and β' mAb NT73 (Thompson *et al.*, 1992). Antibodies 5RA1 and 3RA1 were prepared following essentially the same method that was used for the other core antibodies. Both 5RA1 and 3RA1 were determined to interact with α on immunoblots of core RNA polymerase.

Spectral Analysis. When ANB-NOS is incubated in water, it hydrolyzes at the carbonyl carbon, causing a change in the spectrum of the compound. To avoid confusion between this reaction and photolysis of the azide, ANB-NOS was reacted with Tris·HCl prior to spectrophotometric analysis. This was done by adding 1.3 mg/mL ANB-NOS in DMSO to a Tris·HCl solution, such that the final concentrations were 0.2 M Tris·HCl (pH 7.9), 1.3% DMSO, and 5.5×10^{-5} M ANB-NOS in a 1.0 mL solution. After 30 min at room temperature in the dark, the spectrum of the ANB-Tris sample was measured using a Shimadzu UV160U spectrophotometer. This sample was blanked against 0.2 M Tris·HCl, pH 7.9. Exposure to overhead fluorescent light (approximately 2.3 m above the bench) was accomplished by leaving the cap open on the microfuge tube and letting this sit on the benchtop for 15 min. Exposure to 366 nm UV light was achieved by resting a Model UVGL-25 Mineralight lamp on the top of the microfuge tube for the time specified in the figure legend.

One-Dimensional Polyacrylamide Gel Electrophoresis and Immunoaffinity Assays. One-dimensional slab SDS–PAGE was performed using the method of Laemmli (1970) for 6% polyacrylamide gels. Since boiling σ^{70} in SDS sample buffer can result in proteolysis (Strickland *et al.*, 1988), samples were heated for 10 min in a 90 °C water bath. Transfer to nitrocellulose from polyacrylamide gels was performed using a Bio-Rad Trans-Blot SD semidry electrophoretic transfer cell following the manufacturer's procedure. After transfer, the blots were incubated for at least an hour in 1% nonfat dry milk in PBS, with 0.02% sodium azide (Johnson *et al.*, 1984) as a blocking agent. Primary antibody was diluted into a fresh sample of milk solution at the ratio specified in the figure legend, and the nitrocellulose blot was incubated in this solution for an hour. Excess antibody was then washed off with TBST using four washes of 5 min each. After this step, horseradish peroxidase-conjugated secondary antibody was used as a probe using a 1:1000 dilution into a 1% milk solution. After an hour, four washes of 5 min each in TBST were used to remove excess secondary antibody. The blot was then probed using the enhanced chemiluminescence (ECL) detection system (Amersham) according to the manufacturer's protocol. Finally, the blot was used to expose a piece of X-ray film for the duration necessary to give a clear signal. This time typically ranged from 15 s to 1 min.

Derivatization of σ^{70} with ANB-NOS. Derivatization occurred in a 50 μL solution whose final concentration was PBS, 5% glycerol, 0.1 mM EDTA, 0.1 mM DTT, and 0.01% Triton X-100. To this was added 11 μg of purified σ^{70}, followed by 5.2 μg of ANB-NOS in DMSO. The final concentration of DMSO was 8%. These values translate into 110 mol of ANB-NOS/mol of σ^{70} and 3 mol of ANB-NOS/mol of primary amine. This solution was allowed to incubate at room temperature for 1 h. At this point, 10 μL of a solution consisting of 1.14 M Tris·HCl (pH 7.9), 5% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.01% Triton X-100, and 0.15 M NaCl was added to quench any unreacted ANB-NOS. Following another 1 h incubation, 90 μL of a solution containing 80% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.01% Triton X-100, and 0.15 M NaCl was added. This sample was then frozen at −20 °C until use for cross-linking to core RNA polymerase. This brought the glycerol concentration up to 50% and diluted the σ^{70} to a final concentration of 73 μg/mL.

Transcription Assay of σ^{70} Activity. Transcription reactions were performed in a solution consisting of 40 mM Tris·HCl (pH 7.9), 10 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, 0.1 mM DTT, 0.01% Triton X-100, 0.1 mg/mL BSA, 10 μg/mL core RNA polymerase, 0.2 mM ATP, 0.2 mM CTP, 0.2 mM GTP, 50 μM UTP, 1 nCi of [^{3}H]UTP, and 20 μg/mL T7 phage DNA in a total volume of 100 μL. The σ^{70} concentration was varied from 0.07 to 2.2 μg/mL. Transcription of the T7 template took place at 37 °C for 30 min before being stopped with the addition of 25 μL of 0.2 M EDTA. The incorporation of the label into an acid-insoluble form was determined as previously reported (Strickland *et al.*, 1988).

Cross-Linking of σ^{70} to Core RNA Polymerase. Cross-linking was performed in the same buffer as the transcription assay without the addition of DNA or nucleotides. The final concentration of core RNA polymerase was 57.5 μg/mL, and the final concentration of derivatized σ^{70} was 5.86 μg/mL. This solution was incubated for 30 min at room temperature. The azido group was then activated by a 5 min exposure to 366 nm UV light, using the same method as that used for the ANB-Tris compound. The light was approximately 3.4 cm above the meniscus of the cross-link solution. The sample was then prepared for electrophoresis by the addition of SDS

sample buffer and loaded immediately onto the gel or stored at −20 °C.

Two-Dimensional Polyacrylamide Gel Electrophoresis and Immunoaffinity Assays. Samples were prepared in the same way as for one-dimensional SDS–PAGE and separated on 3 mm diameter tube gels using the Laemmli (1970) buffer system and 7.5% acrylamide. The gels were then extruded and incubated for 1.5 h at 45 °C in a solution of 2 M hydroxylamine hydrochloride, 0.37% ethanolamine, and 0.1% SDS. The pH of this solution had been adjusted to 9.0 at 45 °C with LiOH. This system is similar to that of a previously published protocol (Saris *et al.*, 1983). However, it used guanidine hydrochloride as a denaturant. We found that proteolysis occurs just as efficiently using SDS as a denaturant. This change avoids the problem of having to elute the SDS before exposure to the guanidine solution, followed by removal of the guanidine before reintroduction of SDS for the second-dimension run. After the 1.5 h incubation, the tube gels were removed from the hydroxylamine solution and placed into tube gel storage buffer consisting of 60 mM Tris·HCl (pH 6.8), 2.3% SDS, 10% glycerol, and 5% 2-ME. The gels were then stored at −70 °C until needed (Duncan & Hersey, 1984). The second-dimension gel was a 3 mm thick slab gel using the same conditions as the first-dimension gel. The tube gels were sealed into a well using molten 1% agarose dissolved in a solution of tube gel storage buffer (Duncan & Hersey, 1984). Two samples to be used as markers were run in parallel. After the bromphenol blue was run to the bottom of the gel, the gel was incubated for 1 h in Towbin buffer (Towbin *et al.*, 1979) plus 0.05% SDS and then transferred to nitrocellulose at 300 mA for 8 h. After this, the blot was probed using 3RA1, 5RA1, or 1H6. 3RA1 is an anti-α mAb whose epitope maps to the 209–329 fragment (data not shown) produced by proteolysis with hydroxylamine. The concentration used was a 1:500 dilution. 5RA1 is an anti-α mAb whose epitope maps to the 1–208 fragment (data not shown). It too was used at a 1:500 dilution. 1H6, the anti-σ^{70} mAb described earlier, was used at a 1:2500 dilution. Because sodium azide inhibits horseradish peroxidase, the secondary antibody was used at a 1:250 dilution. Exposure times for the various immunoblots are stated in the figure legends.

RESULTS

Effect of Room Lighting on Azide Stability. The spectrum of ANB-Tris shows a characteristic local maximum at approximately 320 nm (Figure 1). This is due to the 5-azido-2-nitrobenzoyl moiety. This peak can be used to measure the stability of the azido group under a variety of conditions. To determine the stability of the azido group under overhead lighting, a sample of ANB-Tris was prepared as described. This solution was allowed to sit under the lights in a microfuge tube with the lid open to allow the light from above to reach the sample. After 15 min, its spectrum was compared to the initial spectrum (Figure 1). From these spectra, it can be seen that exposure to overhead lighting for brief periods of time does not have a significant effect on the azido group. Exposure of this sample to 366 nm UV light for 5 min results in complete photolysis and a change in the absorbance at 320 nm from 9.7×10^3 to 3.8×10^3 absorbance units/mol of ANB-NOS. Thus, complete photolysis results in a change equal to 5.9×10^3 absorbance units/mol of ANB-NOS (Figure 1).

Activity of σ^{70} Following a Mock Derivatization. To ensure that the incubations required for the derivatization of σ^{70} did not decrease the activity of the protein, a mock derivatization was performed in which pure DMSO was added in place of ANB-NOS in DMSO. All other steps were as stated earlier. The ability of this protein sample to activate transcription by core RNA polymerase was compared to that of an equal amount of σ^{70} from storage buffer. There did not appear to be a significant drop in the level of tritium incorporation at any of the concentrations of σ^{70} tested (data not shown).

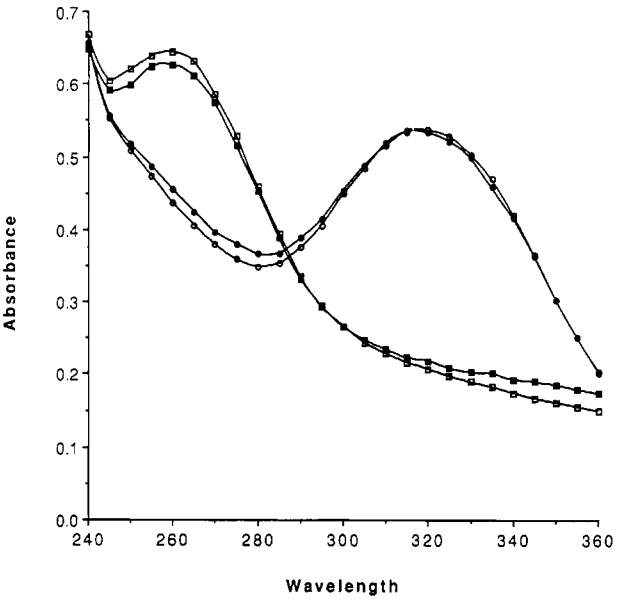

FIGURE 1: Effect of room light and UV light on azide stability. Tris-ANB (5.5×10^{-5} M) was synthesized as described in the Experimental Procedures. The spectrum of this compound was measured (○). It exhibited a maximum at 320 nm equal to 0.54 absorbance unit. This solution was then exposed for 15 min to the overhead light present in the room and the spectrum was remeasured (●). The absorbance at 320 nm was 0.53 absorbance unit. After this, the solution was exposed to 366 nm UV light for 5 min (□) and the spectrum remeasured. The absorbance at 320 nm had dropped to 0.21 absorbance unit. Finally, the solution was exposed to UV light for another 5 min and its spectrum determined (■). The reading at 320 nm in this case was 0.22 absorbance unit.

Activity of σ^{70} Following Derivatization. Activation of specific transcription of T7 DNA by σ^{70}-ANB was also determined. At the level of derivatization used for the cross-linking experiments, a 2-fold drop in activity can be seen (data not shown). Since the mock derivatization procedure did not result in a loss of σ^{70} activity, this must be due to the actual derivatization of the lysyl side chain and not to some side effect of the protocol used. This is in agreement with previous results (Narayanan & Krakow, 1982), where it was shown that derivatization of lysyl residues with trinitrobenzenesulfonic acid resulted in a loss of function for σ^{70}. Because it was also shown that σ^{70} thus modified still formed a holoenzyme (Narayanan & Krakow, 1982), we believe that the inactivation of σ^{70} by ANB-NOS occurs at some step after holoenzyme formation. Native gel analysis shows that, at the molar ratios used during cross-linking, the vast majority of the derivatized σ^{70} migrates as holoenzyme and not as free σ^{70} (data not shown).

Aggregation of σ^{70} Derivatized with ANB-NOS. When the amount of ANB-NOS added to a constant amount of σ^{70} is increased, a new band is observed following irradiation with UV light in the absence of core enzyme that exhibits a much slower migration rate on SDS–PAGE than σ^{70} but that cross-reacts with an anti-σ^{70} monoclonal antibody (Figure 2). This phenomenon is due to the placement of the 5-azido-2-nitrobenzoyl moiety on solvent-exposed primary amines. This modification is expected to increase the hydrophobicity of the

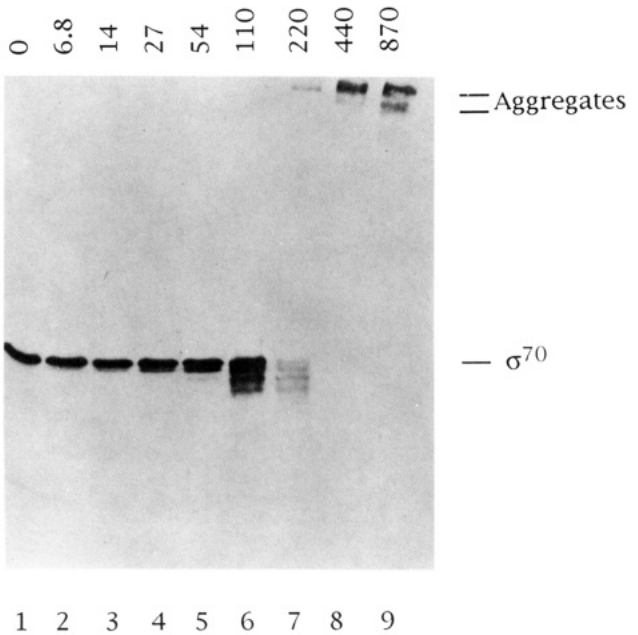

FIGURE 2: Titration of ANB-NOS when derivatizing σ^{70}. σ^{70}-ANB samples were prepared using various concentrations of ANB-NOS. These samples were then run on SDS gels and transferred to nitrocellulose, as described in the Experimental Procedures. The membrane was then probed using 1H6, an anti-σ^{70} mAb, at a 1:10 000 dilution. The exposure time for detection of the ECL signal was 1 min. The moles of ANB-NOS added per mole of σ^{70} were as follows: lane 1, 0; lane 2, 6.8; lane 3, 14; lane 4, 27; lane 5, 54; lane 6, 110; lane 7, 220; lane 8, 440; and lane 9, 870.

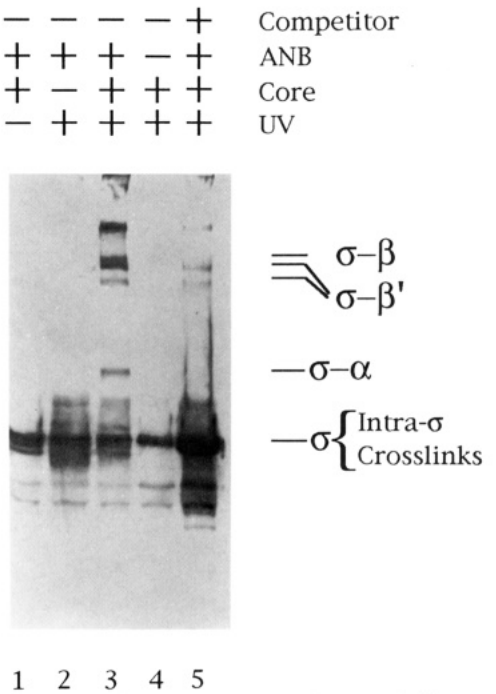

FIGURE 3: Detection of cross-link samples with an anti-σ^{70} monoclonal antibody. An immunoblot of the cross-linked samples was prepared and probed as described for Figure 2. Lane 1 contains a control in which the sample was not exposed to UV. Lane 2 contains a control to which no core enzyme was added. Lane 3 contains the cross-link sample. Lane 4 contains a control to which no ANB-NOS was added during the derivatization of σ^{70}. Lane 5 contains a control to which a 15-fold excess of underivatized σ^{70} was added as a competitor of the σ^{70}-ANB.

side chain. The formation of an aggregate could act to bury this hydrophobic group in a solvent-excluded core. This effect can occur at a 10-fold increase in ANB-NOS, even without activation of the azide (data not shown). However, when the azide group is activated with UV light, all of the σ^{70} detected is present as the aggregate, indicating that at the higher derivatization level no monomeric σ^{70} exists in solution. An alternative interpretation of this result is that the higher levels of azide oxidize the DTT and protein, forming disulfide bonds between protein molecules. UV irradiation could then cross-link these multimers before the 2-mercaptoethanol in the sample buffer reduced them back to monomers. If this were the case, the use of a nonreducing sample buffer for nonirradiated samples would result in the same band pattern as that of the irradiated sample. This is not the case (data not shown).

Cross-Linking of σ^{70} to Core RNA Polymerase. By using an amount of ANB-NOS that did not cause detectable aggregation, it was possible to cross-link σ^{70} to core RNA polymerase (Figures 3 and 4). The cross-link sample, defined as a sample that has been processed according to the procedure detailed in the Experimental Procedures section, exhibits protein bands that are dependent on the presence of core RNA polymerase, exposure to UV light, and derivatization of σ^{70} with ANB-NOS. In addition, these products can be competed down with an excess of underivatized σ^{70}. From the various antibodies used, it is possible to identify the fastest migrating cross-link species as σ^{70}–α (which is sometimes resolved as a doublet as in Figure 6). The next fastest cross-link band is σ^{70}–β'. Slightly slower than this is another σ^{70}–β', which in this gel system runs at about the same position as the first band of a σ^{70}–β doublet.

In addition to the cross-linking results, there are two interesting phenomena that are evident in Figure 3. The first is the appearance of σ^{70}-ANB bands running slightly faster than that of the underivatized σ^{70} in lane 4. When run on SDS–PAGE, σ^{70} exhibits a mobility of approximately 95 kDa (Burgess *et al.*, 1969). Apparently, derivatization of σ^{70} with ANB-NOS results in σ^{70} migrating at a rate more consistent with its actual size. Secondly, in lanes 2, 3, and 5 there seems to be a broadening of the σ^{70} band. Since these lanes and only these lanes contain derivatized σ^{70} exposed to UV light, this is probably due to intramolecular cross-linking with a resultant change in mobility. The bands detected by anti-σ^{70} monoclonal antibody that appear at approximately the same size as the σ^{70}–β and σ^{70}–β' bands in the excess σ^{70} sample are small multimers of σ^{70}. This conclusion is based on the lack of reaction with any core antibodies and the fact that they can be generated in the absence of core, but with excess underivatized σ^{70} added (data not shown). We believe it is unlikely that these bands are due to contamination of the σ^{70} sample with core since we did not detect any core subunits in the no core control when probing with mAbs to core subunits (Figure 4). It should also be noted that due to the sensitivity of the assay used, some breakdown products of σ^{70} are evident.

Effect of Reducing Agents on Azide Stability. It has previously been reported that aryl azides can be reduced by DTT and 2-mercaptoethanol (Cartwright *et al.*, 1976; Staros *et al.*, 1978). Unfortunately, these compounds are needed to maintain enzymatic activity for a number of proteins, including σ^{70}. To determine which agent should be used to generate the best cross-link signal, the 0.1 mM DTT in the derivatization buffer, the cross-link buffer, or both was replaced with 5 mM 2-mercaptoethanol. This corresponds to the levels used when working with σ^{70} (Burgess *et al.*, 1969; Cassani *et al.*, 1970). When this was done, a marked decrease in cross-link efficiency was observed (Figure 5). It can be seen that replacing the DTT in the cross-link buffer only has the least detrimental effect and that replacing the DTT in both buffers has the

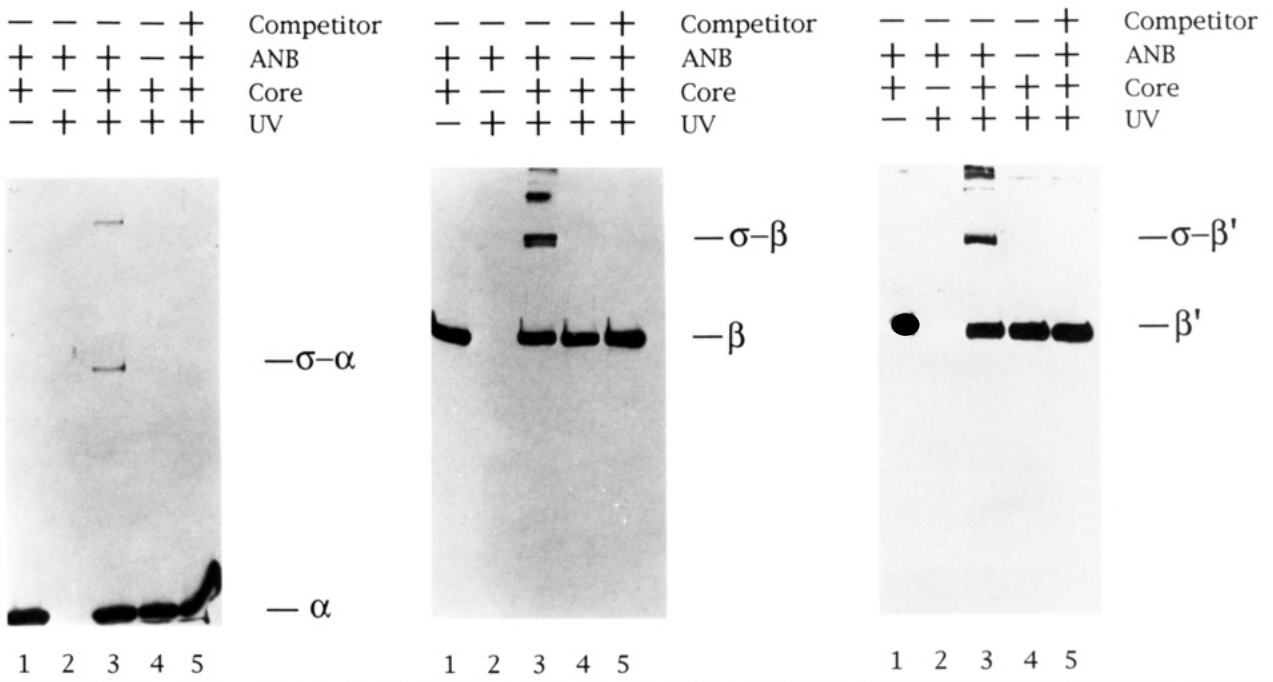

FIGURE 4: Detection of cross-link samples with mAb's to core subunits. An immunoblot of the cross-link samples was prepared as described for Figure 2 with different primary mAb's and ECL exposure times as detailed below. The lanes correspond to the same samples as in Figure 3. (A, left) The primary antibody used was a 1:500 dilution of 5RA1, an anti-α mAb. The ECL exposure time used was 1 min. (B, middle) The primary antibody used was a 1:10 000 dilution of NT63, an anti-β mAb. The ECL exposure time used was 15 s. (C, right) The primary antibody used was a 1:500 dilution of NT73, an anti-β' mAb. The ECL exposure time used was 1 min.

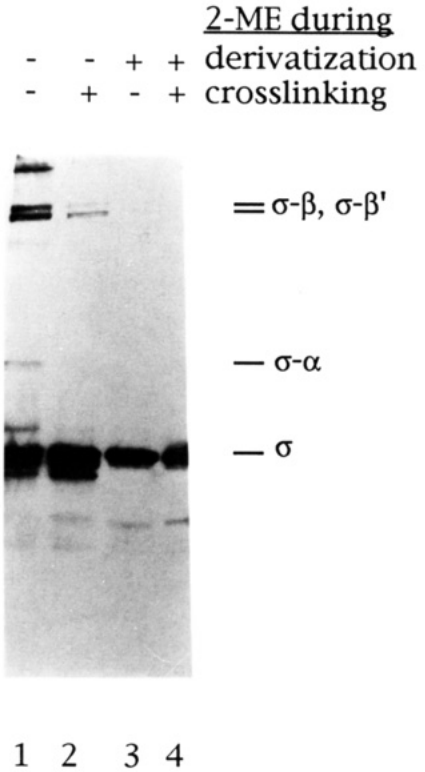

FIGURE 5: Effect of reducing agent on cross-linking efficiency. An immunoblot of cross-link samples was prepared as described for Figure 2. Lane 1 is a control sample in which σ^{70} was cross-linked to core RNA polymerase in the buffer detailed in the Experimental Procedures. Lane 2 is a sample in which the 0.1 mM DTT in the cross-link buffer was replaced with 5 mM 2-mercaptoethanol. Lane 3 is a sample in which the 0.1 mM DTT in the derivatization buffer was replaced by 5 mM 2-mercaptoethanol. Lane 4 is a sample in which the 0.1 mM DTT in both buffers was replaced by 5 mM 2-mercaptoethanol.

greatest loss of efficiency. Spectral analysis reveals that the azide is only 76% as stable in cross-link buffer with 5 mM 2-mercaptoethanol when compared to the cross-link buffer with 0.1 mM DTT after the 30 min incubation time employed prior to irradiation (data not shown). The loss of cross-link efficiency may be, therefore, due to a greater reduction of the azide by the higher concentration of 2-mercaptoethanol. To ensure that the protein was not oxidizing in the 2-mercaptoethanol sample, both samples were separated by SDS–PAGE using a nonreducing sample buffer. This did not reveal any intermolecular cystines, suggesting that the proteins are not being inactivated via oxidation in the 2-mercaptoethanol buffer.

To determine the loss in azide activity due to reduction by DTT, we measured the change in absorbance of the cross-linking solution at 320 nm due to exposure to 366 nm UV light. Prior to exposure to UV light, the absorbance at 320 nm was equal to 0.039 absorbance unit. After exposure to UV light, the absorbance at this wavelength was equal to 0.008. This decrease was found to equal 3.4×10^3 absorbance units/mol of ANB-NOS in this system since the concentration of ANB-NOS is 9.12×10^{-6} M. As previously mentioned, complete photolysis of ANB-NOS in the absence of a reducing agent results in a decrease of 5.9×10^3 absorbance units/mol. This means that 58% of the azido groups in this system can still be photolyzed after exposure to DTT. Because an increase in the percent of active azide is expected to result in less than a 2-fold increase in cross-link efficiency, we decided to continue to use 0.1 mM DTT in order to protect σ^{70} activity.

Cross-Linking σ^{70} to Core RNA Polymerase Using NHS-ASA. To test the effect of different cross-linkers on the products generated, σ^{70} was derivatized with NHS-ASA instead of ANB-NOS (Figure 6). This resulted in a banding pattern similar to that of ANB-NOS. However, some differences can be seen. The σ^{70}–α formed with NHS-ASA is a single band, not the doublet seen with ANB-NOS. The faster migrating σ^{70}–β' band is much darker when NHS-ASA is used instead of ANB-NOS. Finally, when probed with the anti-β monoclonal antibody NT63, the σ^{70}–β cross-

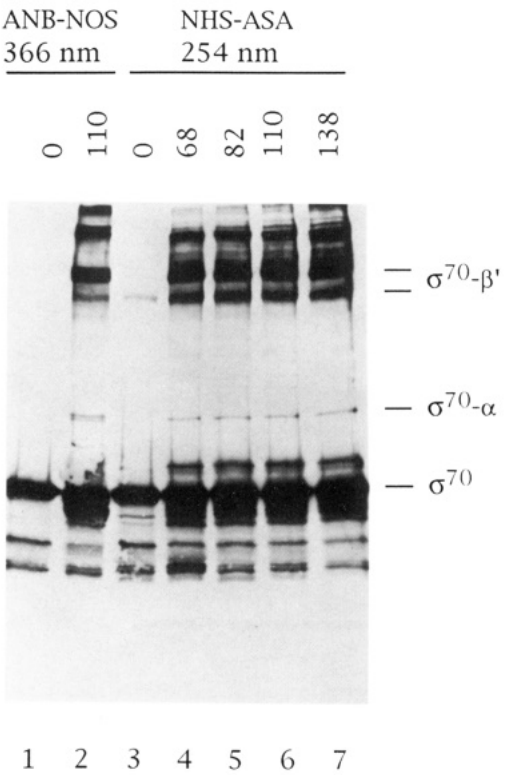

FIGURE 6: Comparison of cross-link products generated using ANB-NOS or NHS-ASA. An immunoblot of protein samples was prepared as described earlier. It was then probed using 1H6, an anti-σ^{70} mAb, at a 1:10 000 dilution. The exposure time used was 1 min. Lane 1 is a control sample in which no ANB-NOS was added to the σ^{70}. Lane 2 is a cross-link sample generated according to the procedure in the Experimental Procedures. Lanes 3–7 were exposed to UV light at a wavelength of 254 nm for 15 min. Lane 3 is a control to which no NHS-ASA was added during the derivatization of σ^{70}. Lanes 4–7 are σ^{70} derivatized with NHS-ASA at 0.62, 0.75, 1.0, and 1.25 times the concentration of ANB-NOS used, respectively.

link is seen to be reduced dramatically to a level approximately one-tenth of that seen with ANB-NOS by visual estimation (data not shown).

Mapping the Site of the σ^{70}–α Cross-Link. When the α subunit of *E. coli* RNA polymerase is exposed to hydroxylamine, it is cleaved in one place. This site of cleavage is between the asparagine at position 208 and the glycine at position 209. The σ^{70} subunit is not cleaved. Using the technique of off-diagonal mapping, it is possible to determine whether σ^{70} cross-links to the first 208 residues of α, the last 121 residues, or both. Probing with an anti-α mAb whose epitope has been mapped to residues 209–329 (Figure 7A) shows a signal that had the same mobility as σ^{70}–α before cleavage, but a faster mobility after cleavage. Its mobility is now intermediate between those of σ^{70}–α and σ^{70} (Figure 7b). We believe that this new signal is σ^{70} cross-linked to the 209–329 fragment of α. When an anti-σ^{70} mAb is used (Figure 7B), it can be seen that this new signal also reacts with an anti-σ^{70} mAb. In addition, a band that seems to correspond to free σ^{70} is also visible. Apparently, some fraction of the cross-linker is also cleaved by this reaction. This is not surprising since it is expected that the carbonyl carbon of the 5-azido-2-nitrobenzoyl would be reactive toward strong nucleophiles such as hydroxylamine. The effective result of this reaction would be cleavage of the cross-linker. Probing with 5RA1, an anti-α mAb mapped to the 1–208 fragment, does not show any reactivity with the cleavage product (Figure 7B). Signals corresponding to the full-length α produced by cleavage of the cross-linker and to the 209–329 fragment of α produced by cleavage of the cross-linker and cleavage of the α protein are not detected by 3RA1. We believe that this is because their signals are below the detection limit of the assay.

DISCUSSION

Optimization of σ^{70}–Core RNA Polymerase Cross-Linking. To avoid loss of cross-link signal due to loss of protein activity, we chose to perform the reaction under conditions that would maintain the stability of the protein. This involved using a buffer system similar to the one in which the protein was purified. To prevent any competing reactions for the ANB-NOS, the buffering species was changed from Tris·HCl to phosphate since ANB-NOS reacts with the primary amines of Tris. After the protein had reacted with the cross-linker, excess Tris·HCl was added to quench any unreacted ANB-NOS still present in the sample. Because σ^{70} activity is lost if stored at –20 °C in 5% glycerol, the glycerol concentration was raised to 50% before storage.

The choice of pH was influenced by two factors. First, the reaction of primary amines with ANB-NOS involves the unprotonated species. A higher pH thus leads to more efficient derivatization. However, the reaction of azide with thiol involves the deprotonated form of the thiol ($pK_a \sim 8$) (Cartwright *et al.*, 1976), and thus a higher pH is undesirable when thiols are present. Because overderivatization of σ^{70} with ANB-NOS led to aggregation, the efficiency of derivatization was not as important a concern as the reduction of the azide. For this reason we kept the pH below 8.0.

It has been previously suggested that 2-mercaptoethanol should be used instead of DTT to prevent reduction of the azido group (Cartwright *et al.*, 1976; Staros *et al.*, 1978; Bartholomew *et al.*, 1990). In this study we show that, at least for σ^{70}, this is not the case. Spectral analysis of σ^{70} derivatized with ANB-NOS indicates that less than half of the azido groups are reduced in this system using 0.1 mM DTT after 8 days of storage at –20 °C. After approximately 6 months of storage, the 320 nm peak has decreased to a level intermediate between the unirradiated and the completely photolyzed spectra (data not shown). This may be due to a slow reduction of the azido group by DTT; however, this may also be due to an alternate mechanism. We have found that the use of 2-mercaptoethanol instead of DTT in the cross-link buffer results in a significant loss of cross-linking efficiency. This seems to be caused by a mechanism other than oxidation of the protein. Spectral analyses of samples in cross-link buffer with either 0.1 mM DTT or 5 mM 2-mercaptoethanol show that there is a loss of 24% of the azide absorbance when 2-mercaptoethanol is used. A fraction of the azide seems to be reduced by the higher amount of reducing agent. This would explain the observed order of efficiency. The less time the azide was exposed to 5 mM 2-mercaptoethanol, the higher the signal seen. We believe that the source of the discrepancy between this work and the previous results is due to the concentration of thiol used. The 50-fold increase of 2-mercaptoethanol over DTT used here is much greater than the 2-fold or 5-fold differences used elsewhere (Cartwright *et al.*, 1976; Staros *et al.*, 1978). We feel that this difference is more appropriate from a protein chemistry standpoint since so much less DTT is needed to keep the thiols of a protein reduced (Cleland, 1964).

When working with aryl azides, it is generally mentioned that the samples should be kept from exposure to light during manipulation. To test the importance of this precaution, we left a sample of ANB-NOS exposed to room light for 15 min to determine whether any change in the characteristic

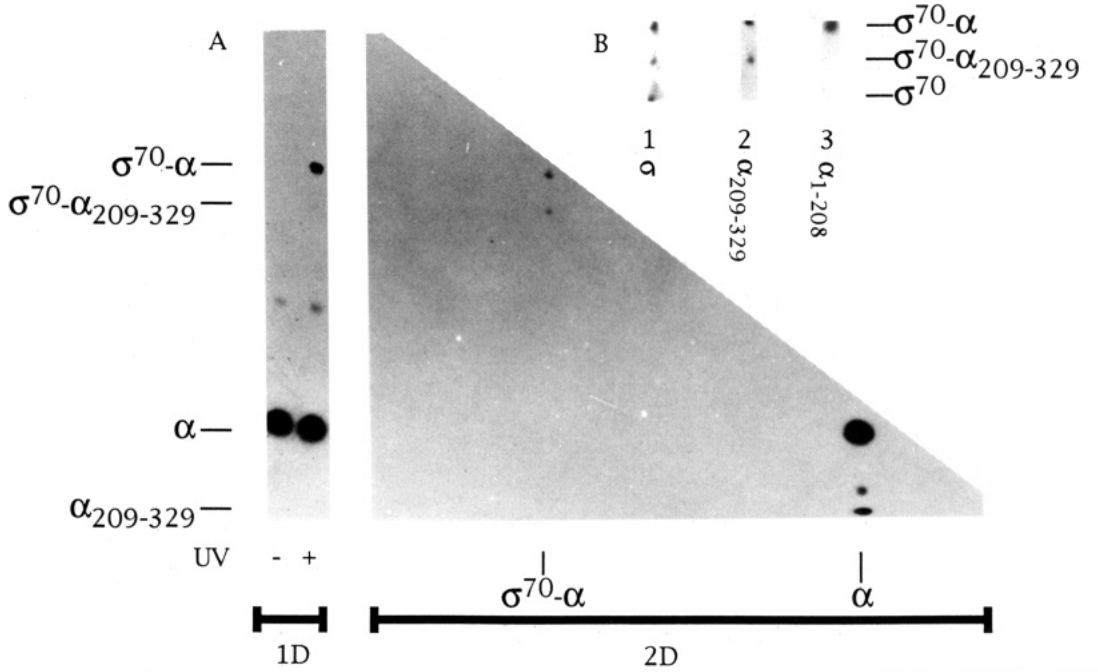

FIGURE 7: Cleavage of the σ^{70}–α cross-link product with hydroxylamine. Cross-link samples were run on SDS–PAGE (from left to right in the figure), cleaved by hydroxylamine while still in the gel, and electrophoresed in a direction perpendicular to the original direction of electrophoresis (from top to bottom in the figure). One-dimensional electrophoresis was performed on markers parallel to the direction of the second electrophoresis run. Lane 1 corresponds to the no UV control from Figure 3, lane 1. Lane 2 corresponds to the cross-linked sample. The first- and second-dimension coordinates of α and σ^{70}–α are as indicated. The samples were then transferred to nitrocellulose and detected by ECL using a 1:250 dilution of secondary antibody. (A) The blot was detected using 3RA1 at 1:500 dilution. The exposure time was 1 min. (B) Similar blots were prepared using 1H6 (ECL exposure time of 1 s) and 5RA1 (ECL exposure time of 1 min). Lane 1 corresponds to the σ^{70}–α region of the 2D gel probed with 3RA1. Lane 2 corresponds to the same region probed with 1H6. Lane 3 corresponds to the same region probed with 5RA1. The proposed identities of the new spots are indicated to the right.

absorption spectrum could be detected (Figure 1). We were thus able to determine that the azido group was resistant to short exposures to overhead lighting, and we used reduced lighting for only those steps that would result in exposure times greater than 15 min.

To prevent the aggregation effect that was seen at higher ratios of cross-linker to σ^{70}, we chose to work with substituted aryl azides instead of an unsubstituted compound such as HSAB. This choice was made to decrease the hydrophobicity of the derivatized side chains and, thus, decrease the driving force for the aggregation phenomenon. In addition, we added the nonionic detergent Triton X-100 to aid in solubilizing the hydrophobic moiety.

Under these conditions, we were able to detect a significant amount of cross-linking between σ^{70} and the core RNA polymerase. These new binary species can be unambiguously identified with the use of subunit specific monoclonal antibodies. The importance of using monoclonal antibodies to identify the resultant complexes is exemplified by the bands identified as σ^{70}–β and σ^{70}–β'. Although it would be expected that σ^{70}–β would migrate slightly faster on SDS–PAGE than σ^{70}–β' because of its smaller size, it actually migrates slower. In addition, there are two distinct bands corresponding to σ^{70}–β', as well as less distinct doublets for σ^{70}–α and σ^{70}–β. This may be a function of the placement of the cross-link site along the polypeptide chains involved. Two proteins cross-linked near their termini may migrate at a different rate than those cross-linked near the center of each protein. Those binary complexes formed by cross-links at the termini should be able to adopt a shape similar to that of a polypeptide equal to the sum of both components. It is not clear what shape would be assumed by a complex formed by cross-linking in the middle of the proteins or what effect this would have on migration rates. Because of this anomalous migration rate, the identity of a cross-linked product cannot be positively determined purely on the basis of its apparent size.

The formation of binary cross-link products between σ^{70} and each core RNA polymerase subunit is in agreement with the results obtained by Hillel and Wu (1977). Coggins *et al.* (1977) did not observe the σ^{70}–α cross-link. Most likely, this is due to the limited specificity of the reagent used. When using a homobifunctional cross-linking reagent such as *N,N'*-bis(2-carboximidoethyl)tartaramide dimethyl ester dihydrochloride, it is necessary for the proper side chains, in this case lysines, to be within the length spanned by the bridge of the cross-linker. If two lysines are not in proper alignment on σ^{70} and α, then no cross-link can be generated. This problem can be avoided by using a variety of cross-linkers with differing specificities, as was done in the study by Hillel and Wu (1977), or a cross-linker with greater reactivity toward amino acid side chains, as is employed in this study.

Even cross-linkers with the same specificities can result in different cross-link patterns. An example of this can be seen by comparing the results obtained using ANB-NOS and NHS-ASA. When considering both bands of the doublet, ANB-NOS generates more σ^{70}–α than does NHS-ASA since each individual band of the doublet is of approximately equal darkness to the singlet seen with NHS-ASA. In contrast, the lower σ^{70}–β' band is stronger in the samples using NHS-ASA. Also, the efficiency of σ^{70}–β formation with NHS-ASA is much weaker than that with ANB-NOS. These differences may be due to the position of the azide group on the benzene ring relative to the site of attachment to the primary amine of the protein. The azide group of ANB-NOS is at the number 5 carbon position relative to the carboxyl group. In NHS-ASA the azide is attached at the number 4 carbon. This slight shift of the second functional group could cause the differences seen in the relative strengths of the cross-

link signals. A second contributing factor could be the functional group on the number 2 carbon. In ANB-NOS this carbon is nitro substituted; in NHS-ASA it is hydroxy substituted. This means that NHS-ASA can donate a hydrogen bond that ANB-NOS cannot. This may result in one compound favoring certain orientations that the other does not. The differences in spatial arrangement could account for the changes in cross-linking results.

Recommended Approach When Using Aryl Azides. When beginning a cross-linking experiment, it is important to keep in mind the advantages and disadvantages of the compound used. With aryl azides the main advantage is the sequential nature of the activation of the functional groups. This feature could be useful in trying to determine which components of a complex or impure mixture react with a protein of interest. The sequential activation is also useful if a specific binary complex of a multisubunit protein is to be studied further since the stepwise nature of the cross-linking reaction limits the cross-links formed, making purification of the desired complex simpler. However, to take advantage of the sequential activation of the functional groups requires that the subunit can be purified and then reconstituted into the complete enzyme. Another potential disadvantage is that derivatization of some sites could block the binding site of the protein. Also, it is theoretically possible that the placement of a hydrophobic group on the surface of the protein could lead to denaturation. Finally, if the protein aggregates upon overderivatization like σ^{70} does, it is possible that aggregation will occur at such low levels of derivatization so as to make cross-linking impossible.

When it has been decided that an aryl azide cross-linker is to be used, we suggest the following course of action. First, determine a mock derivatization protocol that does not adversely affect enzyme activity. When this has been done, perform a titration series on the amount of cross-linker relative to a constant amount of protein to determine whether aggregation of derivatized protein is a problem, and if so at which level of derivatization it occurs. We suggest using a mild nonionic detergent such as Triton X-100 and keeping the salt concentration low to minimize any hydrophobic interactions between aryl azide moieties. Finally, cross-link protein samples with various levels of derivatization below the level that gave aggregation to determine which sample gives the best signal for the desired binary complex or complexes. In addition, it may be productive to try a few cross-linkers. For example, ANB-NOS would be a better cross-linker when studying σ^{70}–α and σ^{70}–β, but NHS-ASA should be used for σ^{70}–β'.

Localizing the Site of the σ^{70}–α Cross-Link. The result of mapping the σ^{70}–α should be of particular interest to those who study *E. coli* transcription. A number of experiments indicate that the C-terminal region of the α subunit is involved in interaction with transcription activators such as CRP (Zou *et al.*, 1992), OmpR (Slauch *et al.*, 1991), and Fnr (Lombardo *et al.*, 1991). In addition, this region has also been implicated as the region involved in interactions with DNA when the enzyme is bound to a class of promoters (Ross *et al.*, 1993). This interaction is believed to cover the region from base pair −60 to base pair −40 relative to the start of transcription. Since σ^{70} contacts the DNA at base pair −35, it would be expected that on these promoters this region of α is in close proximity with σ^{70}. Our results show that even in solution, there is a proximal relationship between σ^{70} and the C-terminal region of α. Further proteolysis experiments could be used to determine whether the cross-link involves the region of σ^{70} implicated in −35 hexamer recognition.

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