Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 9702–9706, October 1995
Biochemistry

Protein crosslinking studies suggest that *Rhizobium meliloti* C_4-dicarboxylic acid transport protein D, a σ^{54}-dependent transcriptional activator, interacts with σ^{54} and the β subunit of RNA polymerase

JOON H. LEE AND TIMOTHY R. HOOVER*

Department of Microbiology, University of Georgia, Athens, GA 30602

Communicated by Sydney Kustu, University of California, Berkeley, CA, July 18, 1995 (received for review April 5, 1995)

ABSTRACT ***Rhizobium meliloti*** **C_4-dicarboxylic acid transport protein D (DCTD) activates transcription by a form of RNA polymerase holoenzyme that has σ^{54} as its σ factor (referred to as Eσ^{54}). DCTD catalyzes the ATP-dependent isomerization of closed complexes between Eσ^{54} and the *dctA* promoter to transcriptionally productive open complexes. Transcriptional activation probably involves specific protein–protein interactions between DCTD and Eσ^{54}. Interactions between σ^{54}-dependent activators and Eσ^{54} are transient, and there has been no report of a biochemical assay for contact between Eσ^{54} and any activator to date. Heterobifunctional crosslinking reagents were used to examine protein–protein interactions between the various subunits of Eσ^{54} and DCTD. DCTD was crosslinked to *Salmonella typhimurium* σ^{54} with the crosslinking reagents succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate and *N*-hydroxysulfosuccinimidyl-4-azidobenzoate. Cys-307 of σ^{54} was identified by site-directed mutagenesis as the residue that was crosslinked to DCTD. DCTD was also crosslinked to the β subunit of *Escherichia coli* core RNA polymerase with succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, but not with *N*-hydroxysulfosuccinimidyl-4-azidobenzoate. These data suggest that interactions of DCTD with σ^{54} and the β subunit may be important for transcriptional activation and offer evidence for interactions between a σ^{54}-dependent activator and σ^{54}, as well as the β subunit of RNA polymerase.**

Initiation of transcription in bacteria is mediated by RNA polymerase holoenzyme, which has the subunit composition $\alpha_2\beta\beta'\sigma$ (for reviews, see refs. 1 and 2). Within a bacterial cell, there are generally several forms of RNA polymerase holoenzyme that differ with respect to the σ factor associated with them and the promoter elements that they recognize. σ^{54}-RNA polymerase holoenzyme (Eσ^{54}) is responsible for transcription of several genes whose products are required for diverse metabolic functions (for reviews, see refs. 3–5). Initiation of transcription by Eσ^{54} requires an activator protein that catalyzes the isomerization of closed complexes between Eσ^{54} and the promoter to transcriptionally competent open complexes (6–9). This isomerization requires the hydrolysis of ATP by the activator (7, 9–11). The activator generally binds to sites located ≈100 bp upstream of the transcriptional start site and must contact Eσ^{54} bound at the promoter through a DNA loop to activate transcription (12–14).

To further dissect the mechanism of activation, it is necessary to understand how the activator engages Eσ^{54} to activate transcription. In electron micrographs, interactions between Eσ^{54} and the σ^{54}-dependent activator nitrogen regulatory protein C (NTRC) were observed in which these proteins contacted each other while bound to their respective sites at the *glnA* promoter regulatory region (13). This interaction between Eσ^{54} and nitrogen regulatory protein C appeared to be transient, however, and no report of a biochemical assay for contact between Eσ^{54} and any activator has been made to date. We report here on the use of chemical crosslinking methods to examine interactions between C_4-dicarboxylic acid transport protein D (DCTD) and either σ^{54} or core RNA polymerase.

Rhizobium meliloti DCTD is a σ^{54}-dependent activator that activates transcription from *dctA*, which encodes a C_4-dicarboxylic acid transport protein (15, 16). Sequence homology between the N-terminal 125 amino acid residues of *R. meliloti* DCTD and other bacterial regulatory proteins indicates that DCTD belongs to a family of regulators that are phosphorylated by histidine protein kinases in response to environmental stimuli (17). Phosphorylation of DCTD presumably causes the protein to assume an active conformation that is capable of activating transcription. $DCTD_{L143}$ is a truncated form of *R. meliloti* DCTD that lacks 142 amino acid residues from the N terminus and is locked in an active conformation (9). We observed that both $DCTD_{L143}$ and wild-type DCTD could be crosslinked to σ^{54} or the β subunit of RNA polymerase, suggesting that DCTD may interact with these subunits to activate transcription and that DCTD does not need to be in an active conformation for these interactions.

MATERIALS AND METHODS

Chemicals. Crosslinking reagents were obtained from Pierce; all other chemicals and proteins were obtained from Sigma unless indicated otherwise.

Protein Preparations. Plasmid pTRH1 bears the *R. meliloti dctD* gene under control of the *lac* promoter and was constructed by cloning ≈1.7 kbp of *Bst*BI–*Eco*RI DNA fragment that carried *dctD* and ≈120 bp from the 3′-end of *dctB* (relevant DNA sequence is found in ref. 17) into the *Acc* I–*Eco*RI sites of pBS (Stratagene). DCTD was overexpressed in four 1-liter cultures of *Escherichia coli* strain JM109 {*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ* ΔM15]} carrying pTRH1, as described for overexpression of $DCTD_{L143}$ (9). Subsequent steps were done at 0–4°C, except the chromatography steps, which were done at room temperature with a Pharmacia LKB fast protein liquid chromatography system. After overexpression of DCTD, cells were harvested by centrifugation at 5000 × *g* for 10 min, resuspended in buffer A [20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid), pH 8/5% (vol/vol) glycerol/50 mM KCl/0.5 mM dithiothreitol] and pelleted again by centrifugation. Cells were resuspended in buffer A (80 ml)/1

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: DCTD, C_4-dicarboxylic acid transport protein D; Eσ^{54}, σ^{54}-RNA polymerase holoenzyme; sulfo-SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; sulfo-HSAB, *N*-hydroxysulfosuccinimidyl-4-azidobenzoate; NHS ester, *N*-hydroxysuccinimide ester; $DCTD_{L143}$, truncated form of *R. meliloti* DCTD.

*To whom reprint requests should be addressed.

mM phenylmethylsulfonyl fluoride and then lysed in a French pressure cell at 6000 psi (1 psi = 6.9 kPa). Extracts were clarified by centrifugation at 17,000 × *g* for 30 min. Ammonium sulfate was added (to 35% saturation) to the clarified extracts, and precipitated proteins were collected by centrifugation. Proteins were resuspended in 5–6 ml of buffer A and dialyzed overnight against the same buffer. The dialysate was centrifuged at 17,000 × *g* for 20 min, and the resulting supernatant was applied to a Q-Sepharose (Pharmacia) anion-exchange column (≈24 ml) that had been equilibrated with buffer A. DCTD was eluted with a gradient to buffer A/350 mM KCl in 138 min at 0.7 ml/min. DCTD eluted from the column at ≈150 mM KCl. Peak fractions containing DCTD were pooled, and proteins were concentrated by ammonium sulfate precipitation (50% saturation). After dialysis against buffer A, samples were applied to a heparin–agarose column (≈16 ml) that had been equilibrated with buffer A. After washing with buffer A, DCTD was eluted from the column with a gradient to buffer A/500 mM KCl in 60 min at 0.7 ml/min. Peak fractions containing DCTD, which eluted from the column at ≈200 mM KCl, were pooled, and proteins were concentrated by ammonium sulfate precipitation. Proteins were resuspended in buffer A, dialyzed against the same buffer, and stored at −80°C.

R. meliloti $DCTD_{L143}$ and *E. coli* core RNA polymerase were purified as described (9). *E. coli* σ^{70} was purified from strain DH5α [*supE44 ΔlacU169* (*ϕ80 lacZ* ΔM15) *hsdR17 recA1 endA1 gyr96 thi-1 relA1*] essentially as described (18). *Salmonella typhimurium* σ^{54} was overexpressed in *E. coli* strain M5219 [M72 *lacZ trpA rpsL* (*λbio252 cI857* ΔH1)] from plasmid pJES259, which carries *S. typhimurium ntrA* under control of the λP_L promoter, and purified as described (19). Mutant forms of σ^{54} were similarly overexpressed in *E. coli* and purified through the Q-Sepharose chromatography step as described for the purification of wild-type σ^{54} (19). Protein concentrations were determined with the bicinchoninic acid protein assay as described by the supplier using purified bovine IgG as a protein standard.

Chemical Crosslinking Assays. DCTD proteins were diluted in 20 mM sodium phosphate buffer, pH 7/150 mM NaCl (PBS) to a final concentration of ≈4.4 μM (DCTD dimer) along with succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) (final concentration, 45 μM) or *N*-hydroxysulfosuccinimidyl-4-azidobenzoate (sulfo-HSAB) (110 μM). All procedures involving sulfo-HSAB were done in the dark. After 15 min at room temperature, reaction mixtures (100-μl vol) were dialyzed against 2 liters of ice-cold PBS for 2–3 hr. At the same time, other proteins used in the crosslinking assays (σ^{54} or core RNA polymerase) were dialyzed against 20 mM potassium phosphate buffer, pH 7/10 mM Na_2EDTA. After dialysis, modified DCTD protein was mixed with an equal volume (10 μl) of σ^{54} or core RNA polymerase, and the proteins were incubated for 5–10 min at room temperature to allow crosslinking. Reactions were quenched by the addition of SDS/PAGE sample buffer and analyzed by immunoblotting using either rabbit antiserum directed against *S. typhimurium* σ^{54} (from Sydney Kustu, University of California, Berkeley, CA), mouse monoclonal antibodies specific for the α, β, or β′ subunits of *E. coli* RNA polymerase (from Joe Krakow, Hunter College of City University of New York, New York), or purified polyclonal antibodies directed against $DCTD_{L143}$. Antibodies directed against $DCTD_{L143}$ were purified from rabbit antiserum, essentially as described by Robinson *et al.* (20). For crosslinking assays with sulfo-HSAB, photoactivatable DCTD proteins (i.e., DCTD treated with sulfo-HSAB) were mixed in a 1.5-ml microcentrifuge tube with the other proteins that had been previously dialyzed against 20 mM potassium phosphate buffer and incubated for 25 min at room temperature (final reaction volume, 20 μl). Reaction mixes were then exposed to six flashes of light from a camera flash (Star 15B; Tianjin Camera, Tianjin, China), which was held at the open end of the microcentrifuge tube. Reactions were mixed with SDS/PAGE sample buffer and analyzed by immunoblotting.

The N-terminal α- and lysinyl ε-amino groups in $DCTD_{L143}$ were quantitated with 2,4,6-trinitrobenzene 1-sulfonic acid, essentially as described (21) before and after modification of the protein with sulfo-SMCC or sulfo-HSAB. These values gave an estimate for the average number of lysine residues of $DCTD_{L143}$ modified upon treatment with these crosslinking reagents. On average, ≈2.5 α- and ε-amino groups of $DCTD_{L143}$ (per monomer) were modified with sulfo-SMCC, whereas ≈3.5 α- and ε-amino groups were modified with sulfo-HSAB ($DCTD_{L143}$ has 11 lysine residues per monomer).

Site-Directed Mutagenesis. Each of the four cysteine residues of *S. typhimurium* σ^{54} were individually replaced with serine residues by site-directed mutagenesis. A ≈1-kbp *Pvu* II–*Sal* I DNA fragment that carried the four cysteine codons from *ntrA* was cloned into the *Sma* I-*Sal* I sites of pUC19. This plasmid was used for *in vitro* site-directed mutagenesis as described (22). Mutagenic primers were as follows: 5′-CGGCAAAAGAcCTTCGCGACaGTCTGCTGATCC-3′ (Cys-198 → Ser); 5′-GGCTCATTATCaGCGATCATCTT-GATCTGCTcGCCAACCACG-3′ (Cys-221 → Ser); 5′-GCCGCCATGaGCAATAGCGCcCGCAACGATG-3′ (Cys-307 → Ser); and 5′-CGTCAGTCGCaGCATCGTCGAG-3′ (Cys-346 → Ser). Bases differing from the original sequence are indicated by lowercase letters, and the altered codons are underlined. In three of the mutagenic primers, additional bases were altered to eliminate restriction sites (but not alter the amino acids corresponding to those positions) to help identify mutagenized plasmids. Restriction enzyme cleavage analysis was done to identify plasmids with the expected mutations, and these regions of *ntrA* were sequenced at the University of Georgia Molecular Genetics Instrumentation Facility to confirm the changes. From each of the mutagenized plasmids, a ≈700-bp *Sal* I–*Eco*RV fragment was isolated and cloned into pJES259 that had been previously digested with the same restriction enzymes. The resulting plasmids, pJHL3 (*ntrA* mutant CS198), pJHL4 (*ntrA* mutant CS221), pJHL5 (*ntrA* mutant CS307), and pJHL6 (*ntrA* mutant CS346), were transformed into *E. coli* strain YMC11 (*endA1 thi-1 hsdR17 Δlac169 hutC$_k$ ΔglnALG2000 ntrA*::Tn*10*) that had plasmid pGP1-2 (23). Plasmid pGP1-2 carries *cI857*, which encodes a temperature-sensitive form of the λ cI repressor. These strains were maintained at 30°C, and mutant σ^{54} proteins were overexpressed at 42°C in these strains, as described for wild-type σ^{54} (19).

RESULTS

Chemical Crosslinking Between DCTD Proteins and σ^{54} with Sulfo-SMCC. Several bifunctional crosslinking reagents were tested to see whether they could crosslink purified $DCTD_{L143}$ with either *S. typhimurium* σ^{54}, *E. coli* core RNA polymerase, or a mixture of these two proteins. We had shown previously that $DCTD_{L143}$ functioned in an *in vitro* transcription system with these proteins (9). Crosslinking reagents included disuccinimidyl suberate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and sulfo-SMCC. Combinations of proteins were mixed with these crosslinking reagents, and the reaction mixtures were analyzed by SDS/PAGE, after which gels were stained with Coomassie blue. Individual proteins treated with the crosslinking reagents were included as controls. In these experiments, a potential $DCTD_{L143}$–σ^{54} crosslinked product was observed when sulfo-SMCC was the crosslinking reagent.

Sulfo-SMCC has a *N*-hydroxysuccinimide ester (NHS ester) and a maleimide joined by a cyclohexane bridge (Fig. 1). The NHS ester reacts principally with ε-amino groups of lysine

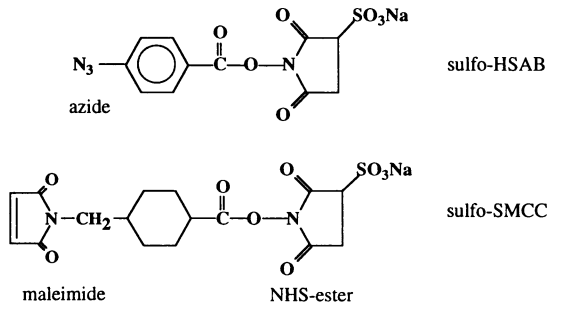

FIG. 1. Chemical structures of sulfo-SMCC and sulfo-HSAB. The spacer arms of sulfo-SMCC and sulfo-HSAB are 11.6 and 9.0 Å, respectively.

residues and the α-amino group at the N termini of proteins, whereas the maleimide reacts primarily with sulfhydryls at neutral pH.

The crosslinking assay with sulfo-SMCC was modified as follows. In the first step, $DCTD_{L143}$ was briefly treated with sulfo-SMCC, then the protein was dialyzed 2–3 hr to remove sulfo-SMCC that had not reacted. In the second step, the modified $DCTD_{L143}$ was incubated with σ^{54} at room temperature to allow crosslinking, after which reaction mixtures were analyzed by immunoblotting. A prominent band with an apparent M_r of ≈150,000 was observed in the crosslinking reaction mixture under these conditions. This band was not present in the control lanes and cross-reacted with affinity-purified antibodies directed against $DCTD_{L143}$ and antiserum directed against σ^{54} (Fig. 2 *A* and *B*), indicating that it was, indeed, a crosslinked product of $DCTD_{L143}$ and σ^{54}.

In the SDS/PAGE used in Fig. 2, the $DCTD_{L143}$ monomer had an apparent M_r of 43,000 [$DCTD_{L143}$ migrates predominantly as a dimer on gel filtration chromatography (9)]. In this gel, σ^{54} migrated with an apparent M_r of 66,000. The size of the crosslinked product suggested a possible stoichiometry of two $DCTD_{L143}$ monomers per σ^{54} for the crosslinked product. The ratio of $DCTD_{L143}$ to σ^{54} was varied in the crosslinking assay, but a $DCTD_{L143}$–σ^{54} crosslinked product with a smaller apparent molecular weight was never seen.

Interactions between wild-type DCTD and σ^{54} were examined to determine whether a nonactive form of DCTD could also be crosslinked to σ^{54} with sulfo-SMCC (DCTD needs to be phosphorylated to be active). Two major DCTD–σ^{54} crosslinked products were observed in immunoblots of these crosslinking assays. Both of these crosslinked products had apparent molecular weights that were slightly larger than that of the $DCTD_{L143}$–σ^{54} crosslinked product (data not shown). The reason for the two bands is unclear, but it may have resulted from an intramolecular crosslink involving the N terminus of DCTD.

Several parameters of the crosslinking assay were examined. A 10-fold molar excess of sulfo-SMCC (relative to $DCTD_{L143}$ dimer) gave maximal formation of the $DCTD_{L143}$–σ^{54} crosslinked product. The optimal time for incubating the modified $DCTD_{L143}$ with σ^{54} was 5–10 min. The amount of crosslinked product increased with increased protein concentrations, but the crosslinked product was seen at protein concentrations as low as 100 nM, the lowest concentration tested. Except for the β subunit of core RNA polymerase (see below), $DCTD_{L143}$ did not crosslink with any other proteins tested at equivalent protein concentrations (up to 2.2 μM). These proteins included *E. coli* σ^{70}, bovine serum albumin, alcohol dehydrogenase, carbonic anhydrase, and cytochrome *c*. For *E. coli* σ^{70}, at least one of its three cysteine residues is near the surface of the protein and reacts rapidly with *N*-ethylmaleimide (24), suggesting that the failure of $DCTD_{L143}$ to crosslink with σ^{70} was not simply due to the absence of a reactive sulfhydryl group near the surface of the protein.

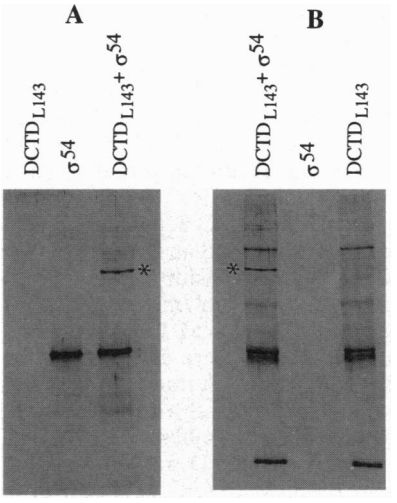

FIG. 2. Crosslinking of $DCTD_{L143}$ and σ^{54} with sulfo-SMCC. *R. meliloti* $DCTD_{L143}$ was modified with sulfo-SMCC and then mixed with *S. typhimurium* σ^{54} as described. For each protein, the final concentration in the crosslinking assay was ≈2.2 μM. Portions of the crosslinking reactions (2 μl) were subjected to SDS/10% PAGE and then transferred to nitrocellulose (NitroBind; Micron Separations, Westboro, MA) for immunoblotting by using either antiserum directed against *S. typhimurium* σ^{54} (*A*) or affinity-purified polyclonal antibodies directed against *R. meliloti* $DCTD_{L143}$ (*B*) as a source of primary antibody. After treatment of the nitrocellulose membranes with the primary antibodies, membranes were treated with a secondary antibody (biotinylated anti-rabbit immunoglobulin) and then a streptavidin–alkaline phosphatase conjugate (both from Amersham). Cross-reacting bands were visualized by incubating the membranes with the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate. Samples shown on the immunoblot include modified $DCTD_{L143}$ ($DCTD_{L143}$), untreated σ^{54} (σ^{54}), and the crosslinking reaction in which modified $DCTD_{L143}$ and σ^{54} were incubated together ($DCTD_{L143}$ + σ^{54}). The asterisk indicates a crosslinked product that cross-reacts with both sources of primary antibody. Treatment of $DCTD_{L143}$ with sulfo-SMCC resulted in crosslinking of $DCTD_{L143}$ monomers. On the basis of their apparent molecular weights, we infer that the major crosslinked $DCTD_{L143}$ products were dimers and tetramers.

Including MgATP, which is hydrolyzed by $DCTD_{L143}$, in the crosslinking reaction did not affect formation of the crosslinked product formation. The ATPase activity of the modified $DCTD_{L143}$, however, was significantly reduced, so that we could not make any inferences from this result.

Crosslinking between $DCTD_{L143}$ and σ^{54} was diminished slightly when a 3-fold molar excess of core RNA polymerase relative to σ^{54} was included in the crosslinking reaction mixture. Given that $DCTD_{L143}$ also crosslinked with the β subunit of RNA polymerase, the slight inhibitory effect on crosslinking between $DCTD_{L143}$ and σ^{54} may have resulted from competition between σ^{54} and the β subunit for crosslinking to $DCTD_{L143}$. Because of the low affinity of σ^{54} for core RNA polymerase (25), we cannot tell from these experiments whether $DCTD_{L143}$ was crosslinked with σ^{54} bound to core RNA polymerase in addition to free σ^{54}.

Cys-307 of σ^{54} Is the Residue That Is Crosslinked to DCTD with Sulfo-SMCC. Because the hydrolysis rate of the NHS ester is greater than that of the maleimide group, much of the NHS ester of sulfo-SMCC attached to $DCTD_{L143}$ through the maleimide group was likely hydrolyzed during the time the modified $DCTD_{L143}$ was dialyzed. We therefore postulated that sulfo-SMCC crosslinked a primary amine in $DCTD_{L143}$ with a cysteinyl sulfhydryl in σ^{54}. Each cysteine within σ^{54} was individually changed to a serine to identify this cysteine residue (*S. typhimurium* σ^{54} has cysteines at positions 198, 221, 307, and 346). The four mutant σ^{54} proteins were overexpressed in *E. coli*, partially purified, and tested in the crosslinking assay

with $DCTD_{L143}$. Each of these σ^{54} mutants were as active as wild-type σ^{54} in a coupled transcription–translation system. Only the mutant CS307 (Cys-307 → Ser) failed to crosslink with $DCTD_{L143}$ (Fig. 3), suggesting that this cysteine residue was involved in the crosslinking. The same results were observed when wild-type DCTD was used in the crosslinking assay. These data indicated that Cys-307 is near the surface of the protein, consistent with the observation that the corresponding region of *Klebsiella pneumoniae* σ^{54} is sensitive to trypsin cleavage (26).

Given the length of the spacer arm of sulfo-SMCC, we do not know the proximity of Cys-307 to its contact site with DCTD. If sulfo-SMCC links the ε-amino group of a lysine residue(s) in $DCTD_{L143}$ to Cys-307 of σ^{54}, the α carbons of these two amino acids could be as much as 27 Å apart in the $DCTD_{L143}$-σ^{54} crosslinked product.

Chemical Crosslinking Between DCTD Proteins and σ^{54} with Sulfo-HSAB. Sulfo-HSAB, which has a NHS ester and an arylazide as functional groups, was tested for its ability to crosslink $DCTD_{L143}$ with σ^{54}. Exposure of the arylazide to light results in formation of an unstable nitrene that reacts nonspecifically with proteins. Sulfo-HSAB was mixed with $DCTD_{L143}$ in the dark to allow the NHS ester to react with primary amines on the protein, and excess sulfo-HSAB was removed by dialysis. Photoactivatable $DCTD_{L143}$ was mixed with σ^{54}, and the mixture was illuminated to activate the arylazide. $DCTD_{L143}$ crosslinked with σ^{54} under these conditions (Fig. 4) but did not crosslink with any of the other proteins tested, which included σ^{70}, core RNA polymerase, bovine serum albumin, alcohol dehydrogenase, carbonic anhydrase, and cytochrome *c*.

The $DCTD_{L143}$–σ^{54} crosslinked product formed with sulfo-HSAB was detected at protein concentrations as low as 600 nM. The amount of crosslinked product was less than that formed with sulfo-SMCC, which was expected, given the shorter half-life of the nitrene compared with that of the maleimide group (a few milliseconds versus several hours). In addition, the crosslinker arm of sulfo-HSAB is slightly shorter and less flexible than that of sulfo-SMCC (Fig. 1).

Crosslinking Between DCTD Proteins and the β Subunit of RNA Polymerase with Sulfo-SMCC. Incubating core RNA polymerase with $DCTD_{L143}$ previously modified with sulfo-SMCC generated a crosslinked product with an apparent M_r >200,000. Like the $DCTD_{L143}$–σ^{54} crosslinked species, this crosslinked product accumulated to its highest level within 10 min of mixing modified $DCTD_{L143}$ with core RNA polymerase. Monoclonal antibodies specific for each of the subunits of core RNA polymerase (from Joe Krakow) were used to identify the subunit of core RNA polymerase crosslinked to $DCTD_{L143}$. The crosslinked product cross-reacted with affinity-purified antibodies directed against $DCTD_{L143}$ and with a monoclonal antibody directed against the β subunit of RNA polymerase (Fig. 5, lanes 2 and 7). Wild-type DCTD could also be crosslinked to the β subunit, and, as expected, the apparent molecular weight of this crosslinked product was slightly larger than that of the $DCTD_{L143}$–β crosslinked product. $DCTD_{L143}$ and the β subunit were crosslinked in reactions in which σ^{54} was present, but a molar excess of σ^{54} relative to core RNA polymerase interfered slightly with formation of the $DCTD_{L143}$-β crosslinked product (data not shown).

We could not demonstrate crosslinking between DCTD proteins and the β subunit of core RNA polymerase with sulfo-HSAB. This result was probably due to the more stringent requirements for crosslinking with sulfo-HSAB and may

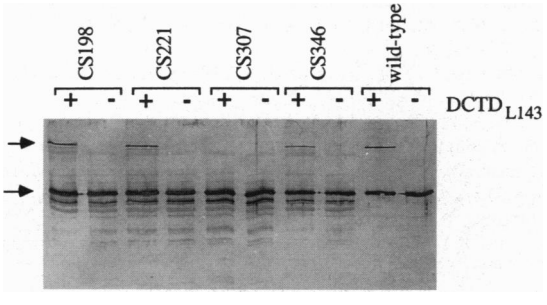

FIG. 3. Identification of cysteine residue in *S. typhimurium* σ^{54} crosslinked with $DCTD_{L143}$. Modified $DCTD_{L143}$ was incubated with partially purified preparations of the various σ^{54} mutants or with purified wild-type σ^{54} as indicated at the top of each lane. Crosslinking reactions were analyzed by immunoblotting with antiserum directed against σ^{54}. Bottom arrow, σ^{54}; top arrow, $DCTD_{L143}$–σ^{54} crosslinked products.

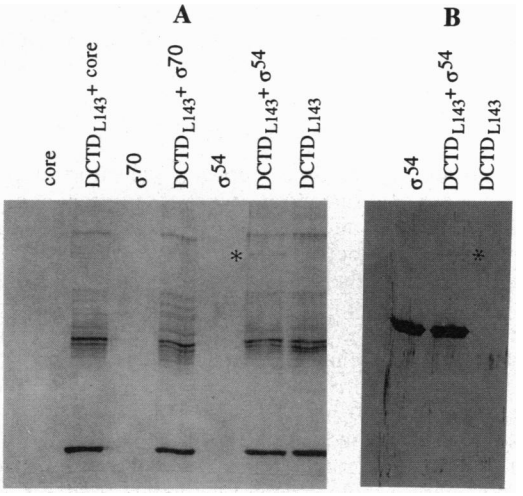

FIG. 4. Crosslinking of $DCTD_{L143}$ and σ^{54} with sulfo-HSAB. $DCTD_{L143}$ was labeled with sulfo-HSAB and mixed in the dark with other proteins as indicated above each lane. The final concentration of each protein in the crosslinking assay was ≈2.2 μM. Crosslinking reaction mixtures were exposed to six flashes of light from a camera flash, and portions of the mixtures (4 μl) were analyzed by immunoblotting with either affinity-purified polyclonal antibodies directed against $DCTD_{L143}$ (*A*) or σ^{54} (*B*) as a source of primary antibody. Samples are indicated above each lane and include core RNA polymerase (core), photoactivatable $DCTD_{L143}$, σ^{70}, σ^{54}, and crosslinking reaction mixtures in which photoactivatable $DCTD_{L143}$ was incubated with the various proteins and then exposed to light. *, $DCTD_{L143}$–σ^{54} crosslinked product.

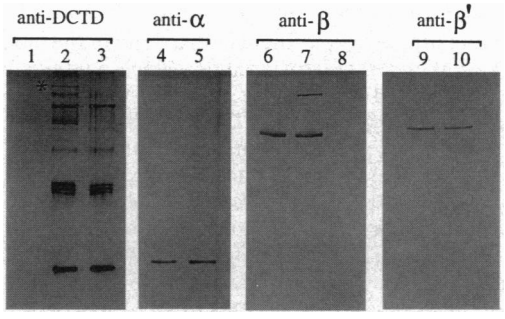

FIG. 5. Crosslinking of $DCTD_{L143}$ and β subunit of RNA polymerase. $DCTD_{L143}$ modified with sulfo-SMCC was incubated with purified *E. coli* core RNA polymerase for 5 min. Crosslinking reactions were subjected to SDS/7% PAGE and then analyzed by immunoblotting with either affinity-purified antibodies directed against $DCTD_{L143}$ (anti-DCTD) or monoclonal antibodies specific for either the α subunit (anti-α; monoclonal antibody 129C4), the β subunit (anti-β; monoclonal antibody 1221C7), or the β′ subunit (anti-β′; monoclonal antibody 371F3) of RNA polymerase. Lanes: 1, 4, 6, and 9, core RNA polymerase alone; 2, 5, 7, and 10, maleimide-activated $DCTD_{L143}$ plus core RNA polymerase; and 3 and 8, maleimide-activated $DCTD_{L143}$ alone. The $DCTD_{L143}$–β subunit crosslinked product is present in lanes 2 (indicated by *) and 7.

suggest that the interaction between $DCTD_{L143}$ and core RNA polymerase is weaker than the interaction between $DCTD_{L143}$ and σ^{54}.

DISCUSSION

Protein–protein interactions between activators and RNA polymerase are often necessary for transcriptional activation in both prokaryotes and eukaryotes. For the major form of RNA polymerase holoenzyme ($E\sigma^{70}$), interaction between the C-terminal region of the α subunit of RNA polymerase and the activator is a common method for transcriptional activation (for review, see ref. 27). A second common mechanism for transcriptional activation with $E\sigma^{70}$ involves interaction between the C-terminal region of the σ^{70} subunit and the activator (27–29).

Interactions between $E\sigma^{54}$ and σ^{54}-dependent activators are also important for transcriptional activation by this form of RNA polymerase holoenzyme. Although previous studies have shown that the C-terminal region of the α subunit is not needed for transcriptional activation by the σ^{54}-dependent activators nitrogen regulatory protein C and nitrogen fixation protein A (30), the subunit(s) of $E\sigma^{54}$ engaged by the activator have not been identified. Our chemical crosslinking data suggest that the σ^{54}-dependent activator DCTD may engage both σ^{54} and the β subunit of RNA polymerase to activate transcription. Both a truncated, constitutively active form of DCTD and wild-type DCTD (a nonactive form) were efficiently crosslinked to σ^{54} and the β subunit, suggesting that activation of DCTD is not needed for interactions with these proteins.

Further biochemical and genetic analysis is needed to determine the physiological relevance of these crosslinking data, but several lines of evidence suggest that the crosslinking results from specific protein–protein interactions. (*i*) Crosslinked products were formed at relatively low protein concentrations with sulfo-SMCC (as low as 100 nM). (*ii*) DCTD did not crosslink with proteins that are not involved in transcription initiation from σ^{54}-dependent promoters. (*iii*) Crosslinking between $DCTD_{L143}$ and σ^{54} was observed with sulfo-HSAB, which forms a short-lived, reactive nitrene upon photoactivation, making it more likely that the crosslinking between these proteins results from specific protein–protein interactions rather than from random collisions. Moreover, the nonspecific manner in which the nitrene reacts with proteins makes the failure of $DCTD_{L143}$ to crosslink with the other proteins that we tested more significant.

These crosslinking experiments were done in the absence of the *dctA* promoter regulatory region, which contains binding sites for both DCTD and $E\sigma^{54}$. As yet, we have not seen enhanced crosslinking of DCTD with either σ^{54} or the β subunit by including DNA that carries the *dctA* promoter regulatory region in the reaction mixtures. We do not believe that the failure to stimulate crosslinking with DNA argues against the physiological relevance of the crosslinking data. Failure to observe stimulation of crosslinking with DNA may have resulted from any one of several reasons, including steric hindrance upon binding of proteins to DNA or low affinity of modified DCTD for its binding sites on the DNA. Moreover, DCTD appears capable of interacting with $E\sigma^{54}$ in a productive manner without binding to its sites at the *dctA* promoter regulatory region. Truncated DCTD proteins that lack all or part of the C-terminal (DNA-binding) domain do not recognize the DCTD-binding sites from the *dctA* promoter region but can still activate transcription from the *dctA* promoter both *in vivo* and *in vitro* at high concentrations (ref. 31; B. Gu, B. S. Wang, M. Liang, D. Scholl, J.H.L., T.R.H., and B. T. Nixon, unpublished work). To activate transcription, these truncated DCTD proteins probably contact $E\sigma^{54}$ directly from solution rather than through DNA looping.

We thank Harry Dailey, Richard Ebright, Sydney Kustu, and Ken Stedman for their helpful suggestions during this work and Joe Krakow and Sydney Kustu for providing antibodies. We also thank Laurie Caslake, Anna Glasgow, Rick Gourse, Karl Klose, Charles Moran, Tracy Nixon, Wilma Ross, and the members of our laboratory for their helpful comments on the manuscript. This work was funded by Award 92-37305-7807 from the U.S. Department of Agriculture.

1. Burgess, R. R., Erickson, B., Gentry, D., Gribskov, M., Hager, D., Lesley, S., Strickland, M. & Thompson, N. (1987) in *RNA Polymerase and the Regulation of Transcription*, eds. Reznikoff, W. S., Burgess, R. R., Dahlberg, J. E., Gross, C. A., Record, M. T. & Wickens, M. P. (Elsevier, New York), pp. 3–15.
2. Gross, C. A., Lonetto, M. & Losick, R. (1992) in *Transcriptional Regulation*, eds. Yamamoto, K. & McKnight, S. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 129–176.
3. Kustu, S., Santero, E., Popham, D., Weiss, D. & Keener, J. (1989) *Microbiol. Rev.* **53,** 367–376.
4. Collado-Vides, J., Magasanik, B. & Gralla, J. (1991) *Microbiol. Rev.* **55,** 371–394.
5. Thony, B. & Hennecke, H. (1989) *FEMS Microbiol. Rev.* **63,** 341–358.
6. Sasse-Dwight, S. & Gralla, J. D. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8934–8938.
7. Popham, D. L., Szeto, D., Keener, J. & Kustu, S. (1989) *Science* **243,** 629–635.
8. Morett, E. & Buck, M. (1989) *J. Mol. Biol.* **210,** 65–77.
9. Lee, J. H., Scholl, D., Nixon, B. T. & Hoover, T. R. (1994) *J. Biol. Chem.* **269,** 20401–20409.
10. Weiss, D. S., Batut, J., Klose, K. E., Keener, J. & Kustu, S. (1991) *Cell* **67,** 155–167.
11. Lee, H.-S., Berger, D. K. & Kustu, S. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2266–2270.
12. Buck, M., Woodcock, J. & Cannon, W. (1987) *Mol. Microbiol.* **1,** 243–249.
13. Su, W., Porter, S., Kustu, S. & Echols, H. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 5504–5508.
14. Wedel, A., Weiss, D. S., Popham, D., Droge, P. & Kustu, S. (1990) *Science* **248,** 486–490.
15. Bolton, E., Higgisson, B., Harrington, A. & O'Gara, F. (1986) *Arch. Microbiol.* **144,** 142–146.
16. Engelke, T. H., Jagadish, M. N. & Puhler, A. (1987) *J. Gen. Microbiol.* **133,** 3019–3029.
17. Jiang, J., Gu, B., Albright, L. M. & Nixon, B. T. (1989) *J. Bacteriol.* **171,** 5244–5253.
18. Hager, D. A., Jin, D. J. & Burgess, R. R. (1990) *Biochemistry* **29,** 7890–7894.
19. Popham, D., Keener, J. & Kustu, S. (1991) *J. Biol. Chem.* **266,** 19510–19518.
20. Robinson, P. A., Anderton, B. H. & Loviny, T. L. F. (1988) *J. Immunol. Methods* **108,** 115–122.
21. Ozols, J. & Strittmatter, P. (1966) *J. Biol. Chem.* **241,** 4793–4797.
22. Deng, W. P. & Nickoloff, J. A. (1992) *Anal. Biochem.* **200,** 81–85.
23. Tabor, S. & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 1074–1078.
24. Narayanan, C. S. & Krakow, J. S. (1983) *Nucleic Acids Res.* **11,** 2701–2716.
25. Lesley, S. A., Brow, M. A. D. & Burgess, R. R. (1991) *J. Biol. Chem.* **266,** 2632–2638.
26. Cannon, W., Claverie-Martin, F., Austin, S. & Buck, M. (1994) *Mol. Microbiol.* **11,** 227–236.
27. Ishihama, A. (1993) *J. Bacteriol.* **175,** 2483–2489.
28. Makino, K., Amemura, M., Kim, S.-K., Nakata, A. & Shinagawa, H. (1993) *Genes Dev.* **7,** 149–160.
29. Mei, L., Moyle, H. & Susskind, M. M. (1994) *Science* **263,** 75–77.
30. Lee, H.-S., Ishihama, A. & Kustu, S. (1993) *J. Bacteriol.* **175,** 2479–2482.
31. Huala, E., Stigter, J. & Ausubel, F. M. (1992) *J. Bacteriol.* **174,** 1428–1431.